Journal of Chemical Ecology, Vol. 27, No. 1, 2001

EVALUATION OF INDIVIDUAL COMPONENTS OF PLUM ODOR AS POTENTIAL ATTRACTANTS FOR ADULT PLUM CURCULIOS

TRACY C. LESKEY,[1,4,*] RONALD J. PROKOPY,[1] STARKER E. WRIGHT,[1] P. LARRY PHELAN,[2] and LEROY W. HAYNES[3]

[1]*Department of Entomology*
University of Massachusetts
Amherst, Massachusetts 01003

[2]*Department of Entomology, OARDC*
Wooster, Ohio 44691

[3]*Department of Chemistry, The College of Wooster*
Wooster, Ohio 44691

(Received June 21, 1999; accepted August 25, 2000)

Abstract—We evaluated olfactory attraction of overwintered plum curculio (PC) adults, *Conotrachelus nenuphar*, to 16 individual volatile components of unripe plum odor in the laboratory using a still-air dual-choice bioassay system and in the field using baited cotton dental wicks attached to boll-weevil traps placed on the ground beneath the canopy of unsprayed apple trees. Two compounds, ethyl isovalerate and limonene, were significantly attractive in both laboratory bioassays and field experiments. In laboratory bioassays, as concentration was decreased across five orders of magnitude, a greater number of compounds elicited responses suggestive of attractancy (except at the lowest concentration). Even so, linalool, 2-hexanone, and 3-hydroxy-2-butanone were the only other compounds showing significant attractiveness in laboratory bioassays, but none of these (nor any other compounds) were significantly attractive in field assays. We suggest that the use of ethyl isovalerate and/or limonene as odor attractants offers potential to increase the efficacy of current traps for monitoring PCs immigrating into fruit orchards during spring.

Key Words—*Conotrachelus nenuphar*, attractants, host plant volatiles, monitoring

* To whom correspondence should be addressed.
[4] Current address: USDA, ARS, Appalachian Fruit Research Station, 45 Wiltshire Road, Kearneysville, West Virginia 25430.

0098-0331/01/0100-0001$19.50/0

INTRODUCTION

Currently, no reliable monitoring device exists to detect initiation and amount of fruit injury by the plum curculio (PC), *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae). This insect is a principal pest of stone and pome fruit in eastern and central North America (Racette et al., 1992) and one of the most destructive pests of peaches and plums in the southeastern United States (Yonce et al., 1995). Growers are forced to use the appearance of fresh egg-laying scars as a sign that PCs pose an imminent threat to fruit in orchards (Prokopy and Croft, 1994).

Various trap designs have been evaluated for monitoring PC adults. They include inverted polyethylene funnels hung beneath tree trunks to capture falling adults (Le Blanc et al., 1981), unbaited sticky-coated apples or plastic spheres hung from host tree branches (Yonce et al., 1995), pitfall traps placed beneath host trees (Yonce et al., 1995), unbaited cotton boll-weevil traps placed on vertical stakes between woods and commercial orchards (Yonce et al., 1995), and cotton boll-weevil traps baited with a component of male PC sex pheromone, grandisoic acid, and placed on cut ends of vertical host tree branches (Eller and Bartelt, 1996). Tall dark-colored unbaited pyramid traps capped with detachable conical boll weevil trap tops, developed originally as supernormal visual mimics of tree trunks for attracting host-seeking pecan weevils, *Curculio caryae* (Horn) (Tedders and Wood, 1994) also have been tested; these traps were placed on the ground in or adjacent to orchards (Prokopy and Wright, 1998; Prokopy et al., 1999). Of these candidates, only the last two methods showed any success, but even they captured small numbers of PCs.

Numerous species of weevils are attracted to host plant volatiles or specific compounds present in host plant volatiles, including the banana weevil, *Cosmopolites sordidus* (Germar) (Budenberg et al., 1993), the cabbage seed weevil, *Ceutorhynchus assimilis Payk.* (Evans and Allen-Williams, 1992, 1993; Bartlet et al., 1997; Smart and Blight, 1997), the pecan weevil, *Curculio caryae* (Horn) (Collins et al., 1997), the pea weevil, *Sitona lineatus* L. (Landon et al., 1997), and the red weevil, *Rhynchophorus ferrugineus* (Olivier) (Gunawardena et al., 1998).

PCs are attracted to host fruit odor over short distances in the laboratory (Butkewich and Prokopy, 1993; Prokopy et al., 1995; Leskey and Prokopy, 2000), and in the field at distances up to 3 m (Butkewich and Prokopy, 1997). A potential method for improving trap efficacy for PCs is to deploy specific attractive compounds present in host plant volatiles in conjunction with a trap. This has been done with the cabbage seed weevil, for which water-bowl traps painted yellow and filled with detergent solution were baited with specific odor compounds from oilseed rape, *Brassica napus* (Smart and Blight, 1997). Although PCs are attracted to host fruit odor, it is not known which particular compounds

present in such odor could potentially be attractive to PCs and provide growers with a means of increasing trap efficacy over unbaited traps (Le Blanc et al., 1981; Yonce et al., 1995; Prokopy and Wright, 1998; Prokopy et al., 1999) or be used in combination with grandisoic acid (Eller and Bartelt, 1996).

Wild plum, *Prunus americana*, is the native host of PCs (Maier, 1990), suggesting that volatiles from cultivated plum potentially could be attractive. We identified 16 individual volatile compounds from odor of unripe host fruit of cultivated plum, *Prunus domestica*, and evaluated responses of PCs to these compounds in the laboratory both in 1998 and in 2000. We used a laboratory bioassay system designed to test PC responses to host fruit odor (Prokopy et al., 1995). During 1998, we also evaluated PC responses to these 16 compounds at a field site using baited boll weevil traps placed on the ground beneath the canopy of unsprayed apple trees.

METHODS AND MATERIALS

Immature Plum Volatiles. Unripe cultivated plums (var. Fellenburg) were gathered approximately two weeks after bloom from an unmanaged orchard at the Ohio Agricultural Research and Development Center. Forty plums were placed in an 8-dr (32 ml) vial, held on ice, and transported to the laboratory for volatile collections. Volatile analysis commenced within 10 min of collecting the fruit. The cap of the vial was outfitted with two rubber septa, which allowed the flow of compressed air into the vial and flow of volatile-laden air out to a trap. Prior to entering the sample vial, the high-purity compressed air was filtered through activated charcoal and humidified by bubbling through distilled water. Fruit volatiles were collected using a trapping device described previously by Phelan and Lin (1991). The device consisted of a 60-cm × 0.1-cm-ID nickel capillary trap packed with Tenax GC (60/80 mesh) and attached to a Hewlett-Packard 5890A gas chromatograph (GC) using a two-position six-port Valco valve (Houston, Texas). This setup allowed volatiles to be collected from the sample and then thermally desorbed at 200°C directly to the capillary GC column. Plum volatiles were collected for 30 min at 20 ml/min. The trap was purged with dry helium for 3 min, and then volatiles were desorbed for 2 min to a DB-1 (0.32 × 30 m, 5-μm film) capillary column. Components were eluted at 30–200°C at 10°C/min, followed by analysis using a HP 5970 Mass Selective Detector interfaced to the GC. Initial identifications were confirmed by comparison of retention times and mass spectra with authentic samples. Blank volatile collections using a clean empty vial were conducted each morning to isolate possible contaminants from the trapping system and GC. Following volatile collection, plums were placed immediately in hexane to permit extraction of possible additional compounds.

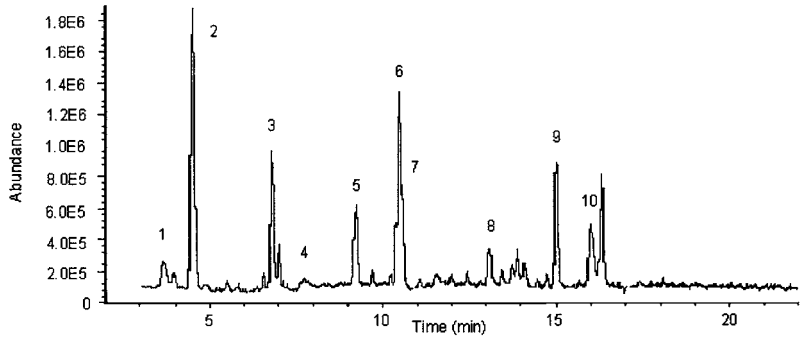


FIG. 1. Total ion chromatogram of natural plum volatiles collected on Tenax for 30 min at 20 ml/min from 40 immature plums (10 g total, avg. = 7.3 mm) at 2 weeks after bloom. Compound identity: **1**, 2-propanol (8 ng); **2**, ethyl acetate (119 ng); **3**, 3-hydroxy-2-butanone (56 ng); **4**, 2-pentanol (4 ng); **5**, ethyl butanoate (40 ng); **6 + 7**, trans-2-hexenal and ethyl isovalerate, respectively (71 ng); **8**, benzaldehyde (11 ng); **9**, limonene (51 ng); **10**, linalool (29 ng). Unmarked peaks were incompletely identified.

Ten compounds were identified from volatile emissions of field-collected plums (Figure 1). Six additional compounds not found in plum volatile profiles were identified from hexane extracts of plums: benzonitrile, 2-hexanol, 3-hexanol, 2-hexanone, 3-hexanone, and isopropyl acetate. All compounds identified from volatile emissions and from hexane extracts were evaluated as potential attractants for PCs in laboratory and field experiments. Compounds were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin). For laboratory bioassays, each compound was diluted in hexane (with the exception of 3-hydroxy-2-butanone, which was diluted in water) to yield five concentrations: 1.00, 0.10, 0.01, 0.001, and 0.0001%. For field experiments, all compounds were diluted in technical grade mineral oil to a 5.00% concentration; those compounds found to exhibit high levels of either attractancy or repellency were diluted again to a 0.50% concentration.

Laboratory Bioassay Procedures. All PCs used here were collected as overwintered adults in the field in late May or early June in 1998 and 2000 from populations present on unsprayed wild plum or apple trees in Hampshire County, Massachusetts. They were assumed to be approximately the same age but of unknown mating status. Within a few hours of collection from the field, PCs were segregated according to sex using criteria described by Thomson (1932). Adults were held for the duration of all experiments in wax-coated paper cups (473 ml) with clear plastic lids. No more than 60 individuals were held in any one cup. PCs were held under a photoregime of 16L : 8D to mimic long-day conditions in nature from late May to late June. Fresh food, either unsprayed

wild plum or apple fruit, was provided along with a wetted cotton wick as a water source. Although some herbivorous insects respond differently to host plant odors according to type of diet provided prior to assay, in preliminary laboratory experiments with PCs, we saw no difference in pattern of PC response to host fruit odors between individuals fed unsprayed apple or plum (Leskey and Prokopy, unpublished data).

A dual-choice still-air system of enclosed Petri dishes, described by Prokopy et al. (1995), was employed to test attractiveness of the 16 volatile components of plum odor. Tests were conducted at ~26°C, 65–80% relative humidity, at the beginning of scotophase of the 16L : 8D cycle, and were carried out from late May to late June in 1998 and 2000. Two pipette tips were attached over holes of the same diameter pierced in the lid of the Petri dish. These tips act as treatment tubes down which the volatiles can pass. A 75-μl aliquot of a solution containing a single compound was pipetted onto a 1-cm square of cotton wick placed next to one of the two pipette tips. A 75-μl aliquot of hexane (or water, in the case of 3-hydroxy-2-butanone) was used as a solvent control and pipetted onto a second cotton square placed next to the end of other pipette tip. During bioassay preparation, volatiles and controls were pipetted beneath a fume exhaust system to prevent cross-contamination between treatments and controls. Hexane-diluted compounds and controls were allowed to evaporate for approximately 4 min, and then each tube was covered with a transparent polystyrene cup. Water-diluted compounds and controls were covered immediately by cups. After cups were placed over both tubes, the bioassay became a closed system, allowing volatiles from the treatment wick to diffuse through the opening of the adjacent pipette tip, into the treatment tube, and then into the Petri dish chamber, presumably creating a concentration gradient of treatment odor that did not, however, exclude treatment odor from entering the control tube. PCs, chosen randomly from the available field-collected pool, were held in groups of 40 males or 40 females, starved for 24 hr, and chilled for 15–20 min in a cold room at 8–9°C just before use. Handling of PCs was kept to a minimum. A single PC was placed gently in the center of each bioassay dish. Each replicate, measuring response to a particular compound, consisted of 12 PCs tested singly in individual bioassay dishes held together on a tray. Dishes were then moved immediately to the testing room. PCs were fed immediately after the bioassay period (2 hr) had ended and were reused in subsequent bioassays no less than four days later.

To assess attractiveness of a particular compound, we used a response index (RI) developed by Phillips et al. (1993). A positive response to either the treatment (a particular compound) or control (corresponding solvent) was considered to have occurred when a PC crawled up the inner surface of one of the two tubes and entered a cup containing either the treatment or control within 2 hr. Few PCs (<3%) leave a treatment or control cup after entering it. PCs were allowed 2 hr to respond because it takes longer than 1 hr for the odor gradient and/or PC

response level to reach an asymptote; after 2 hr, little change in PC response level occurs (Prokopy et al., 1995). The RI was calculated by subtracting the number of PCs responding to the control (C) from the number responding to the treatment (T), dividing the amount by the total number of PCs tested, and multiplying by 100. Thus, $RI = ((T - C)/\text{total}) \times 100$; the greater the RI value, the more attractive the stimulus. We accept a RI value of 9 as the minimum for suggesting attractiveness and a RI value >40 as being highly attractive. This latter is based on the mean ± a single standard deviation unit (mean RI = 25.0 ± 16.0 SD, $N = 120$) obtained for responses to a standard attractive source: a hexane extract of McIntosh fruit collected one week after bloom, previously shown to be the most attractive phenological stage of apples (Leskey and Prokopy, 2000). Under this system, a RI value of −13 or greater can be considered as indicating repellency (according to the subtraction of two standard deviation units from the mean RI for our hexane standard). One replicate (involving assay of 12 PCs) was tested each day that bioassays were conducted. If the RI for the standard fell below a value of 9, data from that particular day were discarded. Discrimination of host odor by PCs is significantly and positively correlated with barometric pressure (Leskey 2000). On test days when barometric pressure was low, PCs did not discriminate well between standard and control. In addition to assessment using RI values, data were subjected to a one-tailed Wilcoxon signed rank test ($P < 0.05$). For each replicate of a particular compound, i.e. a tray of 12 Petri dishes, we subtracted the total number of PCs that entered control cups from the total number of PCs that entered treatment cups to obtain the difference between them and used this value to designate ranks among all replicates. Ranks were ultimately used to calculate the test statistic (Statistix, 1992). Those PCs that remained in the dishes were excluded from this analysis because they did not respond to either the treatment or control.

Field Assay Procedures. For each compound tested, a 150- or 15-μl aliquot of compound in neat solution was diluted to a 5.00 or 0.50% concentration, respectively, in mineral oil (3 ml total volume); 3 ml of mineral oil alone served as a control. Each treatment or control substance was applied to a 7.6-cm piece of cotton dental wick (Absorbal, Wheatridge, Colorado), which was then wrapped with aluminum foil, sealed at one end, and suspended horizontally by a wire attached to the base of a fountain-style boll weevil trap top capping an accompanying solid green boll weevil plastic trap base (Gemplers, Inc., Belleville, Wisconsin).

Release rates were determined by exposing five wrapped wicks (identical to those deployed in field assays) containing each compound at each concentration tested to open-air indoor conditions at 26–28°C for 96 hr and weighing each wick daily to determine milligrams of compound lost per day over the four-day period.

All compounds were evaluated simultaneously in a small unmanaged block

of apple trees of mixed cultivars, each about 3 m in canopy diameter. Grass beneath tree canopies was maintained at 5–10 cm throughout. One trap was placed at each cardinal point approximately 1 m away from the trunk beneath the canopy of an unsprayed apple tree, with the compound- or control-baited wick facing the trunk. For each tree, traps baited with the same compound were placed at north and south positions and control-baited traps at east and west positions (or the reverse orientations determined randomly). After 48 hr, PCs captured in the compound- and control-baited traps were counted, with totals from the two compound-baited traps combined into a single value and totals from the two control-baited traps combined into a single value, each value constituting one replicate. For the next replicate, trap location for each compound was rerandomized among trees, cardinal orientation of compound- and control-baited traps beneath trees was randomly assigned, and compound- and control-baited wicks were replaced.

In the first field experiment, each of the 16 compounds was evaluated in 12 replicates run consecutively over a 24-day period at a 5.00% concentration of each compound. In the second field experiment, the most and least attractive compounds from the first field experiment were tested again, at a 5.00% as well as a 0.50% concentration, with 10 replicates of each. Procedures were essentially the same as in the first field experiment except that each replicate lasted 24 rather than 48 hr.

To measure the attractiveness of a particular compound, a field response index was calculated by subtracting the number of PCs responding to the control (C) from the number responding to the treatment (T) [the particular compound], dividing by the total number of PCs captured in the treatment and control traps, and multiplying by 100. Thus, $RI = [(T-C)/(T+C)] \times 100$; the greater the RI, the more attractive the compound. To analyze the extent of response to traps baited with a particular compound compared to corresponding control traps, data were subjected to a one-tailed Wilcoxon signed rank test ($P \leq 0.05$). For each replicate of a particular compound, we subtracted the total number of PCs that entered control-baited traps from the total number of PCs that entered treatment-baited traps to obtain the difference between them and used this value to designate ranks among all replicates. Ranks were ultimately used to calculate the test statistic (Statistix, 1992).

RESULTS

Laboratory Assays. There were no significant differences between males and females in response to compounds at any of the five concentrations tested. Therefore, results were combined across sex to increase statistical power. For compounds at 1.00% concentration (Table 1), an RI suggesting attraction ($RI >$

TABLE 1. LABORATORY RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR (AT 1.00% IN SOLVENT) AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	PCs assayed (*N*)[a]	PCs responding (%)[b]		RI
		Treament	Control	
Benzaldehyde	72	1	29[b]	−27.8
Benzonitrile	48	2	31[b]	−29.2
Ethyl acetate	48	31	25	6.3
Ethyl butyrate	48	35	21	14.6
Ethyl isovalerate	72	15	19	−4.2
2-Hexanol	48	15	19	−4.2
3-Hexanol	48	21	21	0.0
2-Hexanone	72	25	23	2.1
3-Hexanone	48	4	21[b]	−16.7
3-Hydroxy-2-butanone	48	19	35[b]	−16.7
Isopropyl acetate	48	13	25[b]	−12.5
Limonene	72	22	29	−6.9
Linalool	48	2	29[b]	−27.1
2-Pentanol	48	23	25	−2.1
2-Propanol	48	21	21	0.0
trans-2-Hexenal	48	4	25[b]	−20.8

[a] Each replicate consisted of 12 PCs tested individually in bioassay dishes and held together on a single tray.

[b] Treatments and controls are significantly different from each other at $P \leq 0.05$ according a one-tailed Wilcoxon signed rank test.

9) was recorded for ethyl butyrate ($RI = 14.6$), while RIs suggesting repellency ($RI < -13$) were recorded for benzaldehyde, benzonitrile, 3-hexanone, 3-hydroxy-2-butanone, linalool, and *trans*-2-hexenal. The positive response to ethyl butyrate was not significant, whereas there was a significantly greater response to control over treatment for the latter six compounds plus isopropyl acetate, indicating that PCs were likely repelled at 1.00% concentration. At 0.10% concentration, responses suggesting attraction were recorded for ethyl isovalerate ($RI = 12.5$), 2-pentanol ($RI = 12.5$), and 2-hexanone ($RI = 10.4$), with response to ethyl isovalerate being significantly greater than to control (Table 2). RIs suggesting repellency were recorded for benzaldehyde and *trans*-2-hexenal, just as at the 1.00% concentration, with significantly greater response to control over treatment for each (Table 2). At 0.01% concentration, responses suggesting attraction were recorded for ethyl isovalerate ($RI = 23.6$), limonene ($RI = 18.1$), ethyl acetate ($RI = 12.5$), linalool ($RI = 12.5$), and benzonitrile ($RI = 11.7$), with responses to treatments of ethyl isovalerate, limonene and linalool being significantly greater than to controls (Table 3). A RI suggesting repellency was recorded for 3-hydroxy-2-butanone as was also the case at 1.00% concentration;

TABLE 2. LABORATORY RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR (AT 0.10% IN SOLVENT) AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	PCs assayed (*N*)[a]	PCs responding (%)[b]		RI
		Treatment	Control	
Benzaldehyde	84	13	26[b]	−13.1
Benzonitrile	60	15	25	−10.0
Ethyl acetate	48	21	27	−6.3
Ethyl butyrate	48	35	29	6.3
Ethyl isovalerate	72	26[b]	14	12.5
2-Hexanol	48	23	15	8.3
3-Hexanol	48	23	23	0.0
2-Hexanone	48	31	21	10.4
3-Hexanone	48	10	21	−10.4
3-Hydroxy-2-butanone	48	23	29	−6.3
Isopropyl acetate	48	13	17	−4.2
Limonene	72	33	29	4.2
Linalool	48	13	19	−6.3
2-Pentanol	48	29	8	12.5
2-Propanol	48	19	19	0.0
trans-2-hexenal	48	8	25[b]	−16.7

[a] Each replicate consisted of 12 PCs tested individually in bioassay dishes and held together on a single tray.

[b] Treatments and controls are significantly different from each other at $P \leq 0.05$ according to a one-tailed Wilcoxon signed rank test.

responses to controls of 3-hexanone, 3-hydroxy-2-butanone, and *trans*-2-hexenal were significantly greater than to treatments. For compounds at 0.001% concentration, RIs suggesting attraction were recorded for 2-hexanol (*RI* = 13.3), 3-hexanol (*RI* = 13.3), 2-hexanone (*RI* = 13.3), 3-hydroxy-2-butanone (*RI* = 23.3), linalool (*RI* = 11.7), and *trans*-2-hexenal (*RI* = 10.0), with responses to treatments of 2-hexanone and 3-hydroxy-2-butanone being significantly greater than to controls (Table 4). RIs suggesting repellency were recorded for benzaldehyde, benzonitrile, and isopropyl acetate with significantly greater responses to controls of benzonitrile and isopropyl acetate (Table 4). At 0.0001% concentration, no responses suggesting attraction were recorded and only isopropyl acetate elicited a response indicating repellency, with response to control being significantly greater than to treatment (Table 5).

Field Assays. In the first field experiment, the highest positive RIs were recorded for responses to traps baited with ethyl isovalerate (*RI* = 71.4), limonene (*RI* = 37.5), benzonitrile (*RI* = 33), and 3-hydroxy-2-butanone (*RI* = 26.3), with responses to ethyl isovalerate and limonene being significantly greater than to controls (Table 6). Negative RIs, indicative of more PCs caught in control-baited

TABLE 3. LABORATORY RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR (AT 0.01% IN SOLVENT) AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	PCs assayed (N)[a]	PCs responding (%)[b]		RI
		Treatment	Control	
Benzaldehyde	84	24	24	0.0
Benzonitrile	60	33	22	11.7
Ethyl acetate	48	38	21	12.5
Ethyl butyrate	48	27	19	8.3
Ethyl isovalerate	72	35[b]	11	23.6
2-Hexanol	48	21	13	8.3
3-Hexanol	48	19	21	−2.1
2-Hexanone	48	27	27	0.0
3-Hexanone	48	4	15[b]	−10.4
3-Hydroxy-2-butanone	48	8	25[b]	−16.7
Isopropyl acetate	48	23	19	4.2
Limonene	72	39[b]	25	18.1
Linalool	48	23[b]	10	12.5
2-Pentanol	48	17	23	−6.3
2-Propanol	48	17	21	−4.1
trans-2-Hexenal	48	12	25[b]	−12.5

[a] Each replicate consisted of 12 PCs tested individually in bioassay dishes and held together on a single tray.
[b] Treatments and controls are significantly different from each other at $P \leq 0.05$ according to a one-tailed Wilcoxon signed rank test.

traps than compound-baited traps, were recorded for 2-hexanol, 3-hexanol, 2-hexanone, 3-hexanone, isopropyl acetate, 2-pentanol, and 2-propanol, with significantly greater responses to control-baited traps than to traps baited with 3-hexanol or 3-hexanone. In the second field experiment, highest positive RIs were recorded for responses to limonene ($RI = 100$) and ethyl isovalerate ($RI = 80$) at 5.00% concentration, with response to each compound being significantly greater than to control. A lesser degree of positive response (none significant) occurred to both of these compounds at the 0.50% concentration and to 3-hexanol at either concentration (Table 7).

DISCUSSION

Two compounds, ethyl isovalerate and limonene, were significantly more attractive than controls in both laboratory bioassays and field experiments (Tables 2, 3, 6, and 7). In laboratory bioassays, as the concentration of compound was decreased successively from 1.00% to 0.001%, a successively greater number of compounds elicited a response suggestive of attractancy ($RI > 9$),

TABLE 4. LABORATORY RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR (AT 0.001% IN SOLVENT) AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	PCs assayed (*N*)[a]	PCs responding (%)[b]		RI
		Treatment	Control	
Benzaldehyde	48	21	33	−16.7
Benzonitrile	48	19	35[b]	−16.7
Ethyl acetate	48	27	21	6.3
Ethyl butyrate	60	35	28	6.7
Ethyl isovalerate	60	22	23	−1.7
2-Hexanol	60	35	22	13.3
3-Hexanol	60	30	17	13.3
2-Hexanone	60	33[b]	20	13.3
3-Hexanone	60	23	25	−1.7
3-Hydroxy-2-butanone	60	38[b]	15	23.3
Isopropyl acetate	60	20	35[b]	−15.0
Limonene	60	23	20	3.3
Linalool	60	32	20	11.7
2-Pentanol	60	17	23	−6.7
2-Propanol	60	30	22	8.3
trans-2-Hexenal	60	27	17	10.0

[a] Each replicate consisted of 12 PCs tested individually in bioassay dishes and held together on a single tray.

[b] Treatment and controls are significantly different from each other at $P \leq 0.05$ according to a one-tailed Wilcoxon signed rank test.

including significantly greater response to treatment over control for linalool at 0.01% (Table 3) and 2-hexanone and 3-hydroxy-2-butanone at 0.001% (Table 4). Benzaldehyde, benzonitrile, 3-hydroxy-2-butanone, linalool, and *trans*-2-hexenal were significantly less attractive than controls in laboratory bioassays at 1.00% (Table 1) but yielded positive RIs (although not significantly positive responses) in the field (Table 6). In contrast, the green leaf volatiles 2-hexanol and 2-hexanone showed RI values suggesting attractancy in laboratory assays at 0.001% (Table 4) but showed negative RIs in field assays (Table 6).

Ethyl isovalerate and limonene were the only compounds tested here that yielded positive and significant responses from PCs in both laboratory bioassays (Tables 2 and 3) and field experiments (Tables 6 and 7). Volatiles from host plant odors can be used in conjunction with traps to markedly increase trap efficacy, as has been shown in the case of butyl hexanoate (a component of apple odor) that enhances attraction of apple maggot flies, *Rhagoletis pomonella* (Walsh), to fruit-mimicking red sphere traps (Reissig et al., 1982; Duan and Prokopy, 1992; Reynolds and Prokopy, 1997). Addition of ethyl isovalerate and limonene to traps may provide growers with a means to increase trap efficacy over cur-

TABLE 5. LABORATORY RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR (AT 0.0001% IN SOLVENT) AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	PCs assayed (*N*)[a]	PCs responding (%)[b]		RI
		Treatment	Control	
Benzaldehyde	72	18	19	−1.4
Benzonitrile	72	19	25	−5.5
Ethyl acetate	72	15	21	−5.5
Ethyl butyrate	72	24	22	1.4
Ethyl isovalerate	72	21	26	−5.5
2-Hexanol	72	22	31	−8.3
3-Hexanol	72	21	24	−2.8
2-Hexanone	72	27	19	4.2
3-Hexanone	72	21	31	−9.7
3-Hydroxy-2-butanone	72	26	29	−2.7
Isopropyl acetate	72	17	31[b]	−13.9
Limonene	72	21	29	−8.3
Linalool	72	17	18	−1.4
2-Pentanol	72	19	11	8.3
2-Propanol	72	22	29	−6.9
trans-2-Hexenal	72	24	21	2.8

[a] Each replicate consisted of 12 PCs tested individually in bioassay dishes and held together on a single tray.

[b] Treatment and controls are significantly different from each other at $P \leq 0.05$ according to a one-tailed Wilcoxon signed rank test.

rent unbaited PC traps (Le Blanc et al., 1981; Yonce et al., 1995; Prokopy and Wright, 1998; Prokopy et al., 1999). In combination with grandisoic acid, an attractive component of the male-produced aggregation pheromone (Eller and Bartelt, 1996), these fruit volatiles could be even more useful in enhancing trap efficacy, such as in the case of traps for the palmetto weevil, *Rhynchophorus cruentatus* Fabricius (Giblin-Davis et al., 1994) and the West Indian sugarcane weevil, *Metamasius hemipterus sericeus* (Olivier) (Giblin-Davis et al., 1996), where combinations of host plant tissue or host plant volatile compounds plus synthetic pheromones were deployed.

Green leaf volatiles including, most importantly, certain 6-carbon alcohols and aldehydes, result from metabolism of lipids and are produced by many green plants (Bernays and Chapman, 1994). Chemotactic responses by herbivorous insects to these compounds are considered to be a generalized mechanism of host plant recognition (Metcalf and Metcalf, 1992). In our field experiment, a negative RI was recorded for PC responses to 2-hexanol, 3-hexanol, 2-hexanone, and 3-hexanone with significantly greater responses to control-baited traps for 3-hexanol and 3-hexanone (Table 6). Perhaps at the concentrations tested, PCs

TABLE 6. FIELD RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR (AT 5.00% IN MINERAL OIL) AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	Replicates[a]	Release-rate (mg/day)	Total PCs captured	PCs responding (%)[b]		RI
				Treatment	Control	
Benzaldehyde	12	14.64	16	56	44	12.5
Benzonitrile	12	11.33	15	67	34	33.0
Ethyl acetate	12	30.54	25	56	44	9.1
Ethyl butyrate	12	27.60	44	57	43	13.6
Ethyl isovalerate	12	19.51	21	86[b]	25	71.4
2-Hexanol	12	18.59	16	44	56	−12.5
3-Hexanol	12	18.57	18	17	83[b]	−66.7
2-Hexanone	12	22.43	20	40	60	−18.2
3-Hexanone	12	26.37	26	31	69[b]	−38.5
3-Hydroxy-2-butanone	12	27.29	19	63	37	26.3
Isopropyl acetate	12	33.31	23	43	57	−13.0
Limonene	12	4.99	32	69[b]	31	37.5
Linalool	12	3.71	20	55	45	10.0
2-Pentanol	12	35.33	24	38	62	−25.0
2-Propanol	12	31.97	29	34	66	−31.0
trans-2-Hexenal	12	1.85	26	54	46	7.7

[a] Each replicate consisted of captures by two compound-baited and two control-baited traps placed on the ground beneath the canopy of an unsprayed apple tree for 48 hr.
[b] Treatments and controls are significantly different from each other at $P \leq 0.05$ according to a one-tailed Wilcoxon signed rank test.

perceived these volatiles to be from a nonhost green plant and thus avoided them. Similarly, traps baited with the green leaf volatiles 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol, and (*Z*)-3-hexen-1-ol alone or in combination resulted in reduced trap catches of the striped ambrosia beetle, *Trypodendron lineatum* (Olivier), in forests of Douglas fir trees in British Columbia, indicating that these beetles may use these compounds as cues to avoid nonhost angiosperm logs in their search for host coniferous logs (Borden et al., 1997). On the other hand, unlike *T. lineatum*, PCs could be attracted to broad-spectrum green leaf volatile compounds, but at lower concentrations. For example, male PCs were attracted to a 5-mg dose of a known green leaf volatile, (*Z*)-3-hexen-1-ol, in an olfactometer in significantly greater numbers than to higher doses of this compound or to a control (Cormier et al., 1998). Similarly, attractive response was recorded for PCs to 3-hexanol at 0.001% concentration but not to this green leaf volatile at higher concentrations in our laboratory bioassays (Tables 1–4).

Insect response to a particular volatile compound can be markedly influenced by the concentration of that compound. For example, the boll weevil,

TABLE 7. FIELD RESPONSE OF ADULT PCs TO INDIVIDUAL VOLATILE COMPONENTS OF PLUM ODOR AND CORRESPONDING RESPONSE INDICES FOR EACH COMPOUND

Compound	%[a]	Replicates[b]	Release rate (mg/day)	Total PCs captured	PCs responding (%)[c] Treatment	Control	RI
Ethyl isovalerate	5.00	10	19.51	10	90[c]	10	80.0
	0.50	10	2.82	4	75	25	50.0
3-Hexanol	5.00	10	18.57	8	63	37	25.0
	0.50	10	2.39	10	60	40	20.0
Limonene	5.00	10	4.99	10	100[c]	0	100.0
	0.50	10	1.03	19	63	37	26.0

[a] % compound in mineral oil.
[b] Each replicated consists of captures by two compound-baited and two control-baited traps placed on the ground beneath the canopy of an unsprayed apple tree for 24 hr.
[c] Treatments and controls are significantly different from each other at $P \leq 0.05$ according to a one-tailed Wilcoxon signed rank test.

Anthonomus grandis Boheman, was repelled at the highest concentration and attracted at lower concentrations of β-bisabolol, the major volatile of cotton, evaluated in laboratory experiments in a moving air olfactometer (Dickens, 1986). The concentrations of 1.00% and 0.10% for most compounds tested in our laboratory bioassays were probably too high to elicit an attractive response from a PC in the confined space of a Petri dish chamber and over the short distance between odor source and release point of the PC. However, concentrations were not high enough to anesthetize or immobilize PCs as can be done by holding PCs at cold temperatures. When immobilized, PCs exhibit retracted antennae and legs. We conducted extensive observations of PCs in Petri dishes at these higher concentrations and observed that PCs appeared to be alert (erect antennae and nonretracted legs) during experiments. More importantly, as concentration was decreased to as low at 0.001%, a greater number of compounds elicited responses, suggesting attraction of PCs in our laboratory bioassays. For example, both linalool and 3-hydroxy-2-butanone were significantly less attractive than controls at 1.00% but were significantly more attractive than controls at 0.01% and 0.001%, respectively (Tables 1, 3, and 4), and *trans*-2-hexenal was significantly less attractive than control at 1.00% but elicited a response suggesting attraction at 0.001% (Tables 1 and 4). Thus, concentration of a particular compound appears to have been an important factor influencing PC responsiveness in our laboratory bioassays.

The concentration of a particular compound (or amount released) and the context in which it is evaluated likely have strong effects on the nature of PC responsiveness. As already mentioned, we found that benzaldehyde, benzonitrile, 3-hydroxy-2-butanone, linalool, and *trans*-2-hexenal were significantly less

attractive than controls in laboratory bioassays at 1.00% concentration (Table 1), but yielded positive RIs in the field at 5.00% concentration (Table 6). Laboratory bioassays evaluated PC responses in enclosed chambers over short distances, whereas field assays involved PC responses over longer distances in natural surroundings. The reverse was true for cabbage seed weevils; these weevils were attracted to (*Z*)-3-hexen-1-ol in a linear track olfactometer in laboratory experiments (Bartlet et al., 1997), but in the field, this compound had no effect on trap captures during weevil colonization of crop fields or was slightly depressive to trap captures during dispersal from crop fields (Smart and Blight, 1997). Similarly, male Mediterranean fruit flies, *Ceratitis capitata* (Weidemann), were attracted to limonene, α-terpineol, linalool, and geranyl acetate in laboratory cages and in wind tunnels, but not in the field (Howse and Knapp, 1996). Perhaps PCs, cabbage seed weevils, and male Mediterranean fruit flies express attraction to certain compounds under certain conditions, but do not do so under other conditions because of greater/lesser concentration of compound and/or the context in which a compound is offered. The still-air dual-choice bioassay system used here is a relatively simple method for simultaneously rapid screening of PC response to a large number of compounds, but this method can not be recommended for gaining deeper insight into the true nature of PC behavior during orientation toward or away from compounds of differing concentration.

This study provides the first evidence for PC attraction to specific compounds present in host plant odor, namely ethyl isovalerate and limonene. However, both of these compounds, as well as other compounds tested, might be more attractive at concentrations or release rates different from those used here, or when in combination. Although ethyl isovalerate and limonene significantly increased trap captures under the conditions tested here, further tests are needed to optimize formulation, release rate, type of trap used in association with compound deployment, and positioning of compounds on traps. The indication here of PC attraction to these compounds increases the potential for creating a reliable monitoring device for this important tree fruit pest.

Acknowledgments—We thank Max Prokopy, Keri Tallman, Anthony Minalga, Susan Nixson, Margaret Young, Brian Hogg, Jonathan Black, Stephen Lavallee, Anthea Yannopoulos, Amanda Ross, and E. Fidelma Boyd for their technical assistance. Anne Averill, Juan Rull, and two anonymous reviewers provided helpful comments to an earlier version of this manuscript. These studies were supported by USDA Hatch funding, the New England Tree Fruit Growers Association, and a University Fellowship to T. C. Leskey from the University of Massachusetts at Amherst.

REFERENCES

BARTLET, E., BLIGHT, M. M., LANE, P., and WILLIAMS, I. H. 1997. The responses of the cabbage seed weevil, *Ceutorhynchus assimilis*, to volatile compounds from oilseed rape in a linear track olfactometer. *Entomol. Exp. Appl.* 85:257–262.

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant-Selection by Phytophagous Insects. Chapman and Hall, Inc. New York, 312 pp.

BORDEN, J. H., CHONG, L. J., SAVOIE, A., and WILSON, I. M. 1997. Responses to green leaf volatiles in two biogeoclimatic zones by striped ambrosia beetle, *Trypodendron lineatum. J. Chem. Ecol.* 23:2479–2491.

BUDENBERG, W., NDIEGE, I. O., KARAGO, F. W., and HANSSON, B. S. 1993. Behavioral and electrophysiological responses of the banana weevil, *Cosmopolites sordidus*, to host plant volatiles. *J. Chem. Ecol.* 19:266–277.

BUTKEWICH, S. C., and PROKOPY, R. J. 1993. The effect of short range host odor stimuli on host fruit finding and feeding behavior of PC adults (Coleoptera: Curculionidae). *J. Chem. Ecol.* 19:825–835.

BUTKEWICH, S. C., and PROKOPY, R. J. 1997. Attraction of adult plum curculios (Coleoptera: Curculionidae) to host-tree odor and visual stimuli in the field. *J. Entomol. Sci.* 32:1–6.

COLLINS, J. K., MULDER, P. G., GRANTHAM, R. A., REID, W. R., SMITH, M. W., and EIKENBARY, R. D. 1997. Assessing feeding preferences of pecan weevil (Coleoptera: Curculionidae) adults using a Hardee olfactometer. *J. Kans. Entomol. Soc.* 70:181–188.

CORMIER, D., CHOUINARD, G., and BELANGER, A. 1998. Plum curculio responses to different attractants under laboratory conditions, p. 37, *in* 1998 Joint Annual Meeting Entomological Society of America and American Phytopathological Society Meeting Program, November 8–12, 1998, Las Vegas, Nevada.

DICKENS, J. C. 1986. Orientation of boll weevil, *Anthonomus grandis* BOH. (Coleoptera: Curculionidae), to pheromone and volatile host compound in the laboratory. *J. Chem. Ecol.* 12:91–98.

DUAN, J. J., and PROKOPY, R. J. 1992. Visual and odor stimuli influencing effectiveness of sticky spheres for trapping apple maggot flies. *J. Appl. Entomol.* 113:271–279.

ELLER, F. J., and BARTELT, R. J. 1996. Grandisoic acid, a male-produced aggregation pheromone for the plum curculio, *Conotrachelus nenuphar. J. Nat. Prod.* 59:451–453.

EVANS, K. A., and ALLEN-WILLIAMS, L. J. 1992. Electroantennogram responses of the cabbage seed weevil *Ceutorhynchus assimilis* to oilseed rape, *Brassica napus* ssp. *olifera*, volatiles. *J. Chem. Ecol.* 18:1641–1659.

EVANS, K. A., and ALLEN-WILLIAMS, L. J. 1993. Distant olfactory responses of the cabbage seed weevil, *Ceutorhynchus assimilis*, to oilseed rape odor in the field. *Physiol. Entomol.* 18:251–256.

GIBLIN-DAVIS, R. M., WEISSLING, T. J., OEHLSCHLAGER, A. C., and GONZALEZ, L. M. 1994. Field response of *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) to its aggregation pheromone and fermenting plant volatiles. *Fla. Entomol.* 77:164–177.

GIBLIN-DAVIS, R. M., PEÑA, J. E., OEHLSCHLAGER, A. C., and PEREZ, A. L. 1996. Optimization of semiochemical-based trapping of *Metamasius hemipterus sericeus* (Olivier) (Coleoptera: Curculionidae). *J. Chem. Ecol.* 22:1389–1410.

GUNAWARDENA, N. E., KERN, F., JANSSEN, E., MEEGODA, C., SCHAFER, D., VOSTROWSKY, O., and BESTMANN, H. J. 1998. Host attractants of red weevil, *Rhynchophorus ferrugineus*: Identification, electrophysiological activity, and laboratory bioassay. *J. Chem. Ecol.* 24:425–437.

HOWSE, P. E., and KNAPP, J. J. 1996. Pheromones of Mediterranean fruit fly: Presumed mode of action and implications for improved trapping techniques, pp. 91–99, *in* B. A. McPheron and G. J. Steck (eds.). Fruit Fly Pests. St. Lucie Press, Delray Beach, Florida.

LANDON, F., FERARY, S., PIERRE, D., AUGER, J., BIEMONT, J. C., LEVIEUX, J., and POUZAT, J. 1997. *Sitona lineatus* Host-plant odors and their components: Effect on locomotory behavior and peripheral sensitivity variations. *J. Chem. Ecol.* 23:2161–2173.

LE BLANC, J. P., HILL, S. B., and PARADIS, R. O. 1981. Essais de piegeage du charancon de la prune, *Conotrachelus nenuphar. Ann. Entomol. Soc. Quebec* 26:182–190.

LESKEY, T. C. 2000. Olfactory and visual cues used by plum curculios to locate host plants. PhD dissertation. University of Massachusetts at Amherst, 152 pp.

LESKEY, T. C., and PROKOPY, R. J. 2000. Sources of apple odor attractive to adult plum curculios (Coleoptera: Curculionidae). *J. Chem. Ecol.* 26:639–653.

MAIER, C. T. 1990. Native and exotic rosaceous hosts of apple, plum and quince curculio larvae (Coleoptera: Curculionidae) in northeastern United States. *J. Econ. Entomol.* 83:1326–1332.

METCALF, R. L., and METCALF, E. R. 1992. Plant Kairomones in Insect Ecology and Control. Chapman and Hall, New York, 168 pp.

PHELAN, P. L., and LIN, H. 1991. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Curculionidae). *J. Chem. Ecol.* 17:1253–1272.

PHILLIPS, T. W., JIANG, X. L., BURKHOLDER, W. E., PHILLIPS, J. K., and TRAN, H. Q. 1993. Behavioral responses to food volatiles by two species of stored-product Coleoptera, *Sitophilus oryzae* and *Tribolium castaneum. J. Chem. Ecol.* 19:723–734.

PROKOPY, R. J., and CROFT, B. A. 1994. Apple insect pest management, pp. 543–586, *in* R. Metcalf and W. Luckman (eds.). Introduction to Insect Pest Management, 3rd ed. John Wiley & Sons, New York.

PROKOPY, R. J., and WRIGHT, S. E. 1998. Plum curculio (Coleoptera: Curculionidae) response to unbaited pyramid and cone traps. *J. Econ. Entomol.* 91:226–234.

PROKOPY, R. J., COOLEY, S. S., and PHELAN, P. L. 1995. Bioassay approaches to assessing behavioral responses of plum curculio adults (Coleoptera: Curculionidae) to host fruit odor. *J. Chem. Ecol.* 21:1073–1084.

PROKOPY, R. J., MARSELLO, M. M., LESKEY, T. C., and WRIGHT, S. E. 1999. Evaluation of unbaited traps for monitoring and controlling plum curculio adults (Coleoptera: Curculionidae) in apple orchards. *J. Entomol. Sci.* 34:144–153.

RACETTE, G., CHOUINARD, G., VINCENT, C., and HILL, S. B. 1992. Ecology and management of the plum curculio (Coleoptera: Curculionidae) in apple orchards. *Phytoprotection* 73:85–100.

REISSIG, W. H., FEIN, B. L., and ROELOFS, W. L. 1982. Field tests of synthetic apple volatiles as apple maggot attractants. *Environ. Entomol.* 11:1294–1298.

REYNOLDS, A. H., and PROKOPY, R. J. 1997. Evaluation of odor lures for use with red sticky spheres to trap apple maggot. *J. Econ. Entomol.* 90:1655–1660.

SMART, L. E., and BLIGHT, M. M. 1997. Field discrimination of oilseed rape, *Brassica napus* volatiles by cabbage seed weevil, *Ceutorhynchus assimilis. J. Chem. Ecol.* 23:2555–2567.

STATISTIX. 1992. Analytical Software, Version 4.0. Statistix, St. Paul, Minnesota.

TEDDERS, W. L., and WOOD, B. W. 1994. A new technique for monitoring pecan weevil emergence. (Coleoptera: Curculionidae). *J. Entomol. Sci.* 29:18–30.

THOMSON, R. R. 1932. Sex differentiation of adults of *Conotrachelus nenuphar. J. Econ. Entomol.* 25:807–810.

YONCE, C. E., HORTON, D. L., and OKIE, W. R. 1995. Spring migration, reproductive behavior, monitoring procedures and host preference of plum curculio (Coleoptera: Curculionidae) in *Prunus* species in Central Georgia. *J. Entomol. Sci.* 30:82–92.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

PLANT GROWTH INHIBITORY ACTIVITY OF L-CANAVANINE AND ITS MODE OF ACTION

NAOKO NAKAJIMA,* SYUNTARO HIRADATE, and YOSHIHARU FUJII

National Institute of Agro-Environmental Sciences
3-1-1 Kan-nondai
Tsukuba, Ibaraki 305-8604, Japan

(Received April 3, 2000; accepted August 26, 2000)

Abstract—L-Canavanine is a nonprotein amino acid contained in jack bean [*Canavalia ensiformis* (L.) DC] and shows a plant inhibitory effect. The inhibitory effect was determined by an immersion test and a microdrop test that employed rice seedlings. L-Canavanine inhibited elongation of the second leaf sheath of rice seedlings more than other natural bioactive substances, such as salicylic acid and cinnamic acid. The modified microdrop test revealed that the mode of action of L-canavanine had no relation to gibberellin synthesis. In the microdrop test, the inhibitory effect of L-canavanine was decreased by simultaneous addition of L-arginine, an analog of L-canavanine. Free amino acid analysis of rice shoots clearly showed that L-canavanine induced an unusual accumulation of L-arginine. However, accumulation of L-arginine did not cause the inhibitory effect on plant growth. These results suggest that the mechanism of inhibition of L-canavanine is closely related to the inhibition of arginine metabolism.

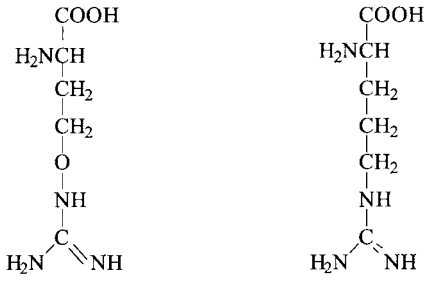
Key Words—L-Canavanine, nonprotein amino acid, plant growth inhibitor, mode of action.

INTRODUCTION

Some chemicals contained in plants have protective functions against insects, microorganisms, and plants. They are variable in their chemical structures and modes of action (Einhellig, 1995b). Some of them may be useful as agricultural chemicals. Many insecticides and fungicides come from natural plants (Benner,

*To whom correspondence should be addressed at present address: Plant Science Center, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hrosawa, Wako, Saitama 351-0198, Japan; e-mail: nnaoko@postman.riken.go.jp

0098-0331/01/0100-0019$19.50/0



L-Canavanine L-Arginine

FIG. 1. Chemical structure of L-canavanine and L-arginine.

1996). On the other hand, few natural plant compounds are used as commercial herbicides because they do not show strong and long-term effects like synthetic herbicides (Benner, 1996). However, it should be possible to reduce the amount of synthetic herbicide applied by using natural products or allelopathic plants or microorganisms that produce those products (Einhellig, 1995a).

L-Canavanine (Figure 1) is a nonprotein amino acid found in legumes such as jack bean [*Canavalia ensiformis* (L.) DC.] (Turner and Harbone, 1967). Jack bean has been shown to inhibit growth of some other plants (Shibuya, 1994), and L-canavanine is referred to as an allelochemical of jack bean (Shibuya, 1994). In this study, the plant inhibitory activity of L-canavanine was determined and compared with other bioactive substances derived from plants. The mode of action of L-canavanine was investigated.

METHODS AND MATERIALS

Comparison of Activity on Elongation Growth. The immersion test (Murakami, 1973) and the microdrop test (Murakami, 1968) were modified as follows.

In the immersion test, 1 ml of 0.5% (w/v) agar medium containing 0.5 mM of the test compound was poured into a glass vial (30 mm ID × 60 mm long). After solidification, five rice seeds (*Oryza sativa* L. cv. Nipponbare) germinated for two days at 30°C were planted on the agar medium in the vial and incubated for five days at 30°C under continuous irradiation with fluorescent lamps (50 μmol/m^2/sec). The length of the second leaf sheath of treated seedlings was measured and compared with that of controls (untreated seedlings).

In the microdrop test, a vial (30 mm ID × 60 mm long) was filled with 30

ml of 0.5% (w/v) agar medium, and five germinated rice seeds (*Oryza sativa* L. cv. Nipponbare) were planted on the medium after solidification. The vial was incubated at 30°C for two days under continuous irradiation with fluorescent lamps (50 μmol/m^2/sec). Test solution (1 μl) was applied on the cross-point of a coleoptile and the first leaf of a seedling by using a microsyringe. After four days, the length of the second leaf sheath was measured. A test solution was prepared by dissolving each test compound in 50% (v/v) aqueous acetone (5 mM). The lighting was changed by covering with one or two layers of black cheesecloth to shade and improve the sensitivity of this method. One or two layers of black cheesecloth decreased light intensity to 20 and 12 μmol/m^2/sec, respectively.

Influence of Gibberellin on Activity. The microdrop test described above was further modified to clarify the relation of the inhibitory effect of allelochemicals with gibberellin synthesis. In this method, rice seedlings grown in the presence of GA_3 were used. To optimize the concentration of GA_3 in agar medium, germinated seeds were placed on medium containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 μM GA_3 and incubated for six days at 30°C under continuous irradiation with fluorescent lamps (50 μmol/m^2/sec). After incubation, the length of the second leaf sheath was measured and the appropriate concentration of GA_3 was determined. Rice seedlings grown under the above conditions were examined for allelochemicals. After application of the test solution, the vial was incubated under the same conditions described above, and length of the second leaf sheath of the seedling was measured after four days.

Effect of L-Arginine on Growth Inhibition Induced by L-Canavanine. In the microdrop test, 0 or 5 nmol/plant of L-canavanine was applied to the coleoptile and the first leaf of a rice seedling, and after drying at room temperature for 1 hr, 0, 25, or 50 nmol/plant of L-arginine was added. After four days of incubation at 30°C, the length of the second leaf sheath of the seedling was measured.

Analysis of Free Amino Acids and Determination of Ammonia. The shoots of rice (0.5–1.0 g) were homogenized in 80% ethanol at 4°C and extracted at 80°C for 20 min. The supernatant was collected by centrifugation at 2000*g* for 10 min. The extraction was repeated twice. Supernatants were combined and evaporated at 40°C under vacuum conditions. They were dissolved in water and washed with ethyl ether to remove pigments. The water fraction was reevaporated and dissolved into 1.5 ml of 0.02 N HCl and analyzed with an amino acid analyzer (Hitachi L-8500) to determine amino acids and ammonia.

Chemicals. GA_3, L-mimosine [α-amino-3-hydroxy-4-oxo-1(4H)-pyridinepropanoic acid], protocatechuic acid (3,4-dihydroxybenzoic acid), cinnamic acid (3-phenyl-2-propenoic acid), oxalic acid (ethanedioic acid) and salicylic acid (2-hydroxybenzoic acid) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol] was obtained from Zeneka

K. K. Agrochemicals (Tokyo, Japan). Vanillic acid (4-hydroxy-3-methoxybenzoic acid) and salicylhydroxamic acid (*N*,2-dihydroxybenzamide) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin USA). Hydroquinone (1,4-benzenediol), arbutin (4-hydroxyphenyl-β-D-glucopyranoside), L-canavanine {*O*-[(aminoiminomethyl)amino] homoserine}, L-DOPA [3-(3,4-dihydroxyphenyl)alanine], and syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Synthetic abscisic acid (ABA) {[*S*-(*Z*,*E*)]-5-(1-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3-methyl-2,4-pentadienoic acid}, coumarin (2H-1-benzopyran-2-one), and sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). A new plant growth inhibitor found in *Duranta repens*, durantanin II, was purified by the method reported by Hiradate et al. (1999).

RESULTS

To evaluate the sensitivity of the bioassay methods for determining inhibitory activity, paclobutrazol, an inhibitor of gibberellin synthesis, was selected as a reference compound. In the immersion test, the inhibitory effect of 1 μM paclobutrazol was detected (Figure 2).

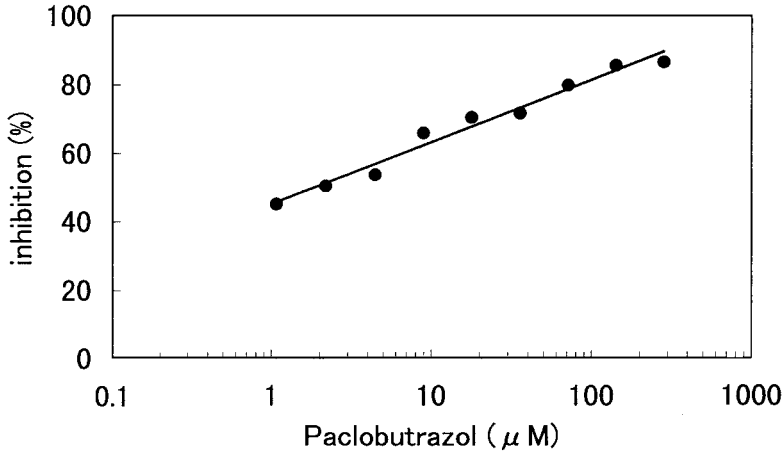


FIG. 2. Effect of paclobutrazol on elongation growth of the second leaf sheath of rice seedlings (immersion test). Germinated seeds were planted on 1 ml of 0.5% agar containing an appropriate concentration of paclobutrazol. Inhibition (%) was calculated as 100 − [(treated/control) × 100].

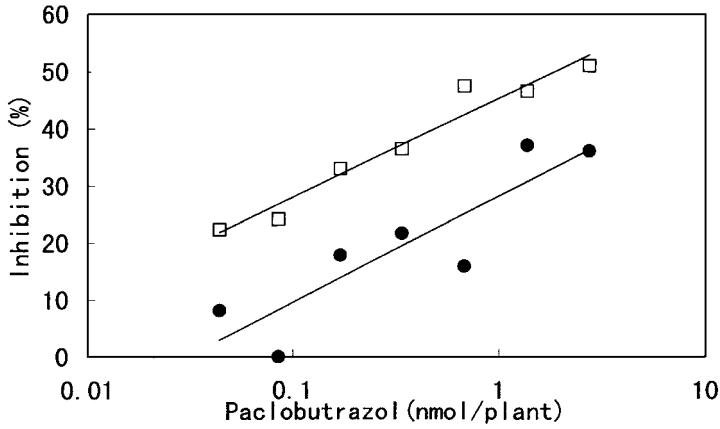


FIG. 3. Effect of irradiance condition on the growth inhibition activity of paclobutrazol on the second leaf sheath of rice (microdrop test). Inhibition (%) was calculated as 100 − [(treated/control) × 100]. ●: 50 μmol/m^2/sec, □: 20 μmol/m^2/sec.

For the microdrop test, the effect of light irradiance on the inhibitory effect of paclobutrazol was studied to optimize sensitivity of this test. Paclobutrazol showed higher inhibitory activity under 20 μmol/m^2/sec of irradiance than under 50 μmol/m^2/sec (Figure 3). The low irradiance condition of 12 μmol/m^2/sec was almost at the same level as that under 20 μmol/m^2/sec (data not shown). In this study, an irradiance condition of 20 μmol/m^2/sec was used.

The immersion test detected strong inhibitory effects of coumarin, L-canavanine, L-mimosine, and a triterpenoid saponin, durantanin II (Figure 4). The microdrop test showed specific strong inhibitory activity of L-canavanine (Figure 5).

The microdrop test was modified to detect inhibitory effects on gibberellin synthesis. Elongation of the second leaf sheath is promoted when the rice seedling is grown in GA_3-containing medium. The rate of the GA_3-induced promotion of elongation became constant when the concentration of GA_3 was higher than 1 μM (data not shown). Paclobutrazol, a gibberellin synthesis inhibitor, inhibited elongation of the second leaf sheaths of rice seedlings grown with no GA_3 in the medium, but not when they were grown in 1 μM GA_3 medium (Figure 6a). On the other hand, ABA, which inhibits gibberellin-induced plant growth, inhibited elongation of the second leaf sheaths of the rice seedlings grown in 1 μM GA_3 medium as well as in medium with no GA_3 (Figure 6b). L-Canavanine had the same effect as ABA (Figure 6c).

When 25 and 50 nmol/plant of L-arginine was applied after application of L-canavanine, the inhibitory effect of L-canavanine was removed in proportion

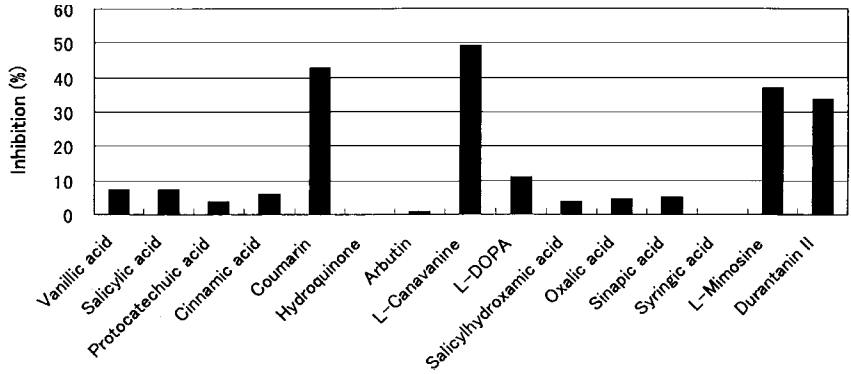


FIG. 4. Effect of allelochemicals on the elongation growth of the second leaf sheath of rice seedlings (immersion test). Five germinated seeds were planted on 1 ml of 0.5% agar each containing 0.5 mM of chemical. Inhibition (%) was calculated as 100 − [(treated/control) × 100].

to the concentration of L-arginine (Table 1). On the other hand, application of L-arginine alone had no effects on the elongation growth (Table 1).

Table 2 shows that almost all amino acids were increased by application of 5 nmol/plant of L-canavanine. L-Arginine content was increased (698% of control), but it was not significant. Application of 5 nmol/plant of L-canavanine and 50 nmol/plant of L-arginine significantly increased the contents of alanine (266% of control) and L-arginine (2885% of control). On the other hand, application of

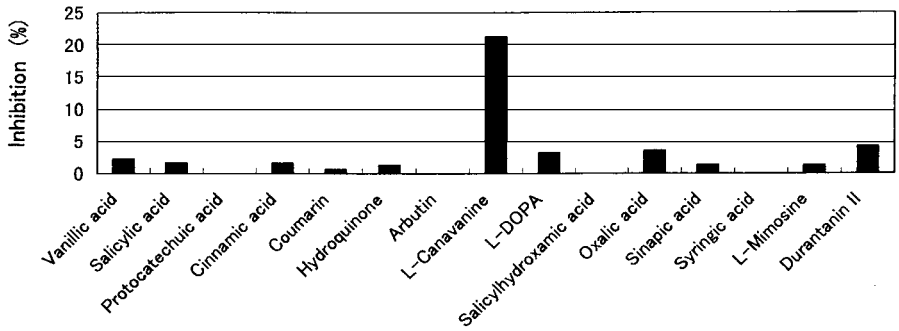


FIG. 5. Effect of allelochemicals on the elongation growth of the second leaf sheath of rice seedlings (microdrop test). One microliter of 5 mM of each test solution (5 nmol) was applied onto the cross point of the coleoptile and the first leaf of a rice seedling by using a microsyringe. Inhibition (%) was calculated as 100 − [(treated/control) × 100].

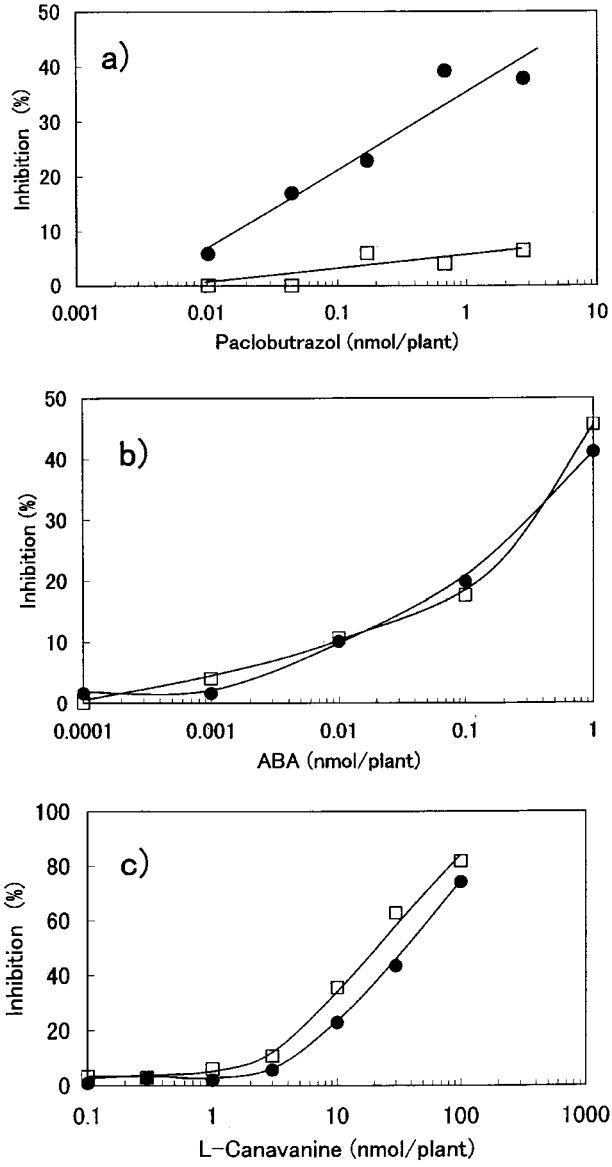


FIG. 6. Effect of 1 μM GA_3 in agar medium on elongation growth of the second leaf sheath of rice seedlings as influenced by (a) paclobutrazol, (b) ABA, and (c) L-canavanine in microdrop test. Inhibition (%) was calculated as 100 − [(treated/control) × 100]. ●: 0 μM GA_3, □: 1 μM GA_3.

TABLE 1. EFFECT OF TREATMENT OF L-CANAVANINE AND L-ARGININE ON LENGTH OF SECOND LEAF SHEATH OF RICE SEEDLINGS IN MICRODROP TEST[a]

Treatment (nmol/plant)		Length of second leaf sheath (mm)
L-canavanine	L-arginine	
0	0	37.2 ± 1.7
0	25	37.4 ± 1.9
0	50	36.9 ± 2.6
5	0	30.0 ± 2.4[b]
5	25	32.1 ± 2.4[b,c]
5	50	35.1 ± 2.9[b,c]

[a] Values are the means with SD ($N = 24$).
[b] Significant difference from treatment of 0 nmol/plant L-canavanine and 0 nmol/plant L-arginine by t test ($P < 0.01$).
[c] Significant difference from treatment of 5 nmol/plant L-canavanine and 0 nmol/plant L-arginine by t test ($P < 0.01$).

50 nmol/plant of L-arginine did not significantly increase arginine content. After four days of treatment (Table 3), glutamic acid and alanine was increased in comparison with controls by application of L-canavanine. The content of L-arginine was reduced to 124% of control. In application of L-canavanine and L-arginine, the contents of glutamic acid, alanine, and L-arginine increased. Glutamic acid also increased by application of L-arginine. Application of L-canavanine and L-arginine had no effect on ammonia content at two and four days after treatment (data not shown).

DISCUSSION

In this study, effects of an allelochemical candidate, L-canavanine, on elongation growth of a plant was investigated in comparison with other bioactive substances derived from plants by using microdrop and immersion tests. In these tests, paclobutrazol, an inhibitor of gibberellin synthesis (Hedden and Graebe, 1985), was used as a reference inhibitor. In the immersion test, inhibitory activity of 1 μM paclobutrazol was seen (Figure 2). To improve the sensitivity of the microdrop test, experimental lighting conditions were optimized prior to the test. Gawaronska et al. (1995) reported that low irradiance promoted gibberellin synthesis and elongation growth of pea seedlings (*Pisum sativum*). Figure 2 shows that the inhibitory activity was more significant under low irradiance (20 μmol/m^2/sec) than under high irradiance (50 μmol/m^2/sec). Acceleration of elongation increases the sensitivity to detect inhibitory effects. By using these tests, inhibitory effects of L-canavanine and the other bioactive substances on elongation growth were compared.

TABLE 2. FREE AMINO ACID CONTENTS IN RICE SHOOTS AT 2 DAYS AFTER TREATMENT WITH L-CANAVANINE AND L-ARGININE[a]

Amino acid	Control	5 nmol canavanine + 0 nmol Arg (%)[b]	5 nmol canavanine + 50 nmol Arg (%)[b]	0 nmol canavaine + 50 nmol Arg (%)[b]
Asp	0.567 ± 0.119 ac	0.621 ± 0.039 a (110)	0.371 ± 0.012 b (65)	0.448 ± 0.029 bc (79)
Thr	0.079 ± 0.012 ac	0.124 ± 0.008 b (157)	0.102 ± 0.011 bc (128)	0.071 ± 0.011 a (89)
Ser	0.316 ± 0.038 ac	0.371 ± 0.005 ab (117)	0.395 ± 0.019 b (125)	0.281 ± 0.049 c (89)
Asn	0.276 ± 0.064 a	0.423 ± 0.080 a (153)	0.356 ± 0.072 a (129)	0.286 ± 0.054 a (103)
Glu	0.450 ± 0.052 a	0.514 ± 0.016 a (114)	0.451 ± 0.037 a (100)	0.318 ± 0.055 a (71)
Gln	0.226 ± 0.048 ab	0.270 ± 0.009 a (119)	0.226 ± 0.025 ab (100)	0.164 ± 0.051 b (73)
Gly	0.046 ± 0.003 a	0.060 ± 0.002 b (132)	0.064 ± 0.005 b (139)	0.044 ± 0.003 a (97)
Ala	0.254 ± 0.046 a	0.395 ± 0.045 b (155)	0.677 ± 0.083 c (266)	0.292 ± 0.033 ab (115)
Val	0.070 ± 0.014 a	0.103 ± 0.005 b (146)	0.094 ± 0.007 ab (133)	0.072 ± 0.015 a (102)
Ile	0.029 ± 0.007 a	0.048 ± 0.003 b (165)	0.034 ± 0.002 a (116)	0.026 ± 0.005 a (90)
Leu	0.028 ± 0.003 a	0.037 ± 0.004 bc (134)	0.043 ± 0.004 b (156)	0.031 ± 0.001 ac (112)
Tyr	0.036 ± 0.003 a	0.039 ± 0.004 a (107)	0.040 ± 0.002 a (112)	0.037 ± 0.002 a (102)
Phe	0.028 ± 0.003 a	0.035 ± 0.002 b (126)	0.036 ± 0.003 b (127)	0.032 ± 0.002 ab (116)
Lys	0.012 ± 0.003 a	0.013 ± 0.002 a (106)	0.017 ± 0.003 a (138)	0.015 ± 0.001 a (124)
His	0.025 ± 0.010 a	0.013 ± 0.010 a (53)	0.009 ± 0.011 a (37)	0.019 ± 0.008 a (75)
Arg	0.005 ± 0.009 a	0.037 ± 0.015 a (698)	0.154 ± 0.068 b (2885)	0.020 ± 0.002 a (383)
Pro	0.069 ± 0.023 a	0.098 ± 0.012 a (143)	0.098 ± 0.030 a (143)	0.087 ± 0.027 a (127)
Total	2.517 ± 0.428 ab	3.203 ± 0.049 a (127)	3.166 ± 0.307 a (126)	2.243 ± 0.307 b (89)

[a] Values are expressed as nmol/g FW and means of three or four replications with SD. Values with different lowercase letters are significantly different from each other ($P < 0.01$, ANOVA followed by Fisher's PLSD test).
[b] Percentage of control.

TABLE 3. FREE AMINO ACID CONTENTS IN RICE SHOOTS AT 4 DAYS AFTER TREATMENT WITH L-CANAVANINE AND L-ARGININE[a]

Amino acid	Control	5 nmol canavanine + 0 nmol Arg (%)[b]	5 nmol canavanine + 50 nmol Arg (%)[b]	0 nmol canavanine + 50 nmol Arg (%)[b]
Asp	0.617 ± 0.048	0.746 ± 0.057 (121)	0.593 ± 0.089 (96)	0.704 ± 0.156 (114)
Thr	0.197 ± 0.007	0.213 ± 0.019 (108)	0.234 ± 0.056 (118)	0.191 ± 0.046 (97)
Ser	0.506 ± 0.020	0.539 ± 0.035 (106)	0.611 ± 0.090 (120)	0.492 ± 0.088 (97)
Asn	0.887 ± 0.090	1.064 ± 0.288 (120)	1.435 ± 0.423 (162)	0.844 ± 0.155 (95)
Glu	0.390 ± 0.175 a	0.799 ± 0.033 b (205)	0.847 ± 0.162 b (217)	0.717 ± 0.115 b (184)
Gln	0.510 ± 0.012	0.517 ± 0.123 (101)	0.618 ± 0.133 (121)	0.496 ± 0.104 (97)
Gly	0.086 ± 0.004 a	0.080 ± 0.007 a (93)	0.105 ± 0.009 b (122)	0.080 ± 0.005 a (93)
Ala	0.396 ± 0.013 a	0.583 ± 0.073 ab (147)	0.947 ± 0.333 b (239)	0.374 ± 0.023 a (95)
Val	0.236 ± 0.012	0.236 ± 0.067 (100)	0.294 ± 0.063 (125)	0.213 ± 0.050 (90)
Ile	0.121 ± 0.008	0.119 ± 0.039 (98)	0.148 ± 0.032 (122)	0.110 ± 0.025 (90)
Leu	0.115 ± 0.036	0.089 ± 0.022 (77)	0.108 ± 0.021 (94)	0.092 ± 0.024 (79)
Tyr	0.092 ± 0.012	0.078 ± 0.004 (85)	0.090 ± 0.006 (98)	0.081 ± 0.006 (88)
Phe	0.069 ± 0.001	0.065 ± 0.004 (94)	0.079 ± 0.005 (113)	0.068 ± 0.006 (98)
Lys	0.061 ± 0.019	0.045 ± 0.002 (74)	0.049 ± 0.008 (80)	0.041 ± 0.008 (68)
His	0.087 ± 0.019 a	0.077 ± 0.007 a (89)	0.140 ± 0.028 b (162)	0.077 ± 0.011 a (89)
Arg	0.050 ± 0.020 a	0.061 ± 0.028 a (124)	0.133 ± 0.042 b (268)	0.034 ± 0.010 a (68)
Pro	0.154 ± 0.020	0.176 ± 0.030 (114)	0.165 ± 0.024 (107)	0.133 ± 0.034 (86)
Total	4.578 ± 0.141	5.489 ± 0.798 (120)	6.596 ± 1.195 (144)	4.749 ± 0.749 (104)

[a] Values are expressed as nmol/g FW and means of three or four replications with SD. Values with different lowercase letters are significantly different from each other ($P < 0.01$, ANOVA followed by Fisher's PLSD test) and values with no letters are not significantly different from each other.
[b] Percentage of control.

In the immersion test, L-canavanine, coumarin, L-mimosine, and durantanin II showed inhibitory effects on elongation of the second leaf sheaths of rice seedlings (Figure 4). In the microdrop test, L-canavanine showed the strongest inhibitory effects, but most of the phenolic acids such as vanillic acid and cinnamic acid had only weak activity (Figure 5). Among the tested compounds, L-canavanine was the strongest plant growth inhibitor.

One mechanism of inhibition of plant elongation is gibberellin synthesis inhibition. It is well-known that most commercial growth retardants inhibit synthesis of gibberellin (Kamiya, 1991). Because L-canavanine inhibited elongation and had growth-retarding activity, the inhibition of gibberellin synthesis is a candidate for the inhibitory mechanism. In order to clarify the mode of action, a bioassay for detecting inhibitory effects of gibberellin synthesis was developed. The microdrop test was modified to detect this inhibitory mechanism. In this method, rice seedlings were grown in the agar medium containing GA_3, resulting in recovery from gibberellin synthesis inhibition. Therefore, it should be possible to distinguish between inhibition of gibberellin synthesis and the other mechanisms by applying this test. The concentration of GA_3 in the agar medium was adjusted to 1 μM, because the gibberellin-induced elongation growth of the second leaf sheath became constant at a GA_3 concentration of more than 1 μM (data not shown). Paclobutrazol and ABA were used as reference substances to evaluate the effectiveness of the test. Paclobutrazol, which is an inhibitor of gibberellin synthesis (Hedden and Graebe, 1985), inhibited elongation of rice seedlings only when grown in the absence of GA_3 (Figure 6a). This is because paclobutrazol specifically inhibits gibberellin synthesis and decreases the concentration of gibberellin in rice seedlings. Paclobutrazol, however, barely inhibited elongation of rice seedlings grown in the presence of 1 μM GA_3. This is because GA_3 was supplied to the rice seedlings from the medium and paclobutrazol was not effective in lowering the concentration of gibberellin in the plants (Figure 6a). On the other hand, ABA is not an inhibitor of gibberellin synthesis, but an inhibitor of elongation growth of plants. ABA showed inhibitory activity on elongation of rice seedlings in the presence of GA_3 as well as in absence of GA_3 (Figure 6b). Accordingly, in this bioassay done in the presence of GA_3, an inhibitor of gibberellin synthesis shows no inhibitory effects, but other inhibitors with other mechanisms may show inhibitory activity. L-Canavanine showed a similar inhibitory effect to ABA on elongation of rice seedlings in the presence and absence of 1 μM of GA_3 (Figure 6c). This result suggests that the mechanism of inhibition by L-canavanine is not that of a gibberellin synthesis inhibition.

L-Canavanine is a nonprotein amino acid, and its structure is similar to L-arginine (Figure 1). Therefore, L-canavanine is likely to have some effect on the metabolism of arginine and the other related amino acids in plants. Simultaneous application of L-arginine significantly reduced the inhibitory effect of L-canavanine (Table 1). This suggests that the mode of action of L-canavanine

may be closely related to the metabolism of L-arginine. From the free amino acid analysis of rice shoots, it was clear that the L-canavanine increased L-arginine content after two days of treatment when both were applied (Table 2). This can be attributed to the application of exogenous L-arginine. However, in the treatment with only L-arginine, the accumulation of L-arginine was less. Therefore, we concluded that L-canavanine inhibited the metabolism of L-arginine in plants, resulting in its accumulation. This phenomenon is interesting because disturbance of amino acid metabolism is one of the important mechanisms of commercial herbicides, such as sulfonylurea derivatives or glyphosate. It may be that the inhibition of L-arginine metabolism is related to the growth inhibitory effect of L-canavanine. The accumulation of L-arginine seemingly would not cause the growth inhibitory effect, because the accumulation occurred more remarkably as rice seedlings recovered from the inhibitory effect of L-canavanine by application of L-arginine. After four days of treatment, this tendency was less, and amino acid contents including L-arginine changed similar to controls (Table 3). Except for L-arginine, treatment of L-canavanine only increased alanine after two days of treatment, and glutamic acid after four days of treatment. These amino acids derive from biosynthetic processes of L-arginine. Therefore, they may be affected by inhibition of L-arginine metabolism. In contrast, L-canavanine had no relation to the ammonia contents, indicating the inhibitory effect of L-canavanine was not due to ammonia accumulation. Thus, a deficiency of some of the compounds derived from L-arginine may be responsible for the growth inhibitory effect of L-canavanine.

As an analog of L-arginine, L-canavanine can be esterified with tRNA (Mitra and Mehler, 1967). Rosenthal (1991) reported that L-canavanine was incorporated into proteins of insects instead of L-arginine. Incorporation of L-canavanine into proteins alters the conformation and disrupts the function of the proteins. Production of proteins containing L-canavanine affects developmental processes and contributes significantly to expression of canavanine's potent antimetabolic properties in insects (Rosenthal, 1991). In this study, L-canavanine seemed to inhibit the metabolism of L-arginine in plants. Further study is needed in order to clarify whether or not L-canavanine is incorporated into proteins in plants.

Acknowledgments—This work was supported in part by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan. This work is part of the Ministry's project, Development of Novel Weed Control Technology by Applying Metabolic Genes in Plants.

REFERENCES

BENNER, J. P. 1996. Pesticides from nature. Part I: Crop protection agents from higher plants—an overview, pp. 217–229, *in* L. G. Copping (ed.). Crop Protection Agents from Nature. The Royal Society of Chemistry, Cambridge, UK.

EINHELLING, F. A. 1995a. Allelopathy current status and future goals, pp. 1–24, *in* Inderjit, K. M. M. Dakshini and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications, ACS Symposium Series 582. American Chemical Society, Washington, D.C.

EINHELLIG, F. A. 1995b. Mechanism of action of allelochemicals in allelopathy, pp. 96–116, *in* Inderjit, K. M. M. Dakshini and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications, ACS Symposium Series 582. American Chemical Society, Washington, D.C.

GAWARONSKA, H., YANG, Y-Y., FURUKAWA, K., KENDRICK, R. E., TAKAHASHI, N., and KAMIYA, Y. 1995. Effects of low irradiance stress on gibberellin levels in pea seedlings. *Plant Cell Physiol.* 36:1361–1367.

HEDDEN, P., and GRAEBE, J. E. 1985. Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm and *Malus pumila* embryos. *J. Plant Growth Regul.* 4:111–122.

HIRADATE, S., YADA, H., ISHII, T., NAKAJIMA, N., OHNISHI-KAMEYAMA, M., ZUNGSONTIPORN, S., and FUJII, Y. 1999. Three plant growth inhibiting saponins from *Duranta repens. Phytochemistry* 52:1223–1228.

KAMIYA, Y. 1991. Dwarfism of the higher plants, 4. Artificial dwarfism of plants. *Chem. Regul. Plants* 29:472–480 (in Japanese).

MITRA, S. K., and MEHLER, A. H. 1967. The arginyl transfer ribonucleic acid synthetase of *Escherichia coli. J. Biol. Chem.* 242:5490–5495.

MURAKAMI, Y. 1968. A new rice seedling test for gibberellin. "microdrop method" and its use for testing extracts of rice and morning glory. *Bot. Mag. Tokyo* 81:33–43.

MURAKAMI, Y. 1973. A method for detecting bound gibberellin by rice seedling test. *Chem. Regul. Plants* 8:40–44 (in Japanese).

ROSENTHAL, G. A. 1991. The biochemical basis for the deleterious effects of L-canavanine. *Phytochemistry* 30:1055–1058.

SHIBUYA, T. 1994. Effect of canavanine in *Canavalia ensiformis* DC. on lettuce germination and seedling growth. *Weed Res. Japan* 41 (Suppl.):142–143 (in Japanese).

TURNER, B. L., and HARBORNE, J. B. 1967. Distribution of canavanine in the plant kingdom. *Phytochemistry* 6:863–866.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

RESPONSE OF THE LADYBIRD PARASITOID *Dinocampus coccinellae* TO TOXIC ALKALOIDS FROM THE SEVEN-SPOT LADYBIRD, *Coccinella septempunctata*

S. AL ABASSI,[1] M. A. BIRKETT,[2] J. PETTERSSON,[1]
J. A. PICKETT,[2,*] L. J. WADHAMS,[2] and C. M. WOODCOCK[2]

[1]*Department of Entomology*
Swedish University of Agricultural Sciences
Box 7044, 750 07 Uppsala, Sweden

[2]*Department of Biological and Ecological Chemistry*
IACR-Rothamsted
Harpenden, Herts, AL5 2JQ. United Kingdom

(Received February 28, 2000; accepted August 27, 2000)

Abstract—Electrophysiological and behavioral responses of the ladybird parasitoid *Dinocampus coccinellae* to volatiles from the seven-spot ladybird, *Coccinella septempunctata*, were investigated to identify semiochemicals involved in host location. Coupled gas chromatography–electroantennography (GC-EAG) with *D. coccinellae* located a small peak of prominent activity in an extract of volatiles from adult *C. septempunctata*. The active compound was identified by coupled GC-mass spectrometry and by comparison with an authentic sample as the free-base alkaloid precoccinelline, which forms part of the toxic defense of this ladybird. Behavioral studies in an olfactometer showed that *D. coccinellae* was significantly attracted to the volatile extract and also to the alkaloid. Myrrhine, a stereoisomer of precoccinelline found in low amounts in *C. septempunctata* and in other ladybird species, was shown to be electrophysiologically active and significantly attractive. Perception of ladybird alkaloids by *D. coccinellae* is a rare example of toxicants acting as aerially transmitted cues for interactions between the third and fourth trophic levels.

Key Words—Seven-spot ladybird, *Coccinella septempunctata*, Coleoptera, Coccinellidae, electroantennogram, behavior, *Dinocampus coccinellae*, Hymenoptera, Braconidae, alkaloid, precoccinelline, myrrhine, hippodamine, volatile, semiochemical.

*To whom correspondence should be addressed. e-mail: john.pickett@bbsrc.ac.uk

0098-0331/01/0100-0033$19.50/0

INTRODUCTION

Ladybird beetles or ladybugs (Coleoptera: Coccinellidae) are important predators contributing to the natural control of pest aphid populations. However, they are themselves attacked by a range of natural enemies (Majerus, 1994). General predation on ladybirds by vertebrates such as birds is largely prevented by highly toxic alkaloids contained in a reflex bleed released when the ladybird is attacked. The effectiveness of this chemical defense is enhanced by the associated bright warning (aposematic) coloration and corelease of characteristic volatile compounds, e.g., 2-isopropyl-3-methoxypyrazine, which serves as a protective allomone and also as an aggregation pheromone (Al Abassi et al., 1998). The wasp *Dinocampus coccinellae* Schrank (Hymenoptera: Braconidae) is the main parasitoid of a number of ladybird species and can cause substantial reductions in populations of the seven-spot ladybird, *Coccinella septempunctata* L. (Majerus, 1994; Triltsch, 1996). There is a close association with the host, involving oviposition by *D. coccinellae* into the adult ladybird body and subsequent larval development, which indicates a tolerance by the wasp to the toxic alkaloids. Furthermore, *D. coccinellae* is known to utilize a combination of visual and olfactory cues in short-range host location (Orr et al., 1992). Since the associated semiochemicals may provide a means of reducing the impact of *D. coccinellae* on populations of *C. septempunctata* currently being exploited in biological control programs, the antennal and behavioral responses of *D. coccinellae* to volatiles associated with *C. septempunctata* were investigated using coupled gas chromatography–electroantennography (GC-EAG) and olfactometer studies. Our hypothesis was that *D. coccinellae* would exploit 2-isopropyl-3-methoxypyrazine as a kairomone in host location, since this compound had previously been identified as an aggregation pheromone for adult *C. septempunctata* (Al Abassi et al., 1998).

METHODS AND MATERIALS

Insects. *C. septempunctata* adults were collected during July–August from the grounds of IACR-Rothamsted, Hertfordshire, U.K., and kept in ventilated polyethylene boxes at 20°C, with the aphid *Acyrthosiphon pisum* as a food source, prior to vacuum distillation. *D. coccinellae* for electrophysiological studies were obtained from parasitized *C. septempunctata* adults; they were maintained under the same conditions as the ladybirds and kept in Petri dishes lined with moist filter paper and provided with a mixture of honey and water. Parasitoids less than 3 days old were used. For olfactometer studies, parasitized *C. septempunctata* adults were sent by air mail to Sweden and emerging *D. coccinellae* were provided with a diet of whey-cheese (Mild Mesost) and water. Parasitoids more than 1 day old were used.

Isolation of Volatiles. Adult *C. septempunctata* (ca. 1000, not sexed) were cooled with liquid nitrogen and extracted with freshly distilled chloroform (2 × 200 ml) for 24 hr (48 hr in total) at 25°C. The combined extracts were dried using anhydrous magnesium sulfate, filtered, and evaporated to ca. 5 ml. Volatiles were distilled under vacuum (0.03 torr) for 21 hr at 25°C as described previously (Pickett and Griffiths, 1980), and the distillate concentrated under a stream of nitrogen to 100 μl (10 ladybird equivalents/μl) and stored in a tightly capped microvial at −20°C.

Gas Chromatography (GC). The vacuum distilled volatiles were analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a cold on-column injector, a flame ionization detector (FID), and a 50-m × 0.32-mm-ID HP-1 bonded-phase fused-silica capillary column. The oven temperature was maintained at 40°C for 2 min and then programmed at 10°/min to 250°C. The carrier gas was hydrogen.

Electrophysiology. Electroantennogram (EAG) recordings from recently emerged *D. coccinellae* were made using Ag–AgCl glass electrodes filled with saline solution [composition as in Maddrell (1969) but without glucose]. The insect was anesthetized by chilling and an antenna was excised and suspended between the two electrodes. The tip of the terminal process of the antenna was removed to ensure a good contact. The signals were passed through a high-impedance amplifier (UN-06, Syntech) and analyzed using a customized software package (Syntech).

Stimulus Delivery. The delivery system, which employed a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). The stimulus (2-sec duration) was delivered into a purified airstream (1 liter/min) flowing continuously over the preparation. Samples (10 μl) of the standard solutions of test compounds were applied to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the paper strip was placed in the cartridge. The control stimulus was hexane (10 μl). Fresh cartridges were prepared immediately prior to each stimulation. Five individual *D. coccinellae* were used.

Coupled Gas Chromatography–Electroantennography (GC-EAG). The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the vacuum distillate volatiles was achieved on an AI 93 GC equipped with a cold on-column injector and an FID. Two columns were used, a 50-m × 0.32-mm-ID HP-1 column and a 30-m × 0.32-mm-ID HP-Wax column. For the HP-1 column, the oven temperature was maintained at 40°C for 2 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. For the HP-Wax column, the oven temperature was maintained at 40°C for 1 min and then programmed at 10°/min to 220°C. The carrier gas was hydrogen. The outputs from the EAG amplifier

and the FID were monitored simultaneously and analyzed using the software package.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). A capillary GC column (50 m × 0.32 mm ID HP-1) fitted with an on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°/min to 250°C. Tentative identification by GC-MS was confirmed by peak enhancement with authentic samples (Pickett, 1990).

Chemicals. 2-Isopropyl-3-methoxypyrazine (97%) was purchased from the Aldrich Chemical Company (Gillingham, United Kingdom). Coccinelline, i.e., the *N*-oxide of precoccinelline, the major toxic component of the reflex bleed of adult *C. septempunctata*, was isolated by acid–base extraction of a chloroform extract (extraction method as above, from ca. 900 adults), followed by liquid chromatography over neutral alumina (BDH, Brockmann Grade 1), eluting with chloroform and 99 : 1 chloroform–methanol (Pasteels et al., 1973). Precoccinelline (59 mg, 93% pure by GC) was obtained by catalytic hydrogenation [Adams' catalyst (PtO_2), methanol, 25°C] of coccinelline (80 mg) (Tursch et al., 1971). Myrrhine (10 mg, 90% pure by GC) was prepared from coccinelline (40 mg) via the Polonovski reaction (Tursch et al., 1975). Precoccinelline and myrrhine were then further purified (>99% by GC) by repeated liquid chromatography over neutral alumina (BDH, Brockmann grade 1), using 99 : 1 chloroform–methanol as the eluant. For all alkaloids, identity was confirmed by comparison of the IR, MS, ^{1}H NMR, and ^{13}C NMR spectra with literature values (Mueller et al., 1984; Lebrun et al., 1999). A sample of hippodamine (5 mg, >99% by GC) was provided by Professor D. Daloze (see Acknowledgments). Solutions for electrophysiological studies (10^{-5}–10^{-8} g/10 μl) were prepared in redistilled hexane, and for behavioral studies (10^{-6} g/μl) in redistilled diethyl ether.

Olfactometry. Behavioral assays using parthenogenetic *D. coccinellae* adults were carried out in a two-way Perspex olfactometer as described previously (Al Abassi et al., 1998). This comprised a weak airstream being directed towards the center of the olfactometer from two drawn-out arms to which volatile sources were applied at the inlets. For each experiment, one *D. coccinellae* was introduced into the center of the chamber and its position noted every 2 min for 20 min. Each experiment was replicated 8–10 times and the results analysed by paired *t* test; the number of visits into the treatment arm was compared with visits to the control arm. If the insect did not move between observations, the experiment was terminated and the data discarded. Stimuli comprised: (a) a single intact *C. septempunctata* adult; (b) the vacuum distillation extract, applied in a 0.5-μl microcap (i.e., 5 ladybird equivalents); (c) the test compounds (Figure 1) 2-isopropyl-3-methoxypyrazine (I), precoccinelline (II), myrrhine (III), and

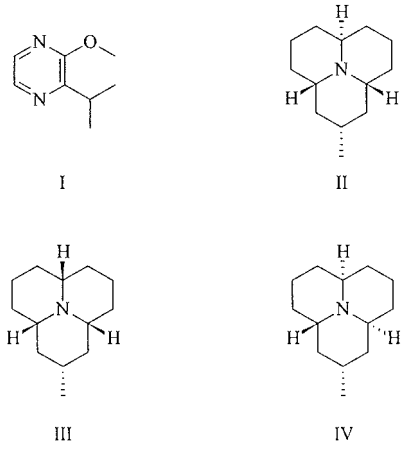


FIG. 1. Structures of compounds I–IV.

hippodamine (IV), diluted to $10^{-6}/\mu$l and applied individually in 0.5 μl microcaps, giving a stimulus approximately equivalent to the level of II found in the ladybird vacuum distillate.

RESULTS

In the olfactometer, adult *D. coccinellae* were not significantly attracted to a single intact *C. septempunctata* adult but showed strong attraction to the vacuum distillation extract (5 ladybird equivalents) (Table 1). Coupled GC-EAG of this extract with *D. coccinellae*, using nonpolar (HP-1) and polar (HP-Wax) capillary columns, showed that 2-isopropyl-3-methoxypyrazine had no electrophysiological activity (I, Figure 2). However, a small peak with a long retention time on both columns was associated with prominent electrophysiological activity (II, Figure 2). This compound was tentatively identified by coupled GC-MS and comparison with published mass spectra (e.g., Tursch et al., 1975; Mueller et al., 1984) as a 2-methylperhydro-9b-azaphenylene alkaloid (M^+ 193, Figure 3). Peak enhancement on coinjection with an authentic sample confirmed the identity of the compound as the free-base precoccinelline (II), a known defense alkaloid of *C. septempunctata* having high toxicity. A minor component of the volatile extract also showed significant electrophysiological activity (III, Figures 2 and 4), and GC-MS analysis gave a spectrum virtually identical to that of II (M^+ 193), suggesting that this component was a diastereoisomer. Coinjection with an authentic sample confirmed the identity of this peak as the ladybird alkaloid myrrhine (III). EAG dose–response curves from *D. coccinellae* confirmed that the pyrazine I was not active, even at high concentrations, while

TABLE 1. RESPONSES OF *Dinocampus coccinellae* IN THE OLFACTOMETER

Stimulus	Observations (mean N)[a]			Replicates (N)
	Treated arm	Control arm[b]	P	
C. septempunctata				
Single adult	5.6 ± 3.7	4.4 ± 3.7	NS[c]	8
Vacuum distillate[d]	5.8 ± 1.8	2.9 ± 1.5	<0.01	10
2-Isopropyl-3-methoxypyrazine[e]	4.8 ± 1.2	5.2 ± 1.2	NS	10
Precoccinelline[e]	5.2 ± 2.5	2.4 ± 1.7	<0.05	10
Myrrhine[e]	5.3 ± 1.4	1.2 ± 1.8	<0.01	10
Hippodamine[e]	4.5 ± 2.0	3.8 ± 2.6	NS	10

[a] Cumulative counts over 20 min (± SD).
[b] Control = solvent (diethyl ether).
[c] NS = not significantly different at $P = 0.05$ (paired t test).
[d] Five ladybird equivalents.
[e] Compounds tested at 10^{-6} g/μl (0.5 μl applied).

precoccinelline (II) and myrrhine (III), and the other known ladybird alkaloid hippodamine (IV), showed similarly high activities (Figure 5). In olfactometer assays, *D. coccinellae* was significantly attracted to the alkaloids II and III but not to the pyrazine I or the alkaloid IV (Table 1).

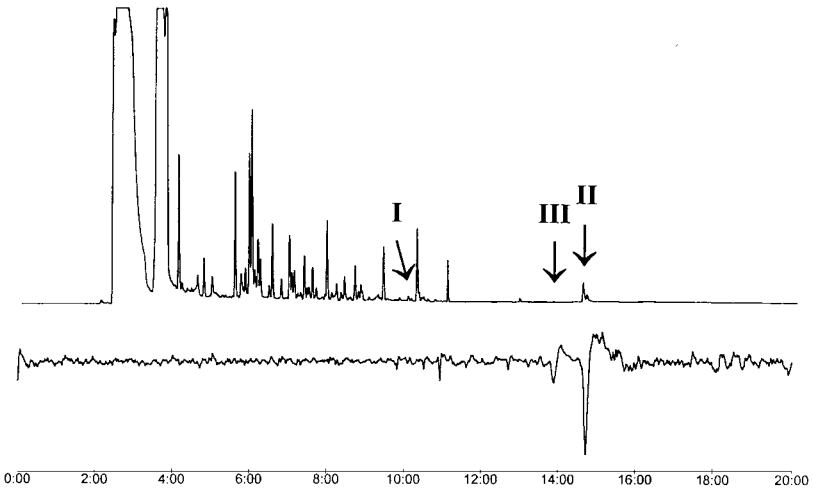


FIG. 2. Coupled GC-EAG with female *Dinocampus coccinellae*. Upper trace: GC of volatiles from a vacuum distillate of adult *Coccinella septempunctata* (HP-1 column); lower trace: EAG response. I = 2-isopropyl-3-methoxypyrazine; II = precoccinelline; III = myrrhine.

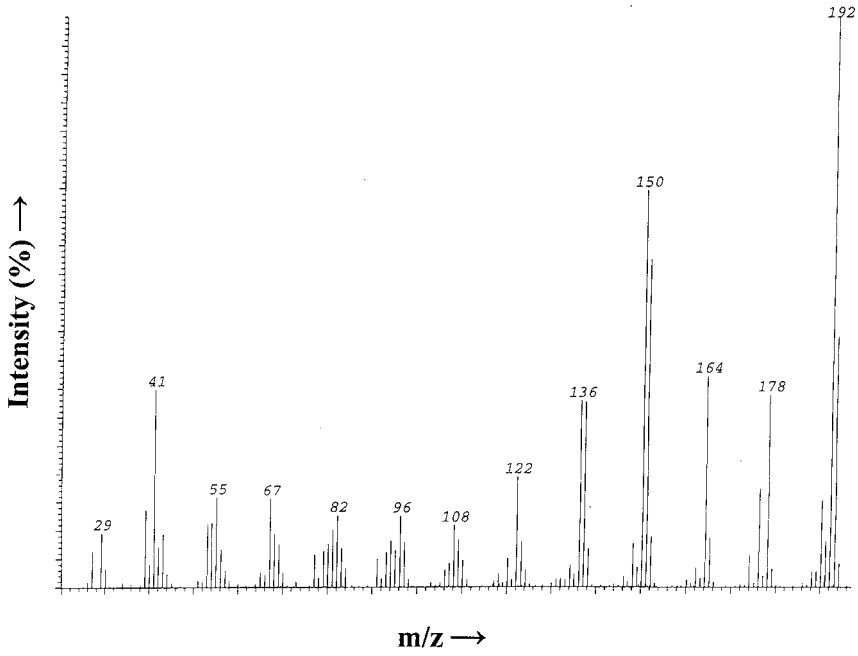


FIG. 3. Mass spectrum from peak II in Figure 2.

DISCUSSION

Although *D. coccinellae* is known to be attracted by *C. septempunctata* (Orr et al., 1992), in this study, single intact ladybirds did not elicit statistically significant attraction. However, in the search for electrophysiologically and behaviorally active material, the volatile sample obtained by extraction and vacuum distillation of *C. septempunctata* gave highly significant responses. This difference may be due to a concentration effect, but it is more likely that the initial extraction in chloroform accessed compounds normally released only on damage, i.e., in the reflex bleed produced during predator attack. *D. coccinellae* showed no EAG or behavioral responses to 2-isopropyl-3-methoxypyrazine (I), despite such activity being anticipated under the hypothesis initiating this study. Surprisingly, the electrophysiologically active components of the vacuum distillation extract were found to comprise the free-base alkaloids precoccinelline (II) and myrrhine (III). When tested at biologically appropriate levels in the olfactometer, both compounds were significantly attractive to *D. coccinellae* and thus confer the kairomonal activity of the *C. septempunctata* volatiles.

Precoccinelline (II), myrrhine (III), and hippodamine (IV) are natu-

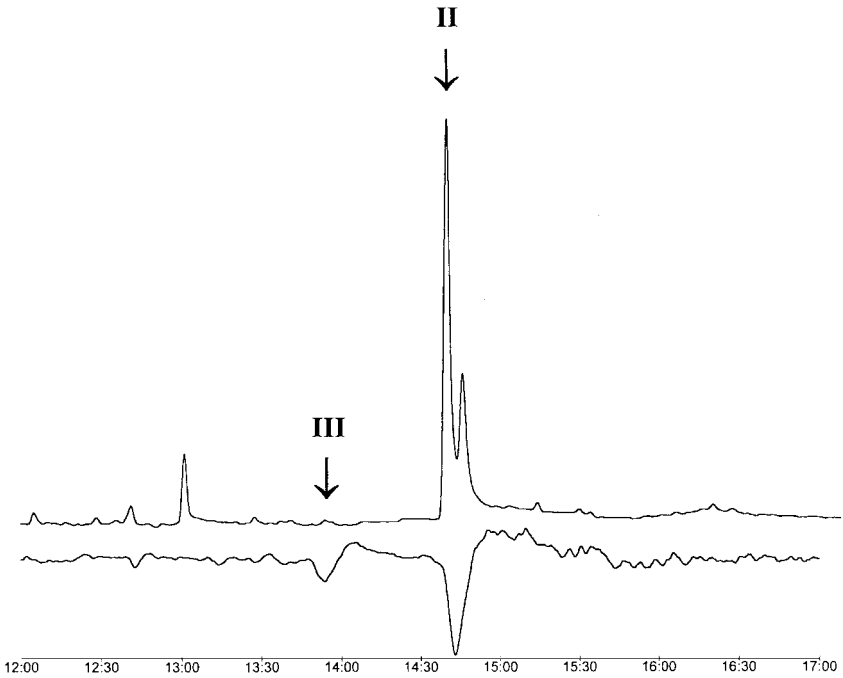


FIG. 4. Expansion of trace from Figure 2 (12–17 min), showing peak for myrrhine (III).

rally occurring 2-methylperhydro-9b-azaphenylene alkaloids that appear to be restricted mainly to ladybirds; of the six possible diastereoisomers, only these three have so far been isolated (Daloze et al., 1994/5; King and Meinwald, 1996). They are also utilized in a similar defense role by cantharid beetles and some amphibious vertebrates (Moore and Brown, 1978; Daly et al., 1993). Their limited occurrence in nature and their importance in the family Coccinellidae suggest that, for a specialist parasitoid such as *D. coccinellae*, these compounds would be reliable indicators for locating suitable hosts (Vet et al., 1991). Contrary to expectations, *D. coccinellae* was not significantly attracted to hippodamine (IV), the free-base defense alkaloid produced by another host species, the convergent ladybird, *Hippodamia convergens* Guerin. However, it was attracted to myrrhine (III), the minor EAG active component in the *C. septempunctata* volatiles; this compound is the major defense alkaloid produced by the eighteen-spot ladybird, *Myrrha octodecimguttata* L., a species that *D. coccinellae* has not been observed to parasitize (Richerson, 1970; Hodek and Honek, 1996; Majerus, 1997). Although further behavioral and ecological studies are necessary to elucidate the issues involved, the identification of toxic alkaloids as volatile

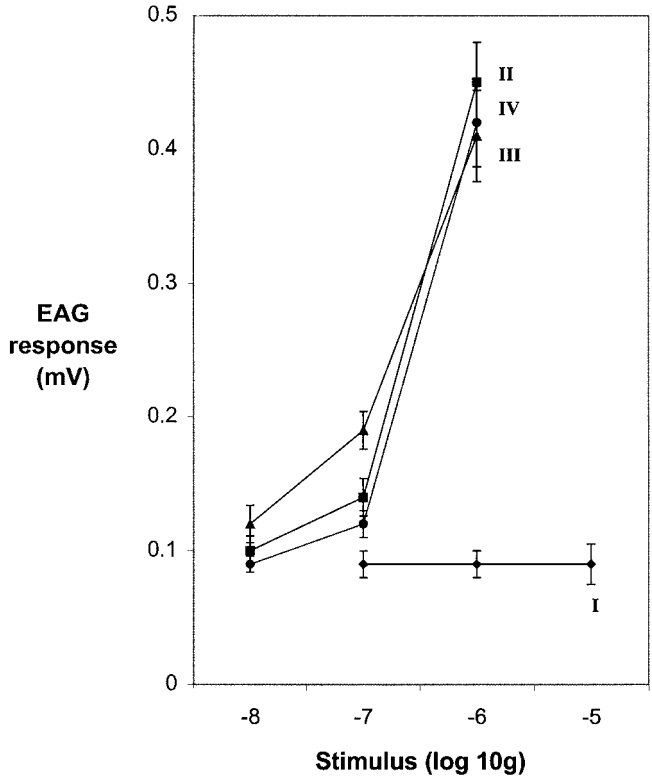


FIG. 5. EAG dose–response of *Dinocampus coccinellae* to 2-isopropyl-3-methoxypyrazine (I), precoccinelline (II), myrrhine (III) and hippodamine (IV). The points on each graph are the means of five preparations ± SE.

kairomones for *D. coccinellae* will form the basis for such work in these laboratories and, it is hoped, elsewhere.

Seminal studies on the defensive chemistry of ladybirds suggested that, of the six possible diastereoisomers, precoccinelline (II) and hippodamine (IV) are unique to *C. septempunctata* and *H. convergens*, respectively (Pasteels et al., 1973), which implies stereochemical control in their biosynthesis. However, with the onset of more advanced analytical techniques, investigations have revealed that such control is not as effective as previously thought, with mixtures of II and IV being found in both species (Daloze et al., 1994/5), although the presence of myrrhine (III) in *C. septempunctata* was not reported. Moreover, hippodamine (IV) was not found in the study of *C. septempunctata* volatiles described here.

The chemical ecology of multitrophic interactions such as those between

plants, aphids and aphid parasitoids has been widely studied with a view to exploiting parasitoids in biological control of pest aphid populations (Wadhams et al., 1999). In contrast, the chemical ecology of interactions between plants, aphids, ladybirds, and their parasitoids has received relatively little attention. The results of this study demonstrate the role of volatile semiochemicals in mediating trophic interactions between the third and fourth levels and represent a rare example of toxicants to which an animal has adapted acting directly as volatile kairomones.

For the development of aphid control programs that involve manipulation of ladybirds, methods of manipulating their natural enemies may also be needed. The significant attraction of *D. coccinellae* to such specific cues as the ladybird alkaloids suggests that there is potential for development of control strategies for this particular natural enemy, thereby enhancing the beneficial role of ladybirds through increased populations. However, as host location is likely to involve a mixture of olfactory and visual cues (Orr et al., 1992), further studies are required to assess the significance of the latter in this process.

Acknowledgments—The authors thank Professor D. Daloze, University of Brussels, Belgium, for kindly providing the sample of hippodamine. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) of the U.K. (ROPA grant MOL04604). IACR receives grant-aided support from the BBSRC. Part of this work was supported by the U.K. Ministry of Agriculture, Fisheries and Food. Investigations in Sweden were supported by the Foundation for Strategic Environmental Research (MISTRA 95065) and the Swedish International Development Co-operation Agency (SIDA/SAREC BIL-CHI-LM-04-K).

REFERENCES

AL ABASSI, S., BIRKETT, M. A., PETTERSSON, J., PICKETT, J. A., and WOODCOCK, C. M. 1998. Ladybird beetle odour identified and attraction between adults explained. *Cell. Mol. Life Sci.* 54:876–879.

DALOZE, D., BRAEKMAN, J. C., and PASTEELS, J. M. 1994/5. Ladybird defence alkaloids: structural, chemotaxonomic and biosynthetic aspects (Col.; Coccinellidae). *Chemoecology* 5/6:173–183.

DALY, J. W., GARRAFFO, H. M., and SPANDE, T. F. 1993. Amphibian alkaloids, pp. 185–288, *in* G. A. Cordell (ed.). The Alkaloids, Vol. 43. Academic Press, San Diego.

HODEK, I., and HONEK, A. 1996. Ecology of Coccinellidae. Series Entomologica 54. Kluwer Academic Publishers, Dordrecht, 464 pp.

KING, G. K., and MEINWALD, J. 1996. Review of the defensive chemistry of coccinellids. *Chem. Rev.* 96:1105–1102.

LEBRUN, B., BRAEKMAN, J. C., and DALOZE, D. 1999. Complete assignment of the ^{1}H and ^{13}C NMR spectra of the Coccinellidae-defensive alkaloids myrrhine, precoccinelline and hippodamine, their *N*-oxides and the corresponding hydrochlorides. *Mag. Res. Chem.* 37:60–64.

MADDRELL, S. H. P. 1969. Secretion by the Malphigian tubules of Rhodnius. The movement of ions and water. *J. Exp. Biol.* 51:71–97.

MAJERUS, M. E. N. 1994. Ladybirds, S. A. Corbet, S. M. Walters, R. West, D. Streeter, and D. A. Ratcliffe (eds.), pp. 125–132. Harper Collins, London.

MAJERUS, M. E. N. 1997. Parasitization of British ladybirds by *Dinocampus coccinellae* (Schrank) (Hymenoptera: Braconidae). *Br. J. Entomol. Nat. Hist.* 10:15–24.

MOORE, B. P., and BROWN, W. V. 1978. Precoccinelline and related alkaloids in the Australian soldier beetle, *Chauliognathus pulchellus* (Coleoptera: Cantharidae). *Insect Biochem.* 8:393–395.

MUELLER, R. H., THOMPSON, M. E., and DIPARDO, R. M. 1984. Stereo- and regioselective total synthesis of the hydropyrido[2,1,6-de]quinolizine ladybug alkaloids. *J. Org. Chem.* 49:2217–2231.

ORR, C. J., OBRYCKI, J. J., and FLANDERS, R. V. 1992. Host-acceptance behaviour of *Dinocampus coccinellae* (Hymenoptera: Braconidae). *Ann. Entomol. Soc. Am.* 85:722–730.

PASTEELS, J. M., DEROE, C., TURSCH, B., BRAEKMAN, J. C., DALOZE, D., and HOOTELE, C. 1973. Distribution et activites des alcaloides defensifs des coccinellidae. *J. Insect. Physiol.* 19:1771–1784.

PICKETT, J. A. 1990. GC-MS in insect pheromone identification: three extreme case histories, pp. 299–309, *in* A. R. McCaffery and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York.

PICKETT, J. A., and GRIFFITHS, D. C. 1980. Composition of aphid alarm pheromones. *J. Chem. Ecol.* 6:349–360.

RICHERSON, J. V. 1970. A world list of parasites of Coccinellidae. *J. Entomol. Soc. B.C.* 67:33–48.

TRILTSCH, H. 1996. On the parasitization of the ladybird *Coccinella septempunctata* L. (Col., Coccinellidae). *J. Appl. Entomol.* 120:375–378.

TURSCH, B., DALOZE, D., DUPONT, M., PASTEELS, J. M., and TRICOT, M. C. 1971. A defense alkaloid in a carnivorous beetle. *Experientia* 27(12):1380–1381.

TURSCH, B., DALOZE, D., BRAEKMAN, J., HOOTELE, C., and PASTEELS, J. M. 1975. Chemical ecology of arthropods X. The structure of myrrhine and the biosynthesis of coccinelline. *Tetrahedron* 31:1541–1543.

VET, L. E., WACKERS, F. L., and DICKE, M. 1991. How to hunt for hiding hosts: The reliability–detectability problem in foraging parasitoids. *Neth. J. Zool.* 41:202–213.

WADHAMS, L. J. 1990. The use of coupled gas chromatography: electrophysiological techniques in the identification of insect pheromones, pp. 289–298, *in* A. R. McCaffery and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York.

WADHAMS, L. J., ANGST, M. E., and BLIGHT, M. M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* (F.) (Coleoptera, Scolytidae) to the stereoisomers of 4-methyl-3-heptanol. *J. Chem. Ecol.* 8:477–492.

WADHAMS, L. J., BIRKETT, M. A., POWELL, W., and WOODCOCK, C. M. 1999. Aphids, predators and parasitoids, pp. 60–73, *in* Insect-Plant Interactions and Induced Plant Defence. Novartis Foundation Symposium 223. Wiley, Chichester, U.K.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

IMPORTANCE OF PREDATOR DIET CUES IN RESPONSES OF LARVAL WOOD FROGS TO FISH AND INVERTEBRATE PREDATORS

DOUGLAS P. CHIVERS[1,*] and REEHAN S. MIRZA[1]

Department of Biological Sciences
University of Maine
5751 Murray Hall, Orono, Maine 04469-5751

(Received May 30, 2000; accepted August 29, 2000)

Abstract—We examined the effects of predator diet on the antipredator responses of larval woodfrogs (*Rana sylvatica*). We found that tadpoles showed stronger responses to fish (*Perca flavescens*) that were fed tadpoles than those fed invertebrates. Similarly, we found that tadpoles responded more strongly to larval dragonflies (*Anax* spp.) fed tadpoles than to dragonflies fed invertebrates. The overall intensity of response of tadpoles to fish was much stronger than that to dragonflies. Predator diet effects are not ubiquitous in predator–prey systems. We discuss possible reasons why predator diet effects are seen in some, but not all, predator–prey systems.

Key Words—Predation, antipredator behavior, chemical cues, diet cues, amphibians, wood frogs, *Rana sylvatica*.

INTRODUCTION

A wide diversity of aquatic organisms use chemical cues to mediate predation risk (reviews in Chivers and Smith, 1998; Kats and Dill, 1998). Chemical cues are particularly important in aquatic environments when visual cues are limited, such as at night, in structurally complex habitats, or in areas of high turbidity (Smith, 1992). Aquatic media are well suited for chemical signals because a large number of compounds can dissolve in water, giving a large number of potential chemical signals to be detected (Kleerekoper, 1969; Hara, 1994).

*To whom correspondence should be addressed. e-mail: doug.chivers@usask.ca
[1] Present address: Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, Saskatchewan S7N 5E2

0098-0331/01/0100-0045$19.50/0

Recently, a number of studies have begun to examine the importance of predator diet cues in the behavioral, morphological, and life historical responses of prey animals to predators. For example, Mathis and Smith (1993a) documented that predator-naïve fathead minnows (*Pimephales promelas*) do not exhibit antipredator behavior to chemical stimuli from predatory pike (*Esox lucius*) if the pike are fed swordtails (*Xiphophorus helleri*), but do respond if the pike are fed fathead minnows. In a similar behavioral study, Chivers et al. (1996) showed that larval damselflies (*Enallagma* spp.) exhibit antipredator behavior to cues of pike fed damselflies, but not cues of pike fed a different diet. Stabell and Lwin (1997) found that crucian carp (*Carassius carassius*) exhibit an adaptive change in body morphology in response to predators fed carp but not predators fed a different fish diet (arctic char, *Salvelinus alpinus*). Moreover, Crowl and Covich (1990) showed that snails (*Physella virgata*) exposed to cues of crayfish feeding on snails were significantly larger and older at the age of first reproduction and at death than snails that were exposed to crayfish that were not feeding on snails.

A few studies have examined the importance of predator diet cues for amphibian prey. For example, Petranka and Hayes (1998) did not observe any differences in the intensity of antipredator behavior of wood frog (*Rana sylvatica*) tadpoles to larval dragonflies (*Anax junius*) that were fed tadpoles versus those that were starved for two days. In contrast, Laurila et al. (1997) showed that tadpoles of the common frog (*Rana temporaria*) responded more strongly to cues of larval dragonflies (*Aeshna juncea*) fed tadpoles than dragonflies fed insects. Similarly, Wilson and Lefcort (1993) showed that tadpoles of the red-legged frog (*Rana aurora*) exhibit antipredator behavior in response to predatory newts (*Taricha granulosa*) fed tadpoles but not to newts fed insects.

Even though diet-dependent antipredator responses are common in aquatic systems, they are not ubiquitous (Petranka and Hayes, 1998; Bryer et al., 2000). Additional studies are needed to understand the importance of diet-dependent antipredator responses in most predator–prey systems, including those involving amphibians. The aim of the current study was to examine if the importance of predator diet were dependent on the type of predator to which the prey is exposed. We examined the responses of wood frog tadpoles to cues from their natural fish predators (perch, *Perca flavescens*) and invertebrate predators (larva dragonflies, *Anax* spp.) that were fed either wood frog tadpoles or a diet of invertebrates.

METHODS AND MATERIALS

We collected six wood frog egg masses from a wetland in Orono, Maine, in April 1998 and transported them to our laboratory at the University of Maine

for testing. Each of the six clutches was maintained in its own 37-liter glass aquarium. The clutches were kept on a 14L : 10D photoperiod at approximately 20°C. After the tadpoles hatched, they were fed ad libitum with ground alfalfa pellets and fish food. Tests began when the tadpoles reached Gosner stage 25 (Gosner, 1960).

We placed one tadpole from each of the six clutches into each of 80 5-liter plastic pails filled with fresh well water. Each pail had a line drawn on the bottom. The line passed through the center of the pail. Tadpoles were kept in the pails for two days before being tested. We recorded the number of times the tadpoles in each pail crossed the centerline for a period of 5 min before and 5 min after adding one of five chemical stimuli to the center of the pail. The stimuli included: (1) cues of fish fed invertebrates, (2) cues of fish fed wood frog tadpoles, (3) cues of larval dragonflies fed invertebrates, (4) cues of larval dragonflies fed wood frog tadpoles, and (5) a control of well water. In each trial 10 ml of stimulus was used. The order of testing was randomized between treatments.

Preparation of Test Stimuli

Fish Cues. A total of four perch (mean ± SD mass = 3.39 ± 0.47 g) were used to prepare the fish cues. Two perch were kept in each of two different 37-liter aquaria and fed ad libitum with either wood frog tadpoles or invertebrates (*Daphnia* and corixids). Twenty-four hours before trials began, we placed one tadpole-fed fish and one invertebrate-fed fish into separate glass jars that held 3 liters of water. The fish conditioned the water for 24 hr prior to the trials. Fish were not fed in the stimulus collection chambers.

Dragonfly Cues. We prepared the dragonfly cues in the same manner as the fish cues except that we used 10 dragonfly larvae to condition the dragonfly-fed tadpole water and 10 dragonfly larvae to condition the dragonfly-fed invertebrate water. The mean (± SD) mass of dragonflies was 0.36 ± 0.03 g. We used 10 dragonflies not one dragonfly because we wanted to match the dragonfly mass with the mass of fish that we used to prepare the stimulus.

For each trial we calculated the change in number of line crosses before versus after the addition of each chemical stimulus. We used a paired *t*-test to compare whether tadpoles changed their behavior after addition of the control stimulus. Given that we found no effect of disturbance related to the addition of the control cue ($t = 0.27$, $d.f. = 15$, $P = 0.791$), we completed a two-way ANOVA testing for effects of predator diet, predator type, and an interaction between predator diet and predator type. We used one-tailed tests because we predicted that tadpoles would decrease activity in response to chemical cues of predators and that the intensity of the response would be stronger if the predator was fed a diet that contained the prey (Kiesecker et al., 1996; Petranka and Hayes, 1998; Chivers and Smith, 1998).

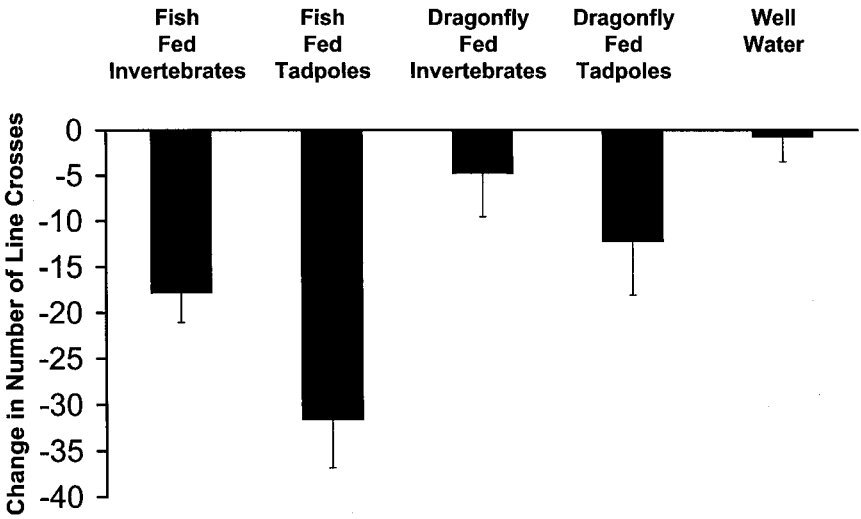


FIG. 1. Mean (+ SE) change in number of line crosses by groups of tadpoles exposed to various chemical cues.

RESULTS

Tadpoles showed a reduction in movement in response to all predator treatments (Figure 1). Our two-way ANOVA revealed that there was a significant effect of predator diet and a significant effect of predator type, but there was no interaction term (Table 1). The intensity of response of tadpoles to fish cues was more than double that of dragonflies.

DISCUSSION

The results of our study demonstrate that predator diet can dramatically influence the intensity of responses of wood frogs to cues of predators. These

TABLE 1. RESULTS OF TWO-WAY ANOVA FOR EFFECTS OF PREDATOR DIET AND PREDATOR TYPE ON ACTIVITY OF WOOD FROG TADPOLES

	F	*d.f.*	*P*
Predator diet	4.784	1	0.033
Predator type	11.081	1	0.001
Predator diet × predator type	0.420	1	0.520

results have important implications. Prey animals should exhibit antipredator responses to cues of predators that are a threat, but they should not waste valuable time and energy to respond to predators that are not a threat (Sih, 1987; Lima and Dill, 1990; Chivers and Smith, 1998).

Several studies have documented that tadpoles exhibit antipredator behavior when exposed to cues of fishes (e.g., Petranka et al., 1987; Kats et al., 1988; Stauffer and Semlitsch, 1993; Feminella and Hawkins, 1994). Some of these studies show that prior experience with fishes is not required for the prey to recognize and respond to the predator. Our results concur with these previous findings. We found that wood frog tadpoles with no prior experience with fishes exhibited antipredator behavior to chemical cues of the fish. Our study extends these findings because we found that the intensity of the response was increased when the fish were fed tadpoles as opposed to when the fish were fed invertebrate prey.

Tadpoles often respond to chemical cues of invertebrate predators even if the individual does not have experience with the predator. For example, Kiesecker et al. (1996) showed that tadpoles of the western toad (*Bufo boreas*), raised from eggs in the laboratory, exhibit responses to cues of backswimmers (*Notonecta* spp.) and giant waterbugs (*Lethocerus americanus*). Similarly, Petranka and Hayes (1998) found that predator-naïve wood frog tadpoles responded to chemical cues of dragonflies that were fed or starved for two days. In our experiment, we found that tadpoles decreased activity when exposed to either chemical cues of dragonflies fed invertebrates or dragonflies fed tadpoles.

We do not know which chemicals are responsible for eliciting the diet responses. In some predatory–prey systems, the prey animals respond to conspecific alarm pheromones in the diet of the predator (Mathis and Smith, 1993a,b). However, this is probably not the case in this system; wood frog tadpoles do not seem to possess a chemical alarm cue system (Petranka and Hayes, 1998).

We observed a significant difference in the intensity of responses to fish versus dragonfly predators. This difference could reflect that fish represent a much stronger threat than do dragonflies. Alternatively, the difference could be attributable to differences in the concentration of cues produced by the two types of predators. This is unlikely because we attempted to match the mass of dragonflies with that of fish.

A few previous studies have examined the effects of predator diet on antipredator responses of tadpoles. Petranka and Hayes (1998) failed to find differences in the way wood frog tadpoles responded to dragonflies that were fed tadpoles versus those that were starved. In contrast, Laurila et al. (1997) documented that tadpoles of the common frog responded with a more intense antipredator response to cues of dragonflies fed tadpoles than dragonflies fed insects. Wilson and Lefcort (1993) showed that tadpoles of the red-legged frogs exhibit antipredator behavior in response to predatory newts fed tadpoles but not

to newts fed insects. We found that wood frog tadpoles showed greater reductions in activity when exposed to both fish and dragonfly predators that were fed tadpoles as opposed to those fed invertebrates.

The fact that diet effects are seen in some predator–prey systems but not others is interesting and worth further investigation. Bryer et al. (2000) documented that slimy sculpins (*Cottus cognatus*) that were experienced with brook trout (*Salvelinus fontinalis*) exhibit antipredator behavior to chemical stimuli from trout regardless of the diet of the trout. They argue that brook trout are always a threat to slimy sculpins in this population and hence knowledge of the last meal eaten by the trout may provide little valuable information to the sculpin. This reasoning could explain why no diet effect was observed in this system. The same logic may apply to other predator–prey systems that have failed to find predator-diet effects. The fact that an effect of diet is observed when using one predator but not when using a different predator may be a consequence of differences in gut physiology of the predators. Some predators may be able to break down the chemicals that elicit the responses. Alternatively, if we see a diet effect when using one predator but not another, then this may indicate that the intensity of predation by one predator is less predictable than that of the other. If predators specialize on specific prey types during some times of the year, the actual threat posed by the predator is context dependent. Prey could use predator diet cues to respond more strongly to the predator only if predation on conspecifics was occurring. Additional studies are needed in order to elucidate why predator diet effects are seen in some, but not all, predator–prey systems.

Acknowledgments—We thank Pam Bryer, Joya Kobu and Matt Puttlitz for providing technical assistance. Funding was provided by the University of Maine, the University of Saskatchewan and NSERC of Canada.

REFERENCES

BRYER, P. J., MIRZA, R. S., and CHIVERS, D. P. 2000. Chemosensory assessment of predation risk by slimy sculpins, *Cottus cognatus*: responses to alarm, disturbance and predator cues. *J. Chem. Ecol.*

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator/prey interactions: A review and prospectus. *Ecoscience* 5:338–352.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, R. J. F. 1996. Damselfly larvae learn to recognize predators from chemical cues in the predator's diet. *Anim. Behav.* 52:315–320.

CROWL, T. A., and COVICH, A. P. 1990. Predator-induced life history shifts in a freshwater snail. *Science* 247:949–951.

FEMINELLA, J. W., and HAWKINS, C. P. 1994. Tailed frog tadpoles differentially alter their feeding behavior in response to non-visual cues from four predators. *J. North Am. Benthol. Soc.* 13:310–320.

GOSNER, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16:183–190.

HARA, T. J. 1994. The diversity of chemical stimulation in fish olfaction and gustation. *Rev. Fish Biol. Fish.* 4:1–35.

KATS, L. B., and DILL, L. M. 1998. The scent of death: chemosensory assessment of predation risk by prey animals. *Ecoscience* 5:361–394.

KATS, L. B., PETRANKA, J. W., and SIH, A. 1988. Anti-predator defenses and the persistence of amphibian larvae with fishes. *Ecology* 69:1865–1870.

KIESECKER, J. M., CHIVERS, D. P., and BLAUSTEIN, A. R. 1996. The use of chemical cues in predator recognition by western toad tadpoles. *Anim. Behav.* 52:1237–1245.

KLEEREKOPER, H. A. 1969. Olfaction in Fishes. Indiana University Press, Bloomington.

LAURILA, A., KUJASALO, J., and RANTA, E. 1997. Different antipredator behaviour in two anuran tadpoles: Effects of predator diet. *Behav. Ecol. Sociobiol.* 40:329–336.

LIMA, S. L., and DILL, L. M. 1990. Behavioral decisions made under the risk of predation: a review and prospectus. *Can. J. Zool.* 68:619–640.

MATHIS, A., and SMITH, R. J. F. 1993a. Fathead minnows, *Pimephales promelas*, learn to recognize northern pike, *Esox lucius*, as predators on the basis of chemical stimuli from minnows in the pike's diet. *Anim. Behav.* 46:645–656.

MATHIS, A., and SMITH, R. J. F. 1993b. Chemical labelling of northern pike (*Esox lucius*) by the alarm pheromone of fathead minnows (*Pimephales promelas*). *J. Chem. Ecol.* 19:1967–1979.

PETRANKA, J. W., and HAYES, L. 1998. Chemically mediated avoidance of a predatory odonate (*Anax junius*) by American toad (*Bufo americanus*) and wood frog (*Rana sylvatica*) tadpoles. *Behav. Ecol. Sociobiol.* 42:263–271.

PETRANKA, J. W., KATS, L. B., and SIH, A. 1987. Predator–prey interactions among fish and larval amphibians: Use of chemical cues to detect predatory fish. *Anim. Behav.* 35:420–425.

SIH, A. 1987. Predator and prey lifestyles: an evolutionary and ecological overview, pp. 203–224, *in* W. C. Kerfoot and A. Sih (eds.) Predation: Direct and Indirect Impacts on Aquatic Communities. University Press of New England, Hanover, New Hampshire.

SMITH, R. J. F. 1992. Alarm signals in fishes. *Rev. Fish Biol. Fish.* 2:3–63.

STABELL, O. B., and LWIN, M. S. 1997. Predator-induced phenotypic changes in crucian carp are caused by chemical signals from conspecifics. *Environ. Biol. Fish.* 49:145–149.

STAUFFER, H. P., and SEMLITSCH, R. D. 1993. Effects of visual, chemical and tactile cues of fish on the behavioural response of tadpoles. *Anim. Behav.* 46:355–364.

WILSON, D. J., and LEFCORT, H. 1993. The effect of predator diet on the alarm response of red-legged frog, *Rana aurora*, tadpoles. *Anim. Behav.* 46:1017–1019.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

ETHYLENE PRODUCTION AND PEROXIDASE ACTIVITY IN APHID-INFESTED BARLEY

V. H. ARGANDOÑA,[1,*] M. CHAMAN,[1] L. CARDEMIL,[1] O. MUÑOZ,[2] G. E. ZÚÑIGA,[3] and L. J. CORCUERA[4]

[1]*Departamento de Biología*
[2]*Departamento de Química*
Facultad de Ciencias, Universidad de Chile
Santiago, Chile
[3]*Departamento de Ciencias Biológicas*
Facultad de Química y Biología
Universidad de Santiago de Chile
Santiago, Chile
[4]*Departamento de Botánica*
Facultad de Ciencias Naturales y Oceanográficas
Universidad de Concepción
Concepción, Chile

(Received December 1, 1999; accepted August 30, 2000)

Abstract—The purpose of this work was to investigate whether ethylene is involved in the oxidative and defensive responses of barley to the aphids *Schizaphis graminum* (biotype C) and *Rhopalophum padi*. The effect of aphid infestation on ethylene production was measured in two barley cultivars (Frontera and Aramir) that differ in their susceptibility to aphids. Ethylene evolution was higher in plants infested for 16 hr than in plants infested for 4 hr in both cultivars. Under aphid infestation, the production of ethylene was higher in cv. Frontera than in Aramir, the more aphid susceptible cultivar. Ethylene production also increases with the degree of infestation. Maximum ethylene evolution was detected after 16 hr when plants were infested with 10 or more aphids. Comparing the two species of aphids, *Schizaphis graminum* induced more ethylene evolution than *Rhopalosiphum padi*. Infestation with *S. graminum* increased hydrogen peroxide content and total soluble peroxidase activity in cv. Frontera, with a maximum level of H_2O_2 observed after 20 min of infestation and the maximum in soluble peroxidase activity after 30 min of infestation. When noninfested barley seedlings from cv. Frontera were exposed to ethylene, an increase in hydrogen peroxide and in total peroxidase activity was detected at levels similar to those of infested plants

* To whom correspondence should be addressed. e-mail: vargando@abello.dic.uchile.cl

0098-0331/01/0100-0053$19.50/0

from cv. Frontera. When noninfested plants were treated with 40 ppm of ethylene, the maximum levels of H_2O_2 and soluble peroxidase activity were at 10 and 40 min, respectively. Ethylene also increased the activity of both cell-wall-bound peroxidases types (ionically and covalently bound), comparable with infestation. These results suggest that ethylene is involved in the oxidative responses of barley plants induced by infestation.

Key Words—*Hordeum vulgare*, *Schizaphis graminum*, *Rhopalosiphum padi*, Poaceae, greenbug, hydrogen peroxide.

INTRODUCTION

Ethylene regulates many physiological processes in plants (Mattoo and Suttle, 1991; Kende and Zeevart, 1997). Ethylene evolution is observed under many situations, including mechanical wounding, hypoxia, environmental pollution, and invasion by pathogens (Osborne, 1978; Boller, 1991; Anderson et al., 1993; Koiwa et al., 1997; Solano and Ecker, 1998). Ethylene induces defense responses in plants against pathogens (Van Loon, 1977) and pathogen resistance genes (PR genes) such as the PI genes, which encode proteinase inhibitors (Bol et al., 1990; Knogge, 1996; Koiwa et al., 1997). Ethylene induction and defense responses in *Nicotiana tobacco* can be elicited by xylanase from the fungus *Trichoderma viridis* (Anderson et al., 1993) or can be induced by jasmonic acid, which in turn is induced by abscissic acid (ABA) (Wasternack and Parthier, 1997; Koiwa et al., 1997). It has been reported that ethylene production is stimulated by a positive feedback loop with activation of ACC synthase (Chalutz et al., 1984; Kende and Zeevart, 1997). However, not all species of plants or cultivars respond to ethylene treatment (Bailey et al., 1993) and/or cell wall-digesting enzymes released by pathogens (Sharon et al., 1993; Knogge, 1996), suggesting specific requirements for ethylene and infection responses. In most plants, ethylene induces the synthesis of compounds that cross-link with cell wall polymers, thus reinforcing the cell wall structure and inhibiting the spread of the pathogen in plant tissues or reducing the injury caused by insect attack. For example, the lignification of Swede root is induced by ethylene (Rhodes and Wooltorton, 1973), possibly through the activation of PAL (Ecker and Davis, 1987) and peroxidases, which considerably reduce the pathogenicity (Ke and Saltveit, 1988; Ingham et al., 1998). In addition, tissues treated with ethylene accumulate cross-linking hydroxyproline-rich glycoproteins in cell walls (Esquerré-Tugayé et al., 1979; Fry, 1982; Cassab, 1998). Peroxidases are also involved in repair of cell walls because they catalyze the biosynthesis of lignin (Ingham et al., 1998; Cassab, 1998) and suberin (Espelie et al., 1986) and induce covalent linkage between lignin and other cell wall polymers (Iiyama, et al., 1994; Cassab et al., 1988; Cassab, 1998). In addition, ethylene induces the accumulation of some mRNAs,

such as those of PAL, coumarate CoA ligase, chalcone synthase, and transcripts of glycoproteins rich in hydroxyproline (Cassab et al., 1988).

Some barley cultivars are more resistant than others to aphid infestation because of morphological and/or biochemical characteristics (Corcuera, 1993). These characteristics can act as defensive barriers against the invader agent (Boller et al., 1983; Jackson and Taylor, 1996). Barley plants of cultivar Frontera are more tolerant to aphid infestation than cultivar Aramir (Casaretto and Corcuera, 1998).

The aphid species and its biotype may cause differences in the degree of damage caused by infestation of a particular wheat genotype (Anderson and Peters, 1994) or barley cultivar (Zúñiga et al., 1989). In barley the combination Frontera/*Schizaphis graminum* (biotype C) gives rise to less damage to the plant than the combination Aramir/*S. graminum* (biotype C). Both cultivars, Frontera and Aramir, are less damaged when they are infested by *Rhopalosiphum padi*. Therefore, the susceptibility or tolerance of a plant to aphid infestation will depend on the specific cultivar–biotype and cultivar–aphid species combinations (Dillwith et al., 1991; Anderson and Peters, 1994).

Aphids induce ethylene production in cereals (Dillwith et al., 1991; Miller et al., 1994; Anderson and Peters, 1994). Ethylene production induced by mechanical injury also influences morphological changes by increasing peroxidase activity (Depége et al., 1997; Ingham et al., 1998; Loukili et al., 1998). This phenomenon is called thigmomorphogenesis and has been observed in a wide variety of plants such as bean, tomatoes, *Bryonia dioica*, etc. (Depége et al., 1997; Loukili et al., 1998). Some aphids release cell wall-digesting enzymes, which could induce defensive responses in plants similar to those induced by pathogens (Runlin et al., 1999).

In this paper we postulate that ethylene is involved in the oxidative responses of barley induced by aphids. To test this hypothesis we examined: (1) ethylene evolution in two barley cultivars that differ in susceptibility to aphids; (2) the effect of infestation and ethylene on the synthesis of hydrogen peroxide; and (3) the effect of ethylene on the activity of soluble peroxidase and ionically and covalently bound peroxidases to the cell wall.

METHODS AND MATERIALS

Plants and Aphids. Two barley cultivars, Frontera and Aramir, which differ in their susceptibility to aphids (Casaretto and Corcuera, 1998), were used to study the effect of aphid infestation on plants. The aphids used in the assays were *Schizaphis graminum* (biotype C) and *Rhopalosiphum padi*. They were obtained from colonies maintained in the laboratory at temperatures of 28°C ± 2°C and under a photoperiod of 12L : 12D.

Ethylene Measurements. For ethylene production, infested and noninfested seedlings cultivated in little pots with vermiculite were placed in 1-liter bottles sealed with rubber caps. The accumulation of ethylene was measured by taking samples of air from the bottles with a syringe after 0, 1, 3, and 5 h. Samples of 1 ml were injected in a Shimadzu gas chromatograph equipped with a flame ionization detector (FID) using an alumina column. The temperature was 110°C for the column and 200°C for the FID detector. A standard of ethylene with a concentration of 77.2 ppb was used for calibration. Samples of air taken from a bottle without plants were used for controls. A control sample also was obtained from a bottle containing 20 aphids without plants. In each sample the fresh weight of the seedlings was used to express the ethylene production per kilogram of fresh weight. The values expressed in the results are the mean of three samples each obtained from independent bottles.

Hydrogen Peroxide and Peroxidase Activity Measurements. Nine-day-old barley plants (cv. Frontera) were incubated with 20 and 40 ppm of ethylene for a period of 6 hr. Samples were taken immediately after incubation (0 time) and at 5, 10, 15, 20, 25, 30, and 35 min after the end of the incubation period.

Extraction of Peroxidases. One gram of leaf tissue was ground in an ice-cold mortar using a pestle, and 10 ml of buffer phosphate (50 mmol/liter, pH 7) was added. The homogenate was centrifuged at 15,000*g* for 10 min. The supernatant was used as the cytosolic fraction and assayed for peroxidase activity. To extract proteins ionically bound to cell walls, the pellet from the previous extract was incubated in 1 mol/liter KCl at 4°C for 12 hr under constant agitation; this extract was centrifuged at 15,000*g* for 30 min. The proteins previously ionically bound to the cell wall were released into the supernatant. Peroxidases covalently bound to the cell walls were obtained by washing the pellet left from this last centrifugation 3 times with 1 mol/liter KCl. After the washes, the pellet was resuspended in 50 mmol/liter Na acetate, pH 5.0, with 2.5% pectinase (w/v), 0.65% cellulase (w/v), 2 mmol/liter benzamidine, and 1 mmol/liter peptain A and kept under agitation for 12 hr at 25°C. This extract was centrifuged at 15,000*g* for 30 min, and the covalently bound peroxidases were released into the supernatant.

Measurement of Peroxidase Activity. Peroxidase activity was measured against two substrates to determine whether the substrate would make a difference in the quantification of the enzyme activity. However, no differences in activity were found between the methods. In the first method (Chance and Maehly, 1955), guaiacol was used as substrate. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm for 2 min. The assay contained 50 μl of 20 mmol/liter guaiacol, 2.9 ml phosphate buffer (pH 7.0), and 10 μl of enzyme extract. The reaction was started with 20 μl of 40 mmol/liter H_2O_2. An enzyme unit (EU) was defined as the amount of enzyme needed to oxidize 1 μmol of guaiacol per minute. In the second method (Cassab et al., 1988;

modified by Riquelme and Cardemil, 1993) the substrates were 0.1% *o*-fenilendiamine (*o*-PDA) and 0.012% H_2O_2 in 0.1 mol/liter Na citrate, pH 5. After 10 min of incubation of the enzyme extract with the substrates at 25°C, the oxidized *o*-PDA was measured at 450 nm. One enzyme unit (EU) was defined as the amount of enzyme which oxidizes 1 μmol/liter of *o*-PDA/min. No reaction was observed in the absence of H_2O_2. In both assays, peroxidase activities were expressed in EU per gram of dry weight.

Determination of Hydrogen Peroxide. H_2O_2 concentration was measured according to the method of Okuda et al. (1991). The reaction mixture contained 100 μl extract, 200 μl of 12.3 mmol/liter 3-(dimethylamino) benzoic acid in 0.4 mol/liter phosphate buffer (pH 6.5), 40 μliter of 3-methyl-2-benzothiazoline hydrazoline hydrazone, and 20 μl peroxidase (0.25 EU) in a total volume of 1 ml. The reaction was started by the addition of peroxidase at 25°C. The increase in absorbance at 590 nm was monitored in a double-beam spectrophotometer (Shimadzu UV-160).

Statistical Analysis of Data. Descriptive statistics (means, standard deviations and standard errors) were used to characterize the data on a plants basis. One-way ANOVA was used to determine the significance of differences of ethylene and aphid effects on plant hydrogen peroxide and peroxidase activity.

RESULTS

Ethylene Evolution from Two Infested Barley Cultivars. Barley plants (cv. Frontera and Aramir) 6, 9, or 12 days old were infested with *S. graminum*. Ethylene was measured after 0, 1, 3, and 5 hr of infestation and was expressed in parts per million per killigram of fresh weight. Ethylene evolution was higher for infested plants than noninfested plants and was also higher for older than younger plants (Table 1). In 6-day-old plants ethylene evolution values of Aramir and Frontera cultivars were not significantly different ($P \leq 0.05$). However, the ethylene production by 9- and 12-day-old plants was higher for cv. Frontera (the more resistant cultivar). Differences in ethylene production between cultivars increased in 12-day-old plants; cv. Frontera evolved almost twice as much ethylene as cv. Aramir after 3 and 5 hr of infestation ($P \leq 0.05$).

Twelve-day-old Aramir and Frontera plants were infested with different numbers of *S. graminum* nymphs. Evolution of ethylene increased as a function of the number of aphids per plant and as a function of infestation time (Figure 1). Maximum ethylene evolution was detected in both cultivars infested with 10 or more aphids for 16 hr. At this time, the levels of ethylene were 37% higher in cv. Frontera than in cv. Aramir.

Plants of cultivars Frontera and Aramir were infested each with 20 aphids of *S. graminum* or *R. padi*. Controls were noninfested plants. Ethylene level

TABLE 1. EFFECT OF APHID INFESTATION ON ETHYLENE EVOLUTION IN *Hordeum vulgare*[a]

Cultivar	Age (days)	Assay	Amount (ppm)			
			0 hr	1 hr	3 hr	5 hr
Frontera	6	Infested	ND	0.2 ± 0.1	0.6 ± 0.1	1.2 ± 0.1
		Noninfested	ND	0.1 ± 0.1	0.2 ± 0.2	0.4 ± 0.1
	9	Infested	ND	0.5 ± 0.1	4.0 ± 0.5	6.0 ± 0.8
		Noninfested	ND	0.2 ± 0.1	0.5 ± 0.1	0.5 ± 0.5
	12	Infested	ND	7.5 ± 1.2	15.0 ± 2.0	25.0 ± 3.0
		Noninfested	ND	2.5 ± 0.2	6.0 ± 0.7	10.0 ± 1.2
Aramir	6	Infested	ND	0.5 ± 0.1	0.8 ± 0.1	1.2 ± 1.0
		Noninfested	ND	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.4
	9	Infested	ND	0.5 ± 0.1	1.2 ± 0.2	2.2 ± 0.1
		Noninfested	ND	0.3 ± 0.1	0.8 ± 0.1	1.5 ± 0.2
	12	Infested	ND	4.0 ± 0.6	8.0 ± 1.0	12.0 ± 1.0
		Noninfested	ND	2.0 ± 1.0	5.0 ± 1.0	9.0 ± 1.0

[a] Plants were infested with 10 aphids 1 hr before initiated the ethylene measurements. Data on ethylene accumulation represent the mean of four samples (±SD). ND = non detectable.

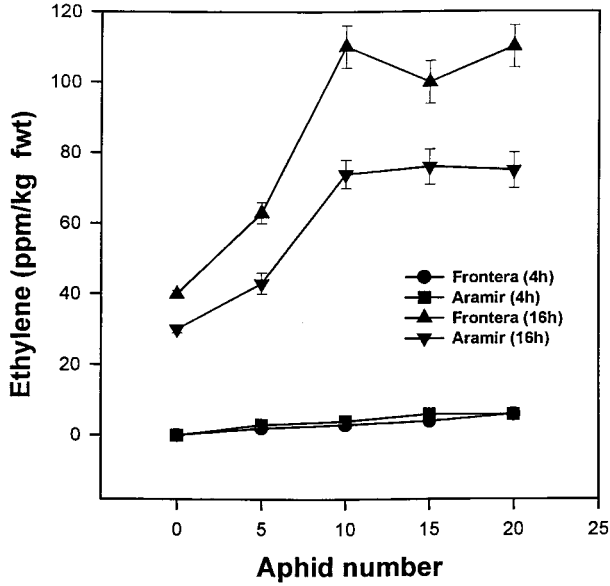


FIG. 1. Effect of aphid infestation on ethylene production in barley. Plants were infested with different numbers of *S. graminum*, one group during 4 hr and another during 16 hr. Each point represents the mean of three measurements (±SE); fwt = fresh weight).

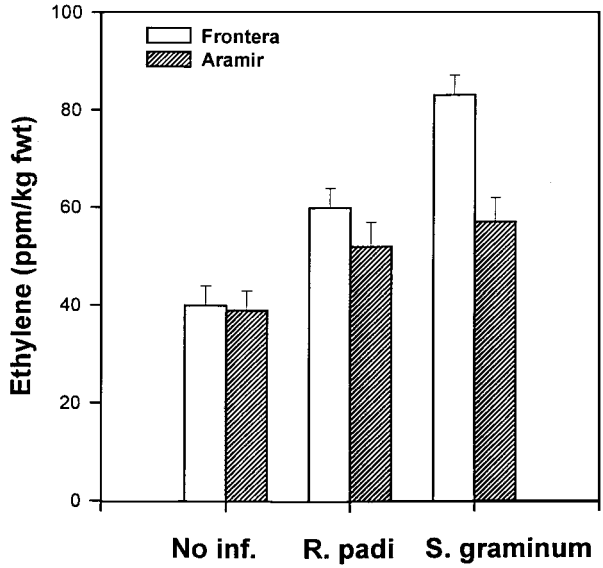


FIG. 2. Effect of two aphid species on ethylene production in barley plants. Plants of Frontera and Aramir cultivars 12 days old were infested with 20 aphids of *S. graminum* or of *R. padi* for 12 hr. Each point represents the mean of three measurements (±SE).

was measured after 12 hr of infestation (Figure 2). Production of ethylene in cv. Frontera plants infested with *S. graminum* was 53% higher with respect to controls and 29% higher than those infested with *R. padi*. In all assays with infested plants, cv. Frontera produced more ethylene than cv. Aramir ($P \leq 0.05$).

H_2O_2 Accumulation and Soluble Peroxidase Activity. H_2O_2 accumulation and soluble peroxidase activity were measured in 10-day-old plants of cv. Frontera infested with 20 aphids of *S. graminum* per plant. The aphids induced an increase in H_2O_2 accumulation with a transient maximum after 20 min of infestation (Figure 3) and was 41% higher than in noninfested plants. *S. graminum* also increased by 64% the soluble peroxidase activity with respect to control plants after 30 min of infestation (Figure 4).

In response to ethylene pulses of 20 and 40 ppm during 6 hr, H_2O_2 and soluble peroxidase activity in noninfested barley plants, cv. Frontera, increased to levels similar to those of plants infested with aphids (Figures 5 and 6). Levels of H_2O_2 were 53% higher in the plants treated with 40 ppm ethylene than in control plants reaching a maximum after 10 min of treatment. With 20 ppm, H_2O_2 accumulation was 43% higher in treated plants than in the controls. A maximum accumulation occurred after 15 minutes of treatment (Figure 5).

The maximum activity of soluble peroxidases occurred at 40 min with 40

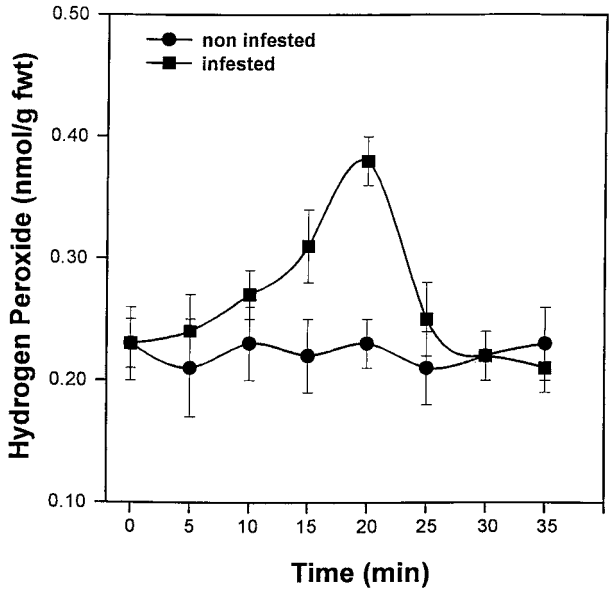


FIG. 3. Effect of aphid infestation on the accumulation of hydrogen peroxide in barley. Plants of Frontera cultivar 10 days old were infested with 20 aphids of *S. graminum* per plant for different periods of time. Each point represents the mean of three measurements (±SE).

ppm of ethylene treatment. However, the maximum activity occurred after 60 min when the treatment was 20 ppm of ethylene. With 40 ppm, soluble peroxidase activity was 53% higher in treated plants than in control ones. With 20 ppm the soluble peroxidase activity was 45% higher than in control plants ($P \leq 0.05$) (Figure 6).

Activity of both cell wall-bound peroxidases, those covalently and ionically bound, also increased under exposure of the plants to 40 ppm of ethylene. In the case of the ionically bound peroxidases, the level of activity was 40% higher than in the control plants after 20 hr of incubation. Covalently bound peroxidases were 24% higher than the control plants after 50 hr of incubation with ethylene (Figure 7).

DISCUSSION

Ethylene has been implicated in the response by plants to wounding (Osborne, 1978) and to pathogen attack (Ecker and Davis, 1987; Kende and Zeevart, 1997). Its biosynthesis is also promoted by many environmental stresses

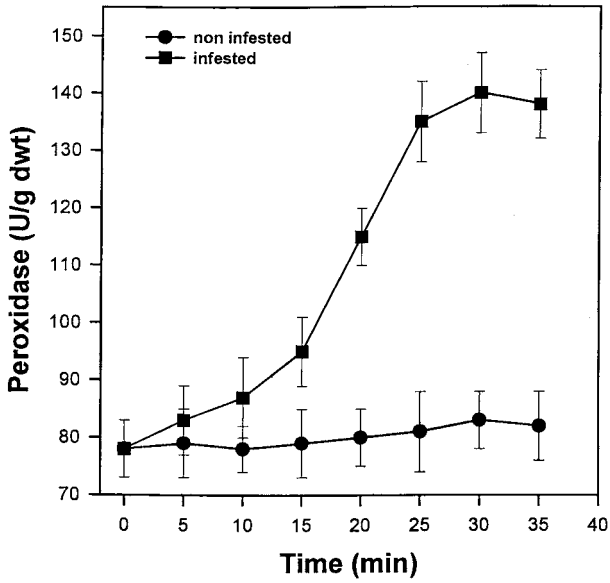


FIG. 4. Accumulation of soluble peroxidases in barley plants cv. Frontera infested with 20 aphids of *S. graminum* per plant for different periods of time. Each point represent the mean of three measurements (±SE).

(Kende and Zeevart, 1997). In pathogenesis, ethylene evolution is correlated with the induction of several PR genes (Dong, 1998). It is still unknown whether aphid infestation induces PR genes in barley as defensive mechanism. Results obtained in this work show that aphids induced more ethylene evolution in Frontera, a more resistant cultivar than Aramir, when infested by *S. graminum* (biotype C). Previous reports indicated that aphid infestation induced higher evolution of ethylene in susceptible than in resistant cultivars of alfalfa, wheat, and barley (Dillwith et al., 1991; Anderson and Peters, 1994; Miller et al., 1994). However, the physiological and molecular responses to infestation will depend on several factors such as plant cultivar, plant age, degree of infestation, aphid species and biotype, and infestation time. Anderson and Peters (1994) found that different combinations of wheat genotype–greenbug biotypes produced different amounts of ethylene.

In this work the infestation also induced an increase in the levels of hydrogen peroxide and peroxidase activity. Moreover, when the plants were subjected to ethylene, this hormone induced an increase in the hydrogen peroxide and in the peroxidase activity. Both hydrogen peroxide and peroxidases are related to the oxidative metabolism triggered by pathogenicity, stress conditions, and infes-

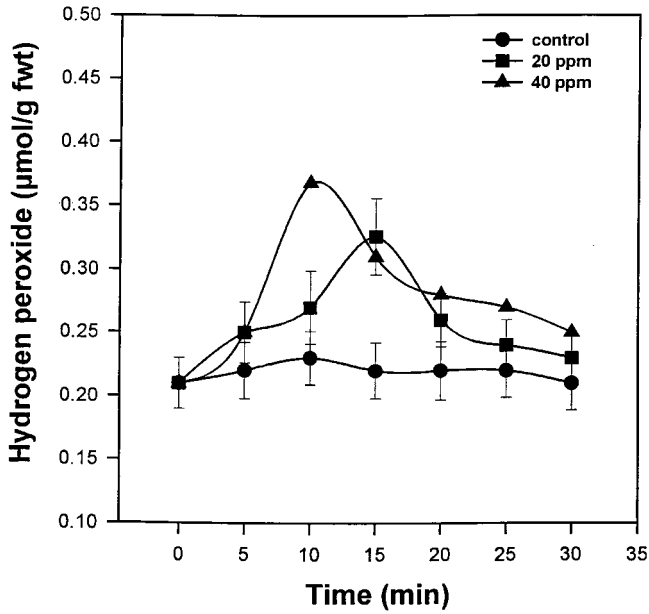


FIG. 5. Accumulation of hydrogen peroxide in cv. Frontera barley plants treated with 20 and 40 ppm of ethylene for 6 hr. After treatment, the hydrogen peroxide was measured at different periods of time. Each point represent the mean of three measurements (±SE).

tation, suggesting that in these cultivars ethylene may induce physiological and molecular responses related to aphid tolerance similar to those found in plant responses to pathogens (Wasternack and Parthier, 1997; Koiwa et al., 1997).

The time and intensity of plant defensive responses to a pathogen are crucial to determine if the infection can be prevented (Koiwa et al., 1997; Dong, 1998). In 12-day-old barley plants of both cultivars, ethylene production became significantly higher after 1 hr of infestation. This ethylene evolution was faster than that detected by Anderson and Peters (1994), suggesting that this initial ethylene evolution may be involved in the oxidative burst of barley plants. This burst may mediate defensive mechanisms against infestation, as has been found for pathogen-infested plants (Koiwa et al., 1997; Dong, 1998). Later production of ethylene by the infested plant may be related to the senescence process (Nakatsuka et al., 1998). Anderson and Peters (1995) found that AVG (2-aminoethoxyvinylglycine), an inhibitor of ethylene biosynthesis, did not reduce chlorophyll degradation or the lesions induced by infestation, indicating that ethylene may not be involved in the development of these symptoms.

The time of production of ethylene was similar in both cultivars of barley,

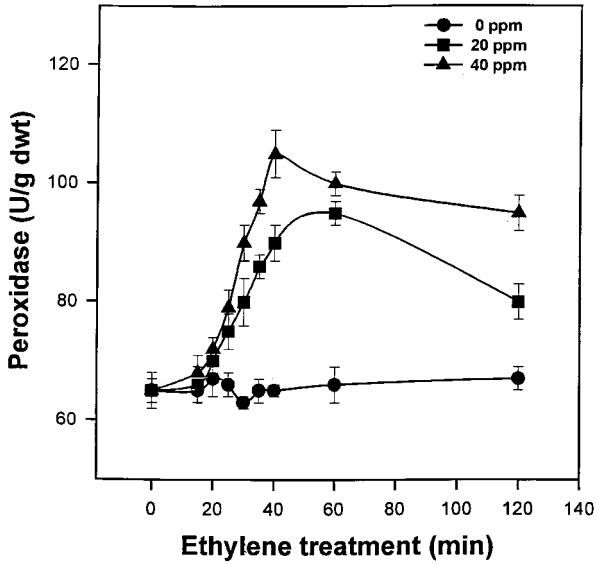


FIG. 6. Effect of ethylene on the activity of soluble peroxidases in barley plants cv. Frontera treated with 20 and 40 ppm of ethylene for 6 hr. After treatment, the peroxidase activity was determined at different periods of time. Each point represent the mean of three measurements (±SE).

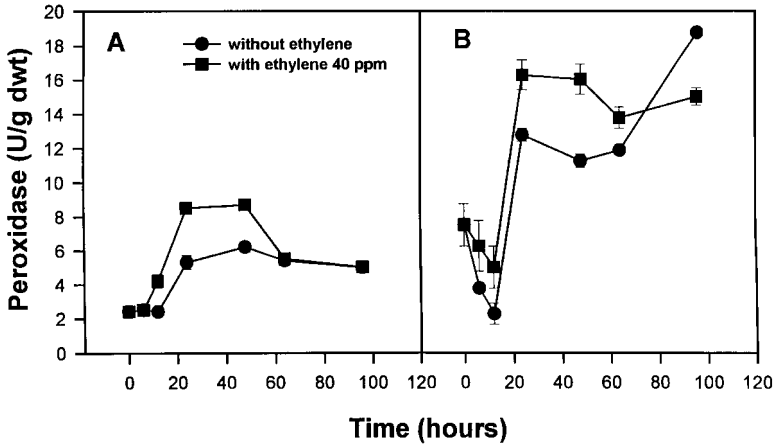


FIG. 7. Effect of ethylene on the activity of peroxidases ionically (A) and covalently bound (B) to the cell wall. The plants of cv. Frontera were treated with 40 ppm of ethylene for 6 hr. After treatment, the activity was measured at different periods of time. Each point represent the mean of three measurements (±SE).

although the levels were higher in cv. Frontera than in cv. Aramir. It seems, therefore, that in the two cultivars it was the magnitude of ethylene evolution, rather than the speed of the response that probably made the difference in their response to infestation.

In both cultivars during the first 4 hr of infestation, ethylene evolution increased slightly with the number of aphids. However, after 16 hr of infestation the level of ethylene evolution was clearly dependent upon the number of aphids per plant reaching a maximum when plants were infested with 10 aphids. This seems to indicate that in plants of this age the threshold of infestation to induce maximum ethylene production requires this number of aphids per plant.

S. graminum (biotype C) induced a higher level of ethylene evolution, indicating that this species was more aggressive in this barley cultivar than *R. padi* and produced more plant damage. This result is in agreement with previously published results, where the amount of chlorophyll loss was higher in barley plants infested with *S. graminum* (biotype C) than with *R. padi* (Casaretto and Corcuera, 1998).

In addition, aphids also induced accumulation of H_2O_2 and increased peroxidase activity. As occurs in the response to plants pathogens, the burst of H_2O_2 production by barley plants preceded the accumulation of soluble and cell wall peroxidases (Lamb and Dixon, 1997; Somssich and Hahlbrock, 1998). In infested barley plants, the maximum level of H_2O_2 was found about 10 min before the maximum accumulation of soluble peroxidase activity. However, in noninfested plants exposed to 40 ppm of ethylene the maximum level of H_2O_2 was reached after 10 min with a maximum induction of the peroxidase activity after 40 min. This suggests that the accumulation of soluble peroxidase activity is a consequence of the H_2O_2 burst and appears to indicate that the accumulation of H_2O_2 could be the beginning of a cascade of events that triggers physiological and molecular plant responses to prevent or minimize insect attack. This H_2O_2 may be produced in the cell wall or in the plasma membrane via a NADPH oxidase associated with this membrane, as has been reported in the case of pathogen attacks on plants (Somssich and Hahlbrock, 1998).

Plant cells produce H_2O_2 in response to both biotic and abiotic stress (Wojtaszek, 1997). H_2O_2 accumulation may be also implicated in the induction of defense genes (Wu et al., 1995). Therefore, the H_2O_2 production is a general response of plants to stress conditions and not specific to plants infested by aphids. While the exact biochemical reaction involved in the production of the H_2O_2 is still being studied, one may speculate that plants may use this compound not only as an inducer of the defensive cascade of metabolic events but rather as a defense mechanism itself (Lamb and Dixon, 1997). However, H_2O_2 can damage plant cells. The prevention of this damage would depend on the efficiency of the antioxidant systems. In plants, H_2O_2 is destroyed specifically by ascorbate peroxidase, and catalases (Asada, 1992; Mehlhorn et al., 1996; Foyer

et al., 1997). It is possible then that the Frontera cultivar has more antioxidant systems, thereby reducing the damage produced by the oxidants.

Infestation induced levels of H_2O_2 and peroxidases similar to those induced in plants by 40 ppm of ethylene, although, the production of H_2O_2 in plants exposed to ethylene was 10 min earlier than in the aphid infested ones. The increase in ethylene evolution induced by aphids is mediated by unknown events. One of them may be an increase in the level of abscissic acid (ABA), since ethylene and jasmonic acid synthesis seems to be mediated by ABA accumulation during pathogenicity (Peña-Cortés et al., 1995; Koiwa et al., 1997). ABA levels have been measured in extracts of infested barley leaves. No changes were found during the first 48 hr of infestation although, a substantial increase was found after 65 hr of infestation (Cabrera et al., 1994). Further research is needed to elucidate the possible role of ABA in ethylene induction in aphid infested barley leaves.

The ionically and covalently cell wall-bound peroxidase activities of barley seedlings increased more under ethylene treatment than in the control seedlings. The activity increase in the control samples was probably due to the experimental stressing conditions such that endogenous ethylene was produced by the seedlings placed in the sealed flasks. However, a small increase in cell wall-bound peroxidases could induce a rapid cell wall reinforcement by oxidative cross-linking and insolubilization of structural cell wall proteins. The extensin cell wall proteins are the best studied and identified. Being a family of proteins, extensins are also known as hydroxyproline-rich glycoproteins (HRGP) (Bradley et al., 1992; Cassab, 1998). Recently, in cotton cell cultures, a covalent linkage has been found between extensin and a pectin fraction, ramnogalacturan I (RG I), (Qi et al., 1995). The role of extensins still remains to be determined, but they probably facilitate cell wall lignification, acting as a primer for lignin deposition and lignin polymerization (Cassab, 1998; Loukili et al., 1998). Whether lignification of the cell walls in vascular bundles also occurs during infestation is unknown, but certainly it would make penetration of the aphid stylet more difficult.

It is interesting to note that the peroxide accumulation seems to occur before ethylene accumulation in infested plants. Thus, aphid attack may first induce H_2O_2 accumulation and H_2O_2 itself may be in part responsible for the induction of peroxidases and ethylene biosynthesis. However, it is not possible at this stage to know exactly the sequence of these events in the plant tissues. Ethylene, in turn, may induce expression of other genes involved in the reinforcement of plant cell walls (Esquerré-Tugayé et al., 1985).

Acknowledgments—The assistance of Angélica Vega is appreciated. This research was supported by FONDECYT (project 1950302) and Red Latinoamericana de Botánica (RLB).

REFERENCES

ANDERSON, J. A., and PETERS, D. C. 1994. Ethylene production from wheat seedlings infested with biotypes of *Schizaphis graminum* (Homoptera: Aphididae). *Physiol. Chem. Ecol.* 23:992–998.

ANDERSON, J. A., and PETERS, D. C. 1995. Inhibitors of ethylene biosynthesis and action do not prevent injury to wheat seedlings infested with *Schizaphis graminum* (Homoptera: Aphididae). *Environ. Entomol.* 24:1644–1649.

ANDERSON, J. D., BAILEY, B. A., TAYLOR, B., SHARON, A., AVNI, A., MATOO, A. K., and FUCHS, Y. 1993. Fungal xylanase elicits ethylene biosynthesis and other defense responses in tobacco, pp. 197–204, *in* J. C. Pech, A. Latché, C. Balagué (eds.). Cellular and Molecular Aspects of the Plant Hormone Ethylene, Vol. 16. Kluwer Academic Publishers, Dordrecht, The Netherlands.

ASADA, K. 1992. Ascorbate peroxidase–a hydrogen peroxide—scavenging enzyme in plants. *Physiol. Plant.* 85:235–241.

BAILEY, B. A., KORCAK, R. F., and ANDERSON, J. D. 1993. Sensitivity to an ethylene biosynthesis-inducing endoxylanase in *Nicotiana tabacum* L. cv Xanthi is controlled by a single dominant gene. *Plant Physiol.* 101:1081–1186.

BOLLER, T. 1991. Ethylene in pathogenesis and disease resistance, pp. 293–313, *in* A. K. Mattoo and J. C. Suttle (eds.). The Plant Hormone Ethylene. CRC Press, Boca Raton, Florida.

BOLLER, T., GEHRI, A., MAUCH, F., and VÖGELI, V. 1983. Chitinase in bean leaves: Induction by ethylene, purification, properties and possible function. *Planta* 15:722–731.

BRADLEY, D. J., KJELLBOOM, P., and LAMB, C. J. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall proteins: A novel rapid defense response. *Cell* 70:21–30.

CABRERA, H. M., ARGANDOÑA, V. H., and CORCUERA, L. J. 1994. Metabolic changes in barley seedlings at different aphid infestation levels. *Phytochemistry* 35:317–319.

CASARETTO, J., and CORCUERA, L. J. 1998. Proteinase inhibitor accumulation in aphid-infested barley leaves. *Phytochemistry* 49:2279–2286.

CASSAB, G. I. 1998. Plant Cell Wall Proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:281–309.

CASSAB, G. I., LIN, J. J., LIN, L. S., and VARNER, J. E. 1988. Ethylene effect on extensin and peroxidase distribution in the subapical region of pea epicotyls. *Plant Physiol.* 88:321–353.

CHALUTZ, E., MATTOO, A. K. SALOMOS, T., and ANDERSON, J. D. 1984. Enhancement by ethylene of cellulysin-induced ethylene production by tobacco leaf disc. *Plant Physiol.* 74:99–103.

CHANCE, B., and MAEHLY, A. C. 1955. Assay of catalases and peroxidases. *Methods Enzymol.* 2:764–775.

CORCUERA, L. J. 1993. Biochemical basis for the resistance of barley to aphids. *Phytochemistry* 33:741–747.

DEPÉGE, N., THONAT, C., COUTAND, C., JULIEN, J. L., and BOYER, N. 1997. Morphological responses and molecular modifications in tomato plants after mechanical stimulation. *Plant Cell Physiol.* 38:1127–1134.

DILLWITH, J. W., BERBERET, R. C., BERGMAN, D. K., NEESE, P. A., EDWARDS, R. M., and MCNEW, R. W. 1991. Plant biochemistry and aphid populations: studies on the spotted alfalfa aphid, *Therioaphis maculata. Arch. Insect Biochem.* 17:235–251.

DONG, X. 1998. SA, JA, ethylene, and disease resistance in plants. *Curr. Opin. Plant Biol.* 1:316–323.

ECKER, J. R., and DAVIS, R. W. 1987. Plant defense genes are regulated by ethylene. *Proc. Natl. Acad. Sci. U.S.A.* 84:5202–5206.

ESPELIE, K. E., FRANCESCHI, V. R., and KOLATTUKUDY, P. E. 1986. Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. *Plant Physiol.* 81:487–492.

ESQUERRÉ-TUGAYÉ, M. T., LAFITTE, C., MAZAU, D., TOPPAN, A., and TOUZA, A. 1979. Cell surfaces in plant-microorganism interactions II. Evidence for the accumulation of hydroxyproline-

rich glycoproteins in the cell walls of diseased plants as a defense mechanism. *Plant Physiol.* 64:320–326.

ESQUERRÉ-TUGAYÉ, M. T., MAZAU, D., PÉLLICIER, B., ROBY, D., RUMEAU, D., and TOPPAN, A. 1985. Induction by elicitors and ethylene of proteins associated to the defense of plants, pp. 459–473, *in* J. L. Key and T. Kosuge (eds.). Cellular Biology of Plant Stress. Liss, New York.

FOYER, C. H., LÓPEZ-DELGADO, H., DAT, J. F., and SCOTT, I. M. 1997. Hydrogen peroxide and glutathione-associated mechanism of acclimatory stress tolerance signaling. *Physiol. Plant.* 100:241–254.

FRY, S. C. 1982. Isodityrosine, a new cross-linking amino acid from plant cell walls glycoprotein. *Biochem. J.* 204:449–455.

IIYAMA, K., LAM, T. B.-T., and STONE, B. A. 1994. Covalent cross-links in the cell wall. *Plant Physiol.* 104:315–320.

INGHAM, L. M., PARKER, M. L., and WALDROM, K. W. 1998. Peroxidases: Changes in soluble and bound forms during maturation and ripening of apples. *Physiol. Plant.* 102:93–100.

JACKSON, A. O., and TAYLOR, C. B. 1996. Plant-microbe interactions: life and death at the interface. *Plant Cell* 8:1651–1668.

KE, D., and SALTVEIT, M. E. 1988. Plant hormone interaction and metabolism in the regulation of russet spotting on iceberg lettuce. *Plant Physiol.* 88:1136–1140.

KENDE, H., and ZEEVART, J. A. D. 1997. The five "classical" plant hormones. *Plant Cell* 9:1197–1210.

KNOGGE, W. 1996. Fungal infection of plants. *Plant Cell* 8:1711–1772.

KOIWA, H., BRESSAN, R. A., and HASEGAWA, P. M. 1997. Regulation of protease inhibitors and plant defense. *Trends Plant Sci.* 2:379–384.

LAMB, C., and DIXON, R. A. 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:251–275.

LOUKILI, A., LIMAM, F., THONAT, C., CAHIBI, W., AYADI, A., BOYER, N., and OUELHAZI, L. 1998. Involvement of phenylalanine ammonia-lyase and cationic peroxidases in tomato thigmomorphogenesis. *Plant Perox. Newslett.* 11:3–12.

MATTOO, A. K., and SUTTLE, J. C. 1991. The Plant Hormone Ethylene. CRC Press, Boca Raton, Florida.

MEHLHORN, H., LELANDAIS, M., KORTH, H. G., and FOYER, C. H. 1996. Ascorbate is the natural substrate for plant peroxidases. *FEBS Lett.* 378:203–206.

MILLER, H. L., NEESE, P. A., KETRING, D. L., and DILLWITH, J. W. 1994. Involvement of ethylene in aphid infestation of barley. *J. Plant Growth Regul.* 13:167–171.

NAKATSUKA, A., MURACHI, S. OKUNISHI, H., SHIOMI, S., NAKANO, R., KUBO, Y., and INABA, A. 1998. Differential expression and internal feedback regulation of 1-aminocyclopropane-11-carboxylate synthase, 1-aminecyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruits during development and ripening. *Plant Physiol.* 118:1295–1305.

OKUDA, T., MATSUDA, Y., YAMANAKA, A., and SAGISAKA, S. 1991. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. *Plant Physiol.* 97:1265–1267.

OSBORNE, D. S. 1978. Ethylene, pp. 265–294, *in* D. S. Letham, P. B. Goodwin and T. J. V. Higgins (eds.). Phytohormones and Related Compounds: A Comprehensive Treatise. Elsevier/North-Holland.

PEÑA-CORTÉS, H., FISAHN, J., and WILLMITZER, L. 1995. Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. *Proc. Natl. Acad. Sci. U.S.A.* 92:4106–4113.

QI, X., BEHERENS, B. X., WESR, P. R., and MORT, A. J. 1995. Solubilization and partial characterization of extensin fragments from cell walls of cotton suspension cultures. *Plant Physiol.* 108:1691–1701.

RHODES, M. J. C., and WOOLTORTON, L. S. C. 1973. Stimulation of phenolic acid and lignin biosynthesis in swede root tissue by ethylene. *Phytochemistry* 12:107–118.

RIQUELME, A., and CARDEMIL, L. 1993. Peroxidases in the cell walls of seeds and seedlings of *Araucaria araucana. Phytochemistry* 32:15–20.

RUNLIN, Z., REESE, J. C., BLACK, W. C., IV, and BRAMEL-COX, P. 1999. Chlorophyll loss in greenbug-susceptible sorghum due to pectinase and pectin fragments. *J. Kans. Entomol. Soc.* 71:51–60.

SHARON, A., FUCHS, Y., and ANDERSON, J. D. 1993. The elicitation of ethylene biosynthesis by a *Trichoderma xylanase* is not related to the cell wall degradation activity of the enzyme. *Plant Physiol.* 102:1325–1329.

SOLANO, R., and ECKER, J. R. 1998. Ethylene gas: Perception, signaling and response. *Curr. Opin. Plant Biol.* 1:393–398.

SOMSSICH, I. E., and HAHLBROCK, K. 1998. Pathogen defense in plants—a paradigm of biological complexity. *Trend Plant Sci.* 2:86–90.

VAN LOON, L. C. 1977. Induction by 2-chloroethylphosphoric acid of viral like lesions, associated proteins and systemic resistance in tobacco. *Virology* 80:417–429.

WASTERNACK, C., and PARTHIER, B. 1997. Jasmonate-signalled plant gene expression. *Trend Plant Sci.* 2:302–307.

WOJTASZEK, P. 1997. Oxidative burst: An early plant response to pathogen infection. *Biochem. J.* 322:681–692.

WU, G., SHORRT, B. J., LAWRENCE, E. B., LEVINE, E. B., FITZSIMMONS, K. C., and SHAH, D. M. 1995. Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. *Plant Cell* 7:1357–1368.

ZÚÑIGA, G. E., ARGANDOÑA, V. H., and CORCUERA, L. J. 1989. Presencia del Biotipo C del áfido *Schizaphis graminum* en la región Metropolitana y su conducta alimentaria. *Agric. Tec.* 49:45–49.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

SECRETIONS OF STINGLESS BEES: THE DUFOUR GLAND OF *Nannotrigona testaceicornis*

LEOPOLDO CRUZ-LÓPEZ,[1] EDA FLAVIA L. R. A. PATRICIO,[2] and E. DAVID MORGAN[3,*]

[1]*El Colegio de la Frontera Sur*
Carretera Antiguo Aeropuerto Km. 2.5
Tapachula, Chiapas, Mexico,
CP 30700, AP 36

[2]*Laboratório de Abelhas, Instituto de Biociências*
Universidade de São Paulo
05508-900 São Paulo SP. Brasil

[3]*Chemical Ecology Group, Lennard-Jones Laboratory*
School of Chemistry and Physics, Keele University
Keele, Staffordshire ST5 5BG, England

(Received May 19, 2000; accepted August 30, 2000)

Abstract—The Dufour gland of *Nannotrigona testaceicornis* is a large, wide, pear-shaped sac. The gland secretion consists chiefly of the diterpene ester all-*trans*-geranylgeranyl acetate (64% of the total), together with a complex mixture of small amounts of cyclic ketals; mono-, sesqui-, and diterpene compounds; acetates; and other oxygenated compounds. Samples of *N. testaceicornis* collected at two sites in Brazil and one in México shared the same composition of their glands, suggesting that the species is uniform over this wide geographical area.

Key Words—Hymenoptera, Apidae, Meliponinae, exocrine secretion, geranylgeranyl acetate, terpene, oxidation products.

INTRODUCTION

An accessory gland of the poison apparatus, first described by Dufour (1834), is found in all aculeate Hymenoptera, which include bees, stinging wasps, and ants. It is a sac, surrounded by a single layer of secretory cells, with a duct which in bees opens into the sting chamber below the sting bulb. No common function has

* To whom correspondence should be addressed. e-mail: e.d.morgan@chem.keele.ac.uk

0098-0331/01/0100-0069$19.50/0

been discovered for the Dufour gland in this wide range of insects (Hölldobler and Wilson, 1990). Among some solitary bees, according to Hefetz (1998), the Dufour gland contents are used to form a waterproof lining to the brood cells, e.g., in the ground-dwelling bees *Colletes thoracicus* and *Andrena haemorrhoa* (Hefetz, 1987), or to mark their nest entrances. In another ground-dwelling eusocial bee, *Lasioglossum* (*Euylaeus*) *malachurum*, Dufour gland secretion is probably used in nest-mate recognition.

The chemistry of the contents of the Dufour gland of solitary bees has already been studied in a number of species. In the Colletidae family, the secretion is dominated by saturated and unsaturated macrocyclic lactones as well as hydrocarbons (Hefetz et al., 1979; Albans et al., 1980; Cane, 1981). The Halictidae also contain macrocyclic lactones (Bergström, 1974; Hefetz, 1987), although Duffield et al. (1981) reported the presence of trace amounts of esters. The Dufour gland secretion of Andrenidae bees is composed of a variety of terpene hydrocarbons and terpene esters (Bergström and Tengö, 1974). In two Melittidae species, the Dufour gland secretion is composed of alkyl butanoates (Tengö and Bergström, 1976). Members of the Anthophoridae exhibited a variety of compounds: terpenoids (Batra and Hefetz, 1979), triglycerides (Norden et al., 1980), saturated and unsaturated hydrocarbons (Vinson et al., 1978). Francke et al. (1984), Williams et al. (1986) and Tengö et al. (1982) documented lactones, esters, hydrocarbons, spiroacetals, ketones, and alcohols in the Dufour gland of the Megachilidae.

Within the bees, social behavior is found chiefly in the family Apidae, and particularly in the subfamilies Meliponinae (stingless bees), Apinae (honeybees) and Bombinae (bumblebees). The Dufour gland of workers of *Apis mellifera* is very small, and although much is known about pheromones of honeybees, neither the chemical composition of the secretion nor its function is known. The Dufour glands of very few species of bumblebees have been studied. The secretions are dominated by straight-chain alkanes and alkenes from C_{21} to C_{31}, with some oxygenated compounds, terpenes, and fatty acids (Oldham et al., 1994a; Tengö et al., 1991). Again, the function is unknown.

In Meliponinae, de Lello (1976) described the single gland that opens at the end of the abdomen as homologous with the Dufour gland. The gland varies considerably in size and shape among species of stingless bees. In spite of the large number of species (about 500 and still increasing), no chemical studies of the Dufour gland secretion of Meliponinae have been made until now.

The genus *Nannotrigona* is found throughout southern México and Central and South America. It contains 10 recognized species (Camargo, 1990). The genus is of some importance because certain species of *Nannotrigona* are considered good pollinators for crops. *N. testaceicornis* (Lepeletier) (Apidae: Meliponinae) is used in Japan for the pollination of strawberries (Maeta et al., 1992). The cephalic secretion of this species has been shown to contain 2-methyl-6-

pentyl-3,4-dihydro-2H-pyran (Francke et al., 1985) (for structure, see Figure 3 below). According of Camargo (1990), *N. testaceicornis* is the only species of *Nannotrigona* found in Mexico.

The aim of this work was to learn something of the chemistry of the Dufour gland secretion of stingless bees. We have studied samples of *N. testaceicornis* collected in Brazil and Mexico, first, to see whether there was any variation in the composition of the secretion, since a single species with such a wide distribution might display geographical variations in its secretions. We have attempted to identify the material in the secretion as a preliminary to studying what might be its function.

METHODS AND MATERIALS

Samples of *N. testaceicornis* were collected at Rio Claro and Cosmopólis, two different regions in the state of São Paulo, Brazil, and in Union Juárez in the state of Chiapas, Mexico.

Scanning Electron Microscopy. The abdominal tergites of workers were carefully removed in a solution of cold 2% glutaraldehyde in sodium cacodylate buffer at pH 7.3, so as to expose the abdominal tissues for observation with a Topcon SM-510 scanning electron microscope. For this purpose, the opened abdomens were critical-point dried and coated with a 30-nm layer of gold.

Transmission Electron Microscopy. Worker bees were immobilized by cooling to 3°C, the Dufour glands were dissected and fixed in a solution of 2% glutaraldehyde in sodium cacodylate buffer (0.15 M), at pH 7.2. After 3 hr the glands were rinsed in cacodylate buffer (0.15 M), postfixed in 1% buffered osmium tetroxide for 2 hr and contrasted with uranyl acetate overnight. The material was dehydrated in an increasing series of alcohol and acetate solutions and embedded in Spurr's resin. Ultra thin sections were obtained with a Porter-Blum MT-2 Ultramicrotome, with glass and diamond knives, mounted on a grid, contrasted with lead citrate, and examined in a Zeiss transmission electron microscope.

Gland Dissection. Individual workers of *N. testaceicornis* were cooled in a refrigerator and dissected under distilled water. The Dufour gland was removed and carefully dropped into a thin-walled soda-glass tube (1.8 × 20 mm) previously sealed at one end, and the open end was then sealed in a micro-flame (Morgan, 1990).

Chemical Analysis. Gas chromatography–mass spectrometry was carried out with a Varian Star 3400 CX gas chromatograph linked to a Varian Saturn 4D mass spectrometer. The samples were analyzed using a fused silica column (30 m × 0.25 mm) coated with poly(5% diphenyl–95% dimethyl)siloxane. The oven was programmed from 50°C to 250°C at 15°C/min. The carrier gas was

helium. The injector port temperature was held at 200°C. The sample was heated in the injector to 200°C for 4 min before crushing the capillary, as described by Morgan and Wadhams (1972).

RESULTS

Dissection of worker abdomens of *N. testaceicornis* revealed a large sac at the end of the abdominal tip, which correspond to the Dufour gland (Figure 1). The reservoir opens into the cloacal chamber, from where the secretion may be deposited on the substratum by the tip of the abdomen. The Dufour glands of Meliponinae, like those of ants and other Hymenoptera, originate from the integument and are composed of class 1 cells according to Noirot and Quennedey (1974). The gland reservoir is lined with a single sheet of epidermal cells. Transmission electron microscopy revealed the fibrillar aspect of the procuticle, but

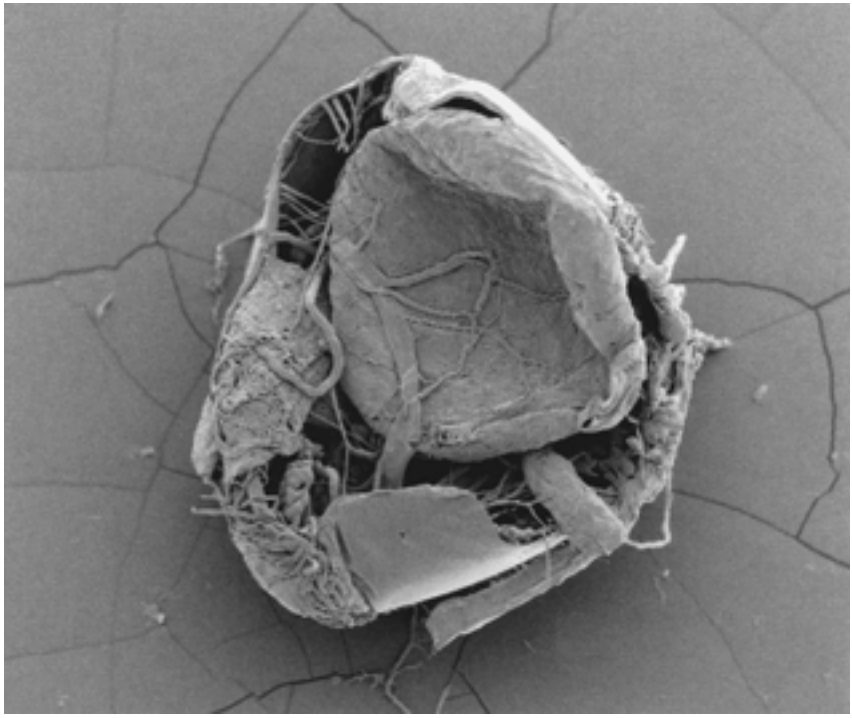


FIG. 1. Scanning electron micrograph of the partially dissected abdominal tip of *N. testaceicornis* (tergites removed) showing the size of the Dufour gland.

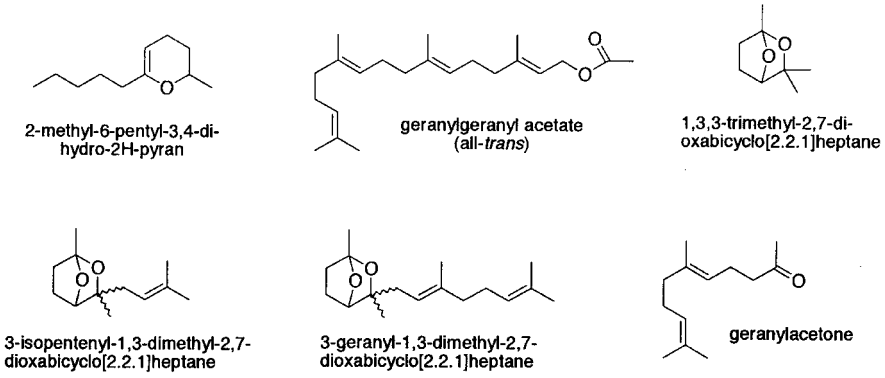


FIG. 2. Structures of some compounds discussed in the text.

contrary to what is found in the Dufour glands of other Meliponinae (Patricio, in preparation), the epicuticle is not smooth, but strongly crenellated. Since no cuticular pores were evident, the secretion from the internal vesicles may cross the cuticle by diffusion, as has been observed in some glands of termites (Quennedey, 1982). The vesiculated smooth endoplasmic reticulum is typical of pheromone-producing glands of invertebrates (Billen, 1987). Multilamellar bodies and storage vesicles are suggestive of intense cellular metabolism of these secretory cells. The epithelium of the glandular duct was composed of thin cells, indicating poor or no secretory activity.

The GC-MS analysis of the Dufour gland secretion of the three samples of *N. testaceicornis* showed that they had approximately the same composition, with the same major compounds and many minor components (Figure 2). Four individuals from each area were analyzed and their chromatograms compared. There was as much variation between individuals as there was between samples from different areas. We therefore concentrated on the identification of the first sample from Rio Claro. As learned from the microscopic examination, the gland forms a relatively large part of the abdominal organs and contains about 5 μg of secretion on average. The most notable feature of the composition was the relatively large amount of one component, all-*trans*-geranylgeranyl acetate (Figure 3), an acyclic diterpene ester (Table 1). There was a very small amount of the corresponding alcohol, *trans*-geranylgeraniol, and the corresponding lower homolog *trans*-farnesyl acetate. Retention times and mass spectra for these compounds were confirmed by comparison with authentic samples. Farnesyl acetate and geranylgeranyl acetate were prepared from acetyl chloride and the respective alcohols. Trace amounts of springene, the hydrocarbon corresponding to dehydrated geranylgeraniol were also seen (peak 30). There was also a set of oxidation products derived from geranylgeraniol (or its acetate), the simplest

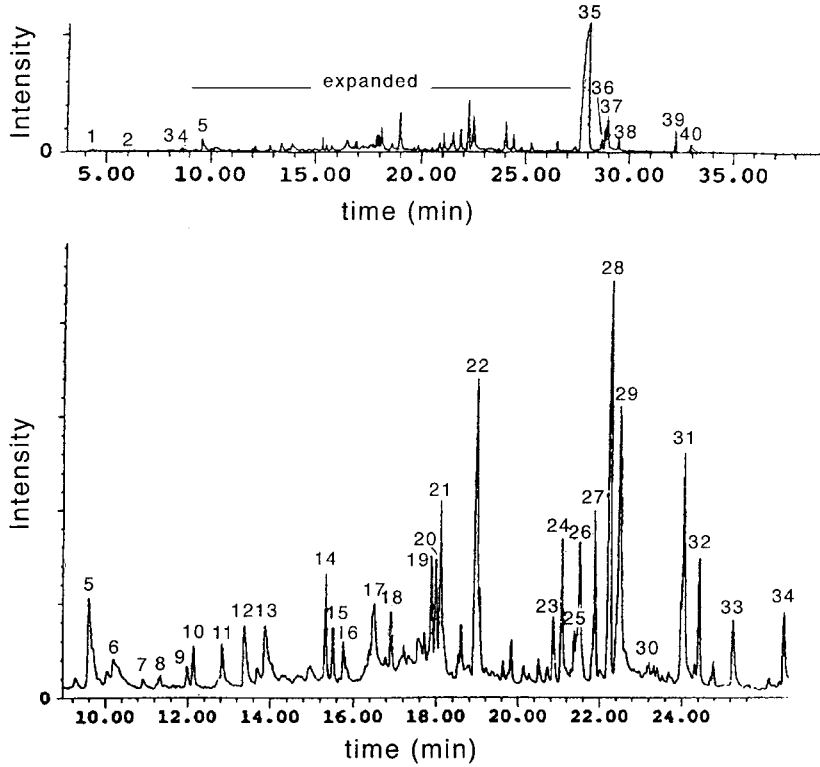


FIG. 3. Total ion current chromatogram of the Dufour gland secretion of *Nannotrigona testaceicornis*. Peak numbers refer to Table 1. The lower figure is an expansion of the region 10–25 minutes with many small peaks.

of these is 1,3,3-trimethyl-2,7-dioxabicyclo[2.2.1]heptane (Figure 3). The higher homologs can exist in two diastereomeric pairs, so two peaks are seen for each homolog of these compounds (peaks 14 and 15 and peaks 19 and 21; Figure 3). Identification of these compounds was provided by the work of Oldham et al. (1994b). Neral, geranial, geranylacetone, and the acetate esters were identified by comparison of their mass spectra and retention times with those of synthetic compounds. Farnesal was identified by comparison with the spectrum of material made earlier. As a sample was not available for retention time comparison, the isomer could not be identified with certainty, but was probably all-*trans*.

The second most abundant substance was identified as hexadecatrienal (M^+ 234). The double-bond positions were unknown, so the exact structure could not be identified, and no mass spectra were available for direct comparison. The next

TABLE 1. QUANTIFICATION OF DUFOUR GLAND SECRETION OF *Nannotrigona testaceicornis* ($N = 4$)

Peak[a]	Compound	Percent (mean ± SD)
1	Unidentified	t
2	1,3,3-Trimethyl-2,7-dioxabicyclo[2.2.1]heptane (isomer 1)	1.34 ± 0.50
3	Unidentified	t
4	$C_8H_{14}O_2$ isomeric with compound 2	t
5	$C_{10}H_{16}$	1.89 ± 1.20
6	Unidentified	0.22 ± 0.18
7	Unidentified	t
8	Unidentified	t
9	$C_9H_{16}O$	0.48 ± 0.15
10	$C_9H_{16}O$	0.73 ± 0.09
11	Neral	0.97 ± 0.40
12	Geranial	1.3 ± 0.13
13	Unidentified	t
14	3-Isopentenyl-1,3-dimethyl-2,7-dioxabicyclo[2,2,1]heptane (isomer 1)	0.55 ± 0.04
15	3-Isopentenyl-1,3-dimethyl-2,7-dioxabicyclo[2,2,1]heptane (isomer 2)	0.28 ± 0.10
16	Unidentified	0.30 ± 0.02
17	Geranylacetone	1.54 ± 0.13
18	Unidentified	0.29 ± 0.05
19	3-Geranyl-1,3-dimethyl-2,7-dioxabicyclo[2,2,1]heptane (isomer 1)	0.46 ± 0.25
20	Bornyl compound (?)	0.30 ± 0.01
21	3-Geranyl-1,3-dimethyl-2,7-dioxabicyclo[2,2,1]heptane (isomer 2)	1.17 ± 0.90
22	Unidentified	0.81 ± 0.03
23	Unidentified	0.38 ± 0.06
24	Farnesal	0.65 ± 0.03
25	Unidentified	t
26	Hexadecatrienal[b]	4.2 ± 1.25
27	Tetradecyl acetate	3.83 ± 0.80
28	Farnesyl acetate	0.39 ± 0.15
29	Hexadecadienal[b]	0.58 ± 0.20
30	Springene	t
31	Hexadecatrienyl acetate[b]	4.40 ± 1.20
32	Terpenoid	0.88 ± 0.03
33	Unidentified	0.83 ± 0.40
34	Geranylgeraniol	0.83 ± 0.36
35	Geranylgeranyl acetate	64.04 ± 9.47
36	Isomer of compound 35	1.08 ± 0.09
37	Isomer of compound 35	1.63 ± 0.30
38	Isomer of compound 35	1.58 ± 0.60
39	Terpenoid	t
40	Squalene	t
Total amount (μg/gland)		4.8

[a] Peak numbers refer to Figure 2.
[b] Unconfirmed identification.

in quantity was peak 31 (Figure 3), identified as hexatrienyl acetate (M^+ 278), with the ions characteristic of a long-chain triunsaturated alkene, plus a strong ion at m/z 43 and a weak ion at m/z 61, characteristic of an acetate group. The next in quantity were tetradecyl acetate (peak 27) followed by geranylacetone (peak 17), which can also be regarded as an oxidation product of geranylgeraniol. The monoterpene aldehydes geranial and neral, together referred to as citral, comprised about 2.25% of the secretion. Peak 29 had a molecular mass two units greater than hexadecatrienal, and a mass spectrum very similar to that of peak 26. We deduce it is a hexadecadienal, but the difference in retention times should be much less if these two differ only in the presence of one double bond. Compound 4 is a structural isomer of compound 2. It has the same molecular mass (M^+ 142) and a similar mass spectrum [m/z 109 (5%), 100 (3), 82 (18), 72 (35), 71 (32), 67 (26), 57 (25), 43 (100)]. Its spectrum does not correspond to that of frontalin, which has the same mass and similar structure to compound 2. Compound 5 has a molecular mass of 138 and a mass spectrum close to that of geraniol. It must therefore be a 2,6-dimethyloctadiene or dihydroocimene, but we lack the synthetic standards to determine the double-bond positions. Compounds 9 and 10 are an isomeric pair of mass 140 ($C_{10}H_{16}O$), probably dimethylnonadienols, *cis* and *trans*, with a $(CH_3)_2COH$—because of the strong m/z 59 ion [m/z 140 (<5%), 110 (30), 95 (45), 69 (42), 59 (80), 43 (80% in compound, 100% in compound 10), 41 (100% in compound 9, 95% in compound 10)].

The component labeled 20 gave a mass spectrum with base peak of m/z 95, which resembles the mass spectrum of isoborneol, but the substance has a higher molecular weight than isoborneol. The structure of the unknown may be an isoborneol derivative with a long-chain alkyl substituent. Compounds 36, 37, and 38 are all geometric isomers of geranylgeranyl acetate. Their spectra are all similar to that of compound 35. They all differ from geranylgeraniol by having significantly strong ions at m/z 43, which is virtually absent in geranylgeraniol. The remaining 3.6% of the total consisted of seven unidentified terpenes (Table 1).

DISCUSSION

Unlike the tiny Dufour gland of honeybee workers, that of the stingless bee *N. testaceicornis* is a relatively large organ, filling the posterior part of the abdomen and containing micrograms of liquid secretion. The most important component by far is geranylgeranyl acetate. This compound has been found before in labial glands of males of several species of bumblebees (Kullenberg et al., 1973; Bergström et al., 1996; Bergman and Bergström, 1997), where it is used for territorial marking. The territorial marking pheromone from the male bee-wolf *Philanthus pulcher* (Hymenoptera, Sphecidae) contains a mixture of ethyl oleate and geranylgeranyl acetate as major components (McDaniel et al.,

1992). The Dufour gland of some species of *Formica* ants also contains geranylgeranyl acetate (Bergström and Löfqvist, 1973). All-*trans*-geranylgeranyl acetate and geranylgeraniol are components of the recruitment pheromone of the ponerine ant *Ectatomma ruidum* (Bestmann et al., 1995). The compounds have also been found in the female sex pheromone of click beetles (*Agriotes sputator*, *A. obscurus*, *Sinaptus*, and *Melanotus* species, Coleoptera, Elateridae) (Yatsynin et al., 1996) and even in a mammalian secretion, from the dorsal glands of the collared peccary, *Tayassu tajacu* (Mammalia, Tayassuidae) (Waterhouse et al., 1996). We have found geranylgeraniol in the Dufour glands of the Old World army ant *Aenictus rotundatus* and the isomer geranyllinalool in the Dufour glands of the New World army ant *Eciton burchelli* (Keegans et al., 1993).

Oldham et al. (1994b) found a series of bicyclic ketals accompanying geranylgeraniol, the principal component in the Dufour gland of the army ant *Aenictus rotundatus*. The simplest of these ketals is 1,3,3-trimethyl-2,7-dioxabicyclo[2.2.1]heptane (Figure 2). It was accompanied by higher homologs containing an isopentenyl or geranyl group (Figure 2). Two isomers were seen for each. We deduced that these compounds were oxidation products, either biochemical or atmospheric, of the geranylgeraniol. The same series of ketals has been observed in this work, always in very small quantity. 1,3,3-Trimethyl-2,7-dioxabicyclo[2.2.1]heptane was first identified in the aroma of Granny Smith apples (Stanley et al., 1986), and then identified in several species of ants of the subfamily Dolichodorinae (Tomalsky et al., 1987; Cox et al., 1989, see also Francke and Schröder, 1999). The complete series of homologs was recognized by Oldham et al. (1994b). Reexamination of the chromatograms from Keegans et al. (1993), Oldham et al. (1994b), and the present work shows that the first peak of each isomeric pair of homologs is the larger one. Study of molecular models indicates this must be the 3*S*,4*S*, 3*R*,4*R* enantiomeric pair. There are no reports of the presence of this substance in the secretion of bees that contain geranylgeraniol or its acetate. We also identified 6-methyl-5-hepten-2-one, geranylacetone, and farnesylacetone, the supposed biosynthetic precursors of these compounds, in *A. rotundatus* (Oldham et al., 1994b) and *E. burchelli* (Keegans et al., 1993), which adds support to the proposed structures and origins.

We found no differences in the analysis of the Dufour gland secretion of *N. testaceicornis* samples from Brazil and Mexico in the present study, although geographical variation is frequently encountered in other insect secretions with pheromonal activity. For example, the green stink bug, *Nezara viridula* has a worldwide distribution in the tropics and subtropics. The male pheromone includes various sesquiterpene compounds. Bugs from Brazil lack a sesquiterpene component that is present in populations from the southern United States and southern France, and sesquiterpene isomer ratios differ between French and North American bugs (Aldrich, 1988). In Lepidoptera, also, geographical variation of pheromone blends has been found. Hansson et al. (1990) found that

the sex pheromone of *Agrotis segetum* include three main components, (*Z*)-5-decenyl acetate, (*Z*)-9-dodeceyl acetate, and (*Z*)-9-tetradecenyl acetate. The ratio of these compounds in populations from Sweden, Britain, France, Armenia, and Bulgaria show marked differences. On the other hand, *Pectinophoroa gossipiella*, the pink bollworm, shows little variation among insects from California, Argentina, Brazil, China, Egypt, Mexico and Pakistan (Haynes and Baker, 1988).

Acknowledgments—We thank C. Mansfield, International Flavours and Fragrances Ltd., Suffolk, U.K., for the sample of geranylgeraniol; M.C. Guadalupe Nieto for helping with the scanning electron micrographs; and M.C. Manuel Rabanales for providing the *Nanotrigona* samples collected in México. This work was partly supported by CONACYT, México through a studentship to L.C.L.

REFERENCES

ALBANS, K. R., APLIN, R. T., BREHCIST, J., MOORE, J. F., and O'TOOLE, C. 1980. Dufour's gland ant its role in secretion of nest cell lining in bees of the genus *Colletes* (Hymenoptera: Colletidae). *J. Chem. Ecol.* 6:549–564.

ALDRICH, J. R. 1988. Chemical ecology of Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

BATRA, S. W., and HEFETZ, A. 1979. Chemistry of the cephalic and Dufour's secretion of *Melissodes* bees. *Ann. Entomol. Soc. Am.* 72:514.

BERGMAN, P., and BERGSTRÖM, G. 1997. Scent marking, scent origin, and species specificity in male premating behaviour of two Scandinavian bumblebees. *J. Chem. Ecol.* 23:1235–1251.

BERGSTRÖM, G. 1974. Studies on natural odoriferous compounds X. Macrocyclic lactones in the Dufour gland secretion of the solitary bees *Colletes cunicularius* L. and *Halictus calceatus* Scap. (Hymenoptera, Apidae). *Chem. Scr.* 5:39–46.

BERGSTRÖM, G., and LÖFQVIST, J. 1973. Chemical congruence of the complex odoriferous secretion from Dufour's gland in three species of ant of the genus *Formica. J. Insect Physiol.* 19:877–907.

BERGSTRÖM, G., and TENGÖ, J. 1974. Studies on natural odoriferous compounds IX. Farnesyl and geranyl esters as main volatile constituents of the secretion from Dufour's gland in six species of *Andrena* (Hymenoptera, Apidea). *Chem. Scr.* 5:28–38.

BERGSTRÖM, G., BERGMAN, P. APPELGREN, M., and SCHMIDT, J. O. 1996. Labia; gland chemistry of three species of bumblebees (Hymenoptera: Apidae) from North America. *Bioorg. Med. Chem.* 3:515–519.

BESTMANN, H. J., JANSSEN, E., KERN, F., LIEPOLD, B., and HÖLLDOBLER, B. 1995. All-*trans*-geranylgeranyl acetate and geranylgeraniol, recruitment pheromone components in the Dufour gland of the ponerine ant *Ectatomma ruidum. Naturwissenschaften* 82:334–336.

BILLEN, J. P. J. 1987. Morphology and ultrastructure of the exocrine glands in social Hymenoptera, pp. 81–84, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Peperny, Munich.

CAMARGO, J. M. F. 1990. Stingless bees of the Amazon. *In* G. K. Veeresh, B. Mallik, and C. A. Viraktamath (eds.). Social Insects and the Environment, Proceedings of the 11th International Congress of the International Union for the Study of Social Insects, 1990. Oxford and IBH Publishing, New Delhi.

CANE, J. H. 1981. Dufour's gland secretion in the cell lining of bees (Hymenoptera: Apoidea). *J. Chem. Ecol.* 7:403–410.

COX, M. F., BROPHY, J. J., and TOIA, R. F. 1989. Chemotaxonomy of the Australian Dolichoderinae: volatile constituents of *Iridomyrmex discors. J. Nat. Prod.* 52:75–80.

DE LELLO, E. 1976. Adnexal glands of the sting apparatus in bees: Anatomy and histology, V. (Hymenoptera: Apidae). *J. Kans. Entomol. Soc.* 49:85–99.

DUFFIELD, R. M., FERNANDEZ, A., LAMB, C., WHEELER, J. W., and EICKWORT, G. C. 1981. Macrocyclic lactones and isopentenyl ester in the Dufour's gland secretion of halictine bees (Hymenoptera: Halictidae). *J. Chem. Ecol.* 7:319–331.

DUFOUR, L. 1834. Recherches anatomiques et physiologiques sur les orthoptères, les hymenoptéres et les névroptères. Memoir présentés par divers savants à l'Academie Royale des Sciences de l'Institute de France, et imprimé par son ordre. *Sci. Math. Phys.* 7:265–647.

FRANCKE, W., and SCHRÖDER, W. 1999. Bicyclic acetals in systems of chemical communication. *Curr. Org. Chem.* 3:407–443.

FRANCKE, W., SCHRÖDER, W., BERGSTRÖM, G., and TENGÖ, J. 1984. Pages 127–136, *in* B. Kullenberg, G. Bergström, B. G. Svensson, J. Tengö, and L. Agren (eds.). The Ecological Station of Uppsala University in Öland 1963–1983, Almqvist and Wiksell, Stockholm.

FRANCKE, W., MACKENROTH, W., SCHRÖDER, W., SCHULZ, S., TENGÖ, J., ENGELS, E., ENGELS, W., KITTMANN, R., and SCHNEIDER, D. 1985. Identification of cyclic enol ethers from insects: Alkyldihydropyranes from bees and alkyldihydro-4H-pyran-4-ones from a male moth. *Z. Naturforsch.* 40C:145–147.

HANSSON, B. S., MIKLÓS, T., LÖFSTEDT, C., SZÖCS, G., SUBCHEV, M., and LÖFQVIST, J. 1990. Pheromones variations among eastern european and a western asian population of the turnip moth *Agrotis segetum. J. Chem. Ecol.* 5:1611–1622.

HAYNES, K. F., and BAKER, T. C. 1988. Potential for evolution of resistance to pheromones. Worldwide and Local Variation in Chemical Communication System of Pink Bollworm Moth, *Pectinophora gossypiella. J. Chem. Ecol.* 7:1547–1560.

HEFETZ, A. 1987. The role of Dufour's gland secretion in bees. *Physiol. Entomol.* 12:243–253.

HEFETZ, A. 1998. Exocrine products in non-Apis bees: chemical functional and evolutionary perspectives, pp. 236–256, *in* R. K. Vander Meer, M. D. Breed, M. L. Winston, and K. E. Espelie (eds.). Pheromone Communication in Social Insects, Westview Press, Boulder, Colorado.

HEFETZ, A., FALES, H. M., and BATRA, S. W. T. 1979. Natural polyesters: Dufour's gland macrocyclic lactones in the brood cell laminesters in *Colletes* bees. *Science* 204:415–417.

HÖLLDOBLER, B., and WILSON, E. O. 1990. The Ants. Springer, Berlin, p. 229.

KEEGANS, S. J., BILLEN, J., MORGAN, E. D., and GÖKÇEN, O. A. 1993. Volatile glandular secretions of three species of New-World army ants, *Eciton burchelli*, *Labidus coecus*, and *Labidus praeditor. J. Chem. Ecol.* 19:2705–2719.

KULLENBERG, B., BERGSTRÖM, G., BRINGER, B., CARLBERG, B., and CEDERBERG, B. 1973. Observations on scent marking by *Bombus* latr. and *Psithyrus* lep. Males (Hym., Apidae) and localisation of site of production of the secretion. *Zoon Suppl.* 1:23–30.

MAETA, Y. T., TEZUKA, H., and SUSUKI, K. 1992. Utilisation of the Brazilian stingless bee, *Nannotrigona testaceicornis* as a pollinator of strawberries. *Honeybee Sci.* 13:17–78.

MCDANIEL, C. A., SCHMIDT, J. O., and HOWARD, R. W. 1992. Mandibular gland secretions of the male beewolves *Philanthus crabriformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera, Sphecidae). *J. Chem. Ecol.* 18:27–37.

MORGAN, E. D. 1990. Preparation of small-scale samples from insects for chromatography. *Anal. Chim. Acta* 236:227–235.

MORGAN, E. D., and WADHAMS, L. J. 1972. Gas chromatography of volatile compounds in small samples of biological material. *J. Chromatogr. Sci.* 10:528–529.

NOIROT, C., and QUENNEDEY, A. 1974. Fine structure of insect epidermal glands. *Annu. Rev. Entomol.* 19:61–80.

NORDEN, B., BATRA, S. W. T., FALES, H. M., HEFETZ, A., and SHAW, G. J. 1980. Anthophora bees: Unusual glycerides from maternal Dufour's glands serve as larval food and cell lining. *Science* 207:1095–1097.

OLDHAM, N. J., MORGAN, E. D., and BILLEN, J. 1994a. On the similarity of the Dufour gland secretion and the cuticular hydrocarbons of some bumblebees. *Physiol. Entomol.* 19:115–123.

OLDHAM, N. J., MORGAN, E. D., GOBIN, B., SCHOETERS, E., and BILLEN, J. 1994b. Volatile secretions of Old-World army ant *Aenictus rotuntatus* and chemotaxonomic implications of army ant Dufour gland chemistry. *J. Chem. Ecol.* 20:3297–3305.

QUENNEDEY, A. 1982. Modifications structurales de la cuticule des glands tégumentaires de termites. *Bull. Soc. Zool. Fr.* 107:463–467.

STANLEY, G., ALGIE, J. E., and BROPHY, J. J. 1986. 1,3,3-Tromethyl-2,7-dioxabicyclo[2.2.1]heptane—a volatile oxidation product of alpha-farnesene in Granny Smith apples. *Chem. Ind.* 16:556.

TENGÖ, J., and BERGSTRÖM, G. 1976. Comparative analysis of lemon-smelling secretions from heads of *Andrena* F. (Hymenoptera, Apoidea) bees. *Comp. Biochem. Physiol.* 55B:179–188.

TENGÖ, J., BERGSTRÖM, G., BORG-KARLSON, A.-K., GROTH, I., and FRANCKE, W. 1982. Volatile compounds from cephalic secretions of females in two cleptoparasite bee genera, *Epeolus* and *Coelioxys. Z. Naturforsch.* 37:375–380.

TENGÖ, J., HEFETZ, A., BERTSCH, A., SCHMITT, U., LÜBKE, G., and FRANCKE, W. 1991. Species specificity and complexity of Dufour's gland secretion of bumblebees. *Comp. Biochem. Physiol.* 99B:641–646.

TOMALSKY, M. D., BLUM, M. S., JONES, T. H., FALES, H. M., HOWARD, D. F., and PASSERA, L. 1987. Chemistry and functions of exocrine secretions of the ants *Tapinoma melanocephalum* and *T. erraticum. J. Chem. Ecol.* 13:253–263.

VINSON, S. B., FRANCKE, G. W., BLUM, M. S., and WHEELER, J. W. 1978. Isolation, identification and function of the Dufour's gland secretion of *Xylocopa virginica texana* (Hymenoptera: Anthophoridae). *J. Chem. Ecol.* 4:315–323.

WATERHOUSE, J. S., KE, J., PICKETT, J. A., and WELDON, P. J. 1996. Volatile components in the dorsal gland secretion of the collared peccary, *Tayassu tajacu* (Tayassuidae, Mammalia). *J. Chem. Ecol.* 22:1307–1314.

WILLIAMS, H. J., STRAND, M. R., ELZEN, G. W., VINSON, S. B., and MERITT, S. J. 1986. Nesting behaviour, nest architecture, and use of Dufour's gland lipids in nest provisioning by *Megachile integra* and *Mendica mendica* (Hymenoptera: Megachilidae). *J. Kans. Entomol. Soc.* 59:588–597.

YATSYNIN, V. G., RUBANOVA, E. V., and OKHRIMENKO, N. V. 1996. Identification of female-produced sex pheromones and their geographical differences in pheromone gland extract composition from click beetles (Col. Elateridae). *J. Appl. Entomol.–Z. Angew. Entomol.* 120:463–466.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

PHENOLIC COMPOUNDS IN A SAHELIAN SORGHUM (*Sorghum bicolor*) GENOTYPE (CE_{145-66}) AND ASSOCIATED SOILS

MANIÉVEL SÈNE,[1,3] CHRISTIANE GALLET,[2,*] and THIERRY DORÉ[1]

[1]*INA Paris-Grignon, Laboratoire d'Agronomie INRA/INA P-G*
16 rue C. Bernard, 75231 Paris Cedex 05, France
[2]*University of Savoie, LDEA*
73376 Le Bourget-du-Lac Cedex, France

(Received September 14, 2000; accepted August 30, 2000)

Abstract—CE_{145-66} is an improved early-maturing grain sorghum genotype, increasingly grown by farmers in the Sahelian part of Senegal. This genotype is known to have negative effects on the following groundnut crop, because of the release of allelopathic phenolic compounds into the soil. We have assessed the synthesis of phenolics in sorghum vegetative parts and the variations in synthesis between years and sites. Total phenols and phenolic acids in the aerial parts and roots of flowering sorghum plants from 52 farmers' fields at two sites (Sagnanème and Médina) in Senegal in 1996 and 1997 were measured. Thirty-eight soil samples, collected after the sorghum harvest, from sorghum rows and interrows also were analyzed for their phenolic content. Total phenols reached 1.1–1.5% of root dry weight and 1.1–2.2% of aerial parts dry weight, with little variation between sites, and large variability between years, presumably due to climatic conditions. Eight phenolic acids and three associated aldehydes were identified by HPLC, with *p*-hydroxybenzoic, *p*-coumaric, and ferulic acids the most abundant. Their totals reached 2.9–3.2 mg/g in 1996 and 2.6–2.8 mg/g in 1997 for the aerial part; and 3.3–3.6 mg/g in 1996 and 2.8–3.3 mg/g in 1997 for roots. In soils under sorghum rows, the mean water-soluble total phenols increased from 4.6 in 1997 to 6.7 μg/g in 1998 in Sagnanème, and from 3.8 in 1997 to 5 μg/g in 1998 in Médina. The concentrations of total phenols and phenolic acids were higher in rows than in interrows. All the phenolic monomers identified in vegetative parts were recovered in associated soil samples, with vanillic and *p*-hydroxybenzoic acids the most abundant. Finally, variability in plant phenolic content seemed more due to climatic than to cropping or soil factors,

*To whom correspondence should be addressed.
[3]Permanent Address: ISRA-CNRA, BP 53 Bambey, Senegal.

as differences between years appear more important than differences between or within sites.

Key Words—Agricultural soils, allelopathy, phenolics, sorghum.

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is a widely produced agricultural crop. Its aerial vegetative parts and seeds are used for many purposes. The vegetative parts are used for animal forage and rural construction, and sorghum grains is a basic food source for many. Grain sorghum is the second most important cereal crop grown under pluvial conditions in Senegal. $CE_{145\text{-}66}$ is an improved early-maturing grain sorghum genotype that is well adapted to the Sahelian zone (dryland) growing conditions in Senegal. Hence, more and more acreage is under cultivation in this part of the country.

Sorghum is also known to possess strong phytotoxic potential against weeds (Einhellig and Rasmussen, 1989; Weston, 1996), against itself (Guenzi and McCalla, 1962, 1966; Chopart and Nicou, 1973; Burgos-Leon et al., 1980), and against other crops (Chopart and Nicou, 1973; Delafond and Burgos-Leon, 1978; Burgos-Leon et al., 1980; Nicou, 1980; Ben-Hammouda et al., 1995). This toxicity is due to the production and release of phenolic compounds, including phenolic acids (Burgos-Leon et al., 1980; Ben-Hammouda et al., 1995) and sorgoleone (Einhellig and Souza, 1992; Einhellig et al., 1993; Weston et al., 1997, 1999). Sène et al. (2000) showed that phenolic acids left in the soil after a $CE_{145\text{-}66}$ crop affected the germination of groundnut. However, few studies have been done on phenolic metabolism and its variability in the vegetative parts of sorghum, and the release and decomposition of such compounds have never been studied under field conditions.

This study was done to enhance our knowledge of phenolic production by sorghum vegetative parts, and to establish the phenolic pattern of sorghum aerial parts and roots in agricultural conditions. A second aim was to compare patterns to those of associated soils, in order to assess the influence of this species on soil composition and subsequent fertility. Large sampling sets of plants (N = 52) and of soils (N = 38) were chosen directly in farmer's fields under natural conditions (wet season followed by a dry season) to take in account and estimate the variability of these compounds.

METHODS AND MATERIALS

Study Sites. Two sets of in situ measurements were made in farmers' fields (in 1996 and 1997) to examine the levels of phenolic compounds present in

sorghum aerial parts and roots. The fields were located at two sites in the central groundnut producing region of Senegal, at the villages of Sagnanème (latitude 14°N, longitude 16°15′W) and Médina (latitude 14°20′N, longitude 15°20′W). Twelve fields were monitored in Sagnanème and 15 in Médina in 1996, and 8 and 17, respectively, in 1997. The soils are ferralitic or ferruginous tropical, with 61–78% sand in the 0- to 10-cm layer. The crops were grown without fertilizer, herbicides, or additional irrigation, similar to the farmers' cultivation methods. Weeds were removed by hand.

Sampling Methods. Sorghum was sown by the farmers at the beginning of the rainy season, which lasts from July to October. A plot 25 × 25 m was marked out in each field on day 15 after emergence. These plots were divided into 25 subplots of 5 × 5 m; five of these subplots were randomly chosen for plant measurements and five others for soil measurements.

Ten flowering sorghum plants were collected from each subplot. Aerial parts and roots of the 10 plants were separated and pooled as a sample of aerial parts or a sample of roots. Samples were dried at 70°C for 96 hr and ground to a homogeneous powder. The five samples of ground aerial parts from each field were pooled and a 50-g sample was taken for phenol analysis. The root samples were treated similarly.

Soil samples were collected from the same fields (from five fields in Sagnanème and eight fields in Médina in 1997, but from all the fields in 1998) in June 1997 and April 1998 (during the dry seasons following the sorghum crop), from the prior sorghum rows and interrows. A total of three soil samples were taken from rows and three from interrows at 0–40 cm depth on each soil subplot. The samples (15) from previous sorghum rows at each field were mixed, and a 250-g sample was taken for phenol analysis. The samples taken from interrows were treated similarly. The soil was sampled before the beginning of the rainy season in 1997, but the rainy season started two weeks prior to soil sampling in 1998. The latter samples were taken after a total rainfall of 157 mm in Sagnanème and 91 mm in Médina. The 1997 and 1998 samples were air-dried for three months.

Plant Analysis. Dried ground roots or aerial parts (500 mg) were extracted twice (100 ml/75 ml) by refluxing with 70% (v/v) boiling ethanol for 20 min. The extracts were combined and evaporated under vacuum. The residues were dissolved in a standard volume of hot distilled water, and aliquots were taken for total phenol determination, with the Folin-Ciocalteau reagent and gallic acid as a standard. Results are expressed as milligrams of gallic acid equivalents.

Although most phenolic compounds are in the leaves as glycosides, the glycosidic bonds are rapidly cleaved at the beginning of senescence and the free phenolics accumulate in surface layers. Mild acidic hydrolysis was used to extract phenolic monomers from ground aerial parts. This type of hydrolysis also releases anthocyans from condensed tannins (proanthocyanidins) and allows quantification.

Dried, ground material (1000 mg roots or aerial parts) from each sample was hydrolyzed with 80 ml 2 M HCl (at 100°C, under O_2) for 40 min, and the resulting solutions were then extracted three times with diethyl ether. The residual aqueous extracts were filtered, and the anthocyan concentrations estimated by measuring at the wavelength of maximum absorbance. The organic extract was evaporated to dryness, and the residues were dissolved in ethanol and stored at −18°C until analyzed for phenolic acid content by HPLC.

Soil Analysis. Free (active) phenols were estimated in aqueous extracts to assess the extracellular persistence of phenolic compounds in humus. Water-soluble phenolic compounds were extracted for each sample by shaking approximately 100 g dry weight soil with 200 ml distilled water under N_2 for 14 hr at 10°C.

The solutions were filtered, and one aliquot was used to measure total phenols as above. About 500 ml of each water extract was then acidified with 2 M HCl and extracted three times with diethyl ether. The ether extracts were evaporated to dryness, and the resulting residues were dissolved in a known volume of ethanol and stored at −18°C.

Liquid Chromatography. The phenolic monomers were separated with a Waters 600 Controller, equipped with the diode array detector (Waters 996) and Millenium software. The samples (20 μl) were injected onto a column (250 × 4.6 mm) filled with μBondapak C_{18}, 10 μm, and eluted with a gradient of solvent A (0.5% acetic acid in distilled water) and solvent B (0.5% acetic acid in acetonitrile). The phenolic acids were separated by a linear gradient (flow rate 1.5 ml/min) from 0% to 20% B in 45 min, with 15 min of reequilibration between samples. They were further identified and quantified by comparing their UV spectra and retention times with those of standard compounds. About 90% of the products detected by chromatographic analysis from both plant and soil extracts were identified.

Statistical Analysis. Individual field results were not considered in this paper. Means across sites and across years were compared by the Mann Whitney *U* test. The Wilcoxon signed-ranks test for dependent variables was used to compare data from roots and aerial parts, and soil data from rows and interrows. Proportions (%) of individual phenolic acids were compared across years or sites by the binomial test.

RESULTS

Polyphenols in Aerial Parts of Sorghum. The total phenol concentrations estimated by the Folin-Ciocalteau reagent were 2.0–2.2% of the sorghum DW in aerial portions in 1996 at both sites, but only about half of this value in 1997 (Table 1). Some of these products were identified as phenolic monomers, and

TABLE 1. PHENOLIC COMPOUNDS OF SORGHUM AERIAL PARTS[a]

	Sagnanème		Médina	
	1996 (N = 12)	1997 (N = 8)	1996 (N = 15)	1997 (N = 17)
Total phenols (mg/g)	22.3 ± 2.6aα	10.7 ± 1.8b	19.7 ± 2.9aβ	11.6 ± 1.5b
Phenolic acids (mg/g)	3.2 ± 0.6a	2.6 ± 0.4b	2.9 ± 0.5a	2.8 ± 0.3a
Protocatechuic acid (%)	3.1 ± 1.6	1.0 ± 0.3	2.3 ± 0.7	1.4 ± 0.4
Gentisic acid (%)	3.4 ± 0.9	2.3 ± 0.6	3.8 ± 3.8	2.2 ± 0.5
p-Hydroxybenzoic acid (%)	19.6 ± 3.5	10.6 ± 1.9	16.2 ± 4.0	12.8 ± 2.5
p-Hydroxybenzaldehyde (%)	23.2 ± 5.6	6.7 ± 2.3	14.5 ± 4.6	10.1 ± 2.9
Vanillic acid (%)	2.4 ± 0.4	2.9 ± 0.3	2.4 ± 0.4	2.7 ± 0.4
Caffeic acid (%)	8.7 ± 1.2	5.5 ± 1.6	8.7 ± 3.8	3.1 ± 1.3
Syringic acid (%)	1.7 ± 1.7	2.9 ± 1.6	1.5 ± 1.4	1.8 ± 0.5
Vanillin (%)	0.2 ± 0.3	1.3 ± 0.2	0.4 ± 0.4	1.3 ± 0.2
p-Coumaric acid (%)	8.3 ± 1.7	23.5 ± 2.5	12.6 ± 2.5	22.3 ± 2.3
Ferulic acid (%)	29.3 ± 3.8	43.3 ± 2.1	37.5 ± 4.6	42.4 ± 3.7

[a] Total phenols are expressed in mg gallic acid equivalents/g dry weight; and the sum of all the identified phenolic acids in mg/g dry wt. Letters (a,b) indicate differences between years (Mann-Whitney test; $P < 0.05$). No differences (Mann-Whitney test; $P < 0.05$) were detected between sites except for total phenols in 1996 (α,β). No differences between phenolic acid relative weights (%) for 1996 and 1997 were detected by the binomial test. All values are mean ± SD.

their contribution to the amount of total phenols varied significantly, depending on the year. In 1996, they accounted for 14–15% at both sites and for 24% at both sites in 1997. Phenolic monomers included eight acids and two aldehydes, with some of them in very small amounts, including vanillin and syringic, protocatechuic, gentisic, and vanillic acids. *p*-Hydroxybenzoic acid, *p*-hydroxybenzaldehyde, *p*-coumaric and ferulic acids, and to a lesser extent, caffeic acid, were well represented. Their individual contributions to the total phenolic acids remained relatively stable across years and sites. These five acids accounted for 87–93% of the total acids. There was no difference between the two sites except for total phenols, which were more concentrated in aerial parts from Sagnanème in 1996 than in those from Médina. The lack of absorbance of the acidic extract at 500–600 nm indicated the absence of proanthocyanidins (condensed tannins) from aerial parts.

Sorghum Polyphenols in Roots. The roots also contained more total phenols at Sagnanème (1.6% dry wt) than at Médina (1.1%) in 1996 (Table 2). The concentration of phenolic acids in the roots was also higher at Sagnanème than at Médina in 1997. The proportion of phenolic acids in roots compared to the total phenol compounds varied between 24% and 27% (in 1997 and 1996, respectively). The patterns of phenolic monomers of the roots were the same as those of the aerial parts, except that the former contained less caffeic acid. *p*-Hydroxyben-

TABLE 2. PHENOLIC COMPOUNDS OF SORGHUM ROOTS[a]

	Sagnanème		Médina	
	1996 (N = 12)	1997 (N = 8)	1996 (N = 15)	1997 (N = 17)
Total phenols (mg/g)	15.7 ± 3.7aα	12.2 ± 0.9b	10.9 ± 2.4aβ	12.7 ± 1.1b
Phenolic acids (mg/g)	3.6 ± 0.6a	3.3 ± 0.2aα	3.3 ± 0.8a	2.8 ± 0.4bβ
Protocatechuic acid (%)	0.8 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.6 ± 0.3
Gentisic acid (%)	1.1 ± 0.9	0.9 ± 0.2	0.7 ± 0.3	1.2 ± 0.4
p-Hydroxybenzoic acid (%)	18.1 ± 7.8	13.8 ± 2.0	27.6 ± 10.2	14.5 ± 2.5
p-Hydroxybenzaldehyde (%)	16.3 ± 5.2c	9.0 ± 2.3d	21.3 ± 6.9	11.7 ± 3.7
Vanillic acid (%)	2.1 ± 0.4	2.3 ± 0.3	2.1 ± 0.6	2.2 ± 0.5
Caffeic acid (%)	0.5 ± 0.2	1.0 ± 0.2	0.3 ± 0.1	0.7 ± 0.2
Syringic acid (%)	2.4 ± 0.9	3.6 ± 0.7	1.5 ± 1.4	2.9 ± 0.7
Vanillin (%)	0.1 ± 0.1	0.6 ± 0.3	0.4 ± 0.2	0.8 ± 0.2
p-Coumaric acid (%)	28.9 ± 5.2	33.4 ± 2.2	22.0 ± 6.4	31.6 ± 8.7
Ferulic acid (%)	29.5 ± 5.3	34.9 ± 1.9	23.5 ± 6.5	34.0 ± 4.9

[a] Total phenols are expressed in mg gallic acid equivalents/g dry weight and the sum of all the identified phenolic acids in mg/g dry wt. Letters (a,b) indicate differences between years (Mann-Whitney test; $P < 0.05$). Differences between Sagnanème and Médina (Mann-Whitney test; $P < 0.05$) are indicated by Greek superscripts (for total phenols in 1996 and phenolic acids in 1997). Superscript letters (c,d) indicate differences between phenolic acid relative weights (%) in 1996 and 1997 (binomial test; $P < 0.05$). See values and mean ± SD.

zoic acid, *p*-hydroxybenzaldehyde, *p*-coumaric, and ferulic acids accounted for 91–94% of total phenolic acids. No proanthocyanidins were detected in sorghum roots.

Water-Soluble Compounds in Soil. As previous experiments (data not shown) have shown that amounts of water-soluble polyphenolics extracted from dried soils were inferior compared to frozen soil, this latter process was preferable. Unfortunately, due to field conditions, freezing of soil samples was not possible. For this reason, underestimation of concentrations was foreseeable in our study. As a result, data were analyzed by comparing sites or years rather than in terms of absolute concentration.

The total water-soluble phenols extracted from soils under sorghum rows increased from 4.6 in 1997 to 6.7 μg/g dry wt in 1998 (at Sagnanème), and from 3.8 in 1997 to 5.0 μg/g dry wt in 1998 at Médina (Table 3). Total phenols were more abundant under previous sorghum rows than under interrows in both years and at both sites (Table 3). In 1997, amounts under interrows were 89% of those under rows, and these proportions decreased to 34% and 38% in 1998, respectively, for Sagnanème and Médina. Phenolic acids also increased in concentrations from 1997 to 1998, with concentrations eightfold higher at Sagnanème and 18-fold higher at Médina (Table 3). Differences between rows and

TABLE 3. WATER-SOLUBLE PHENOLIC COMPOUNDS IN SOILS AFTER SORGHUM CROP UNDER SORGHUM ROWS AND UNDER INTERROWS[a]

	Sagnanème				Médina			
	1997 ($N = 5$)		1998 ($N = 8$)		1997 ($N = 8$)		1998 ($N = 17$)	
	Rows	Interrows	Rows	Interrows	Rows	Interrows	Rows	Interrows
Total phenols (μg/g)	4.6 ± 1.9a	4.1 ± 1.2bα	6.7 ± 2.7a	2.3 ± 0.8bβ	3.8 ± 0.6aα	3.4 ± 0.7bα	5.0 ± 1.7aβ	1.9 ± 0.6bβ
Phenolic acids (ng/g)	46 ± 40aα	64 ± 76a	355 ± 157aβ	88 ± 47b	11 ± 3aα	13 ± 8aα	194 ± 134aβ	35 ± 34bβ
Protocatechuic acid (%)	0 ± 0	0 ± 0	3 ± 3	2 ± 5	0 ± 0	0 ± 0	3 ± 7	1 ± 2
Gentisic acid (%)	0 ± 0	0 ± 0	<1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
p-Hydroxybenzoic acid (%)	14 ± 11	13 ± 13	37 ± 15c	23 ± 8d	10 ± 13	11 ± 17	39 ± 15	30 ± 17
p-Hydroxybenzaldehyde (%)	1 ± 2	1 ± 2	14 ± 10c	3 ± 1d	0 ± 0	0 ± 0	9 ± 11	1 ± 1
Vanillic acid (%)	56 ± 34	49 ± 34	29 ± 13c	60 ± 17d	83 ± 18	76 ± 22	26 ± 16c	51 ± 18d
Caffeic acid (%)	0 ± 0	0 ± 0	1 ± 2	<1 ± <1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Syringic acid (%)	1 ± 3	1 ± 1	3 ± 3	3 ± 3	0 ± 0	0 ± 0	2 ± 2	1 ± 2
Vanillin (%)	2 ± 4	3 ± 6	2 ± 2	1 ± 1	6 ± 15	10 ± 21	3 ± 11	4 ± 14
p-Coumaric acid (%)	0 ± 0	0 ± 0	10 ± 10	7 ± 9	1 ± 3	2 ± 5	15 ± 9	5 ± 3
Ferulic acid (%)	0 ± 0	0 ± 0	3 ± 1	2 ± 1	0 ± 0	0 ± 0	3 ± 2	1 ± 1
Syringaldehyde	27 ± 37	34 ± 34	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a] Total phenols are expressed in μg gallic acid equivalent/g dry wt and the sum of all the identified phenolic acids in ng/g dry wt. Letters (a and b) indicate differences between rows and interrows (Wilcoxon test; $P < 0.05$). Differences (Mann-Whitney test; $P = 0.05$) between 1997 and 1998 are indicated by Green letters (α, β). Letters (c and d) indicate differences between phenolic acid relative weights (%) in rows and interrows (binomial test; $P < 0.05$). All values are mean ± SD.

interrows were not significant in 1997, whereas in 1998, phenolic acid concentrations were four to six times higher in soils under sorghum rows than in soils from interrows.

The same phenolic acids found in the soils were all found in sorghum vegetative parts, except for syringaldehyde, which was found only in 1997 in Sagnanème soils. All 10 acids from sorghum vegetative parts were found in at least one soil, but their weight in total acids were very different. Protocatechuic, gentisic, caffeic, and ferulic acids were not found in any soils in 1997, including those from rows or interrows. *p*-Hydroxybenzoic acid, vanillic acid, and syringaldehyde accounted for 87–96% of the acids found in the soils in 1997, and for 65–83% of those found in 1998. *p*-Coumaric acid was more abundant in 1998 than in 1997, accounting for 7–15% of the total phenolic acids in 1998 and 0–2% in 1997.

DISCUSSION

Phenolic Metabolism in Sorghum Vegetative Parts. Although sorghum grain is a major source of condensed tannins (Gupta and Hashlam, 1978), tannins were not detected in the vegetative parts of sorghum tissues. Many of the total phenols extracted (15–30%) were in the form of phenolic monomers, although direct comparisons of these two parameters is difficult because of the relative Folin-Ciocalteau results (in gallic acid equivalents). Other phenolic compounds, including those that were detected by HPLC but not identified (one or two compounds in plant or soil extracts), require further analysis to provide a more complete view of sorghum phenolic metabolism.

Within one site and one year, relatively low variability was observed. Coefficients of variation (mean value) were, for total phenols, 14% in aerial parts and 15% in roots, and for phenolic acids 15% in both aerial parts and roots. Because these results were obtained with a large number of samples, they clearly indicate that the sorghum cultivar $CE_{145\text{-}66}$ contains higher concentrations of phenolics than other species, such as *Dennstaedtia punctilobula* leaves (0.4–1.2 mg/g equivalent tannic acid) (Dustin and Cooper-Driver, 1992), or *Triticum aestivum* roots (0.5 mg/g) and shoots (1.8 mg/g equivalent tyrosine) (Brown et al., 1984), or component parts of three other sorghum cultivars (leaves: 0.208–0.246, culms: 0.060–0.202, glumes: 0.120–0.193 mg/g, and roots: 0.166–0.215 mg/g equivalent acid ferulic) (Ben-Hammouda et al., 1995). The phenolic acid concentrations were also high in roots, where they reached one third of the total phenol concentration. Other genotypes contained (mg/g) 0.012–0.079 in glumes, 0.046–0.728 in leaves, 0.009–0.117 in culms, and 0.026–0.205 in roots (Ben-Hammouda et al., 1995). *Sorghum vulgare* had a total phenolic concentration of 0.421 mg/g in roots (Burgos-Leon et al., 1980). In contrast, Woodhead and Bernays (1978) found a higher phenolic acid level in sorghum leaves (5.4 mg/g). Burgos-Leon

et al. (1980) and Ben-Hammouda et al. (1995) found an allelopathic effect of these sorghum genotypes and species on other crops. The $CE_{145\text{-}66}$ genotype, with which phytotoxicity on groundnut germination has been shown, could be at least as allelopathic as those sorghum types. Sorghum sundangrass hybrid shoot tissues also have been reported to contain high levels of *p*-hydroxybenzoic acid and aldehydes that were well correlated with allelopathic potential of shoot tissues themselves (Weston et al., 1989). In our study, cinnamic phenolic monomers (*p*-coumaric, ferulic, and caffeic) were particularly abundant in $CE_{145\text{-}66}$. The phenolic acids identified in its vegetative parts were more varied than those previously found by the above authors in other sorghum varieties or species, probably because they were restrained by technical limits of detection. For instance, Woodhead and Bernays (1978) found no protocatechuic or syringic acids or vanillin in the sorghum genotype they studied, while Burgos-Leon et al. (1980) identified only *p*-coumaric, protocatechuic, and *o*-hydroxybenzoic acids. The other phenolic compounds they identified occurred at trace levels. More recently, Ben-Hammouda et al. (1995) found ferulic, *p*-coumaric, syringic, *p*-hydroxybenzoic, and vanillic acids.

Total phenol concentration in the aerial plant parts was higher than in the roots in 1996 and 1997, but phenolic acid content in roots was equal to or higher than that in aerial parts. The relatively high concentrations of phenolic acids in roots compared to aerial parts is important for the management of farmers' cropping systems because aerial parts are completely removed from the fields for animal feed or other uses. Decomposing roots are thus likely to be the major source of phenolics and, hence, of allelopathic effects.

Variations Between Sites and Between Years. Comparisons of phenolic concentrations in roots and aerial parts between Sagnanème and Médina revealed small but sometimes significant differences. Environmental and agronomic factors likely explain this variability, including climate and soil fertility, since the same variety was grown at both sites. Médina has a drier climate (324 mm rainfall in 1996 and 403 mm in 1997), compared to Sagnanème (622 mm in 1996 and 932 mm in 1997). Soils are less fertile in Médina than in Sagnanème (Sène, 1999). The drop (by a factor of 2) in phenolic production in aerial parts between 1996 and 1997 could be due mainly to climatic factors (rainfall, radiation), which were very different in the 1996 and 1997 growing seasons. Rainfall was about 1.5 times greater in Sagnanème, and 1.25 times greater in Médina in 1997 than in 1996. As a moderate water deficit is believed to stimulate secondary metabolite synthesis (Horner, 1990; Koricheva et al., 1998), the water stress that occurred in 1996 could have resulted in increased phenolic production and then increased allelopathic potential. The between-sites and between-years differences were far greater for total phenols than for phenolic acids, and for aerial parts than for roots. Nevertheless the same pattern of acids has been found through years and sites, for both aerial and subterranean parts.

Phenolic Compounds in Soils. Although both plant biomass (Sène, 1999) and phenolic production were much lower in 1997 than in 1996, the highest concentration of phenolics in soils associated with sorghum occurred in 1998, rather than 1997. Total phenol concentrations in the soils that supported $CE_{145\text{-}66}$ were about 10- and 100-fold higher than those reported by Ben-Hammouda et al. (1995) from soils under diverse sorghum genotypes (70–200 ng/g). The patterns of phenolic acids found in the soils we assayed were the same as those identified in aerial parts and roots, confirming that sorghum is probably the main source of phenolic acids for its associated soils. The differences in the respective weights of these acids between plant and soil were due to biotransformations that occur during senescence, such as transformation of syringic acid to syringaldehyde, and the emergence of vanillic acid and vanillin following lignin breakdown. These phenolics therefore probably came mainly from sorghum root exudates or root decomposition, although leaching of aerial parts by rain prior to cropping is possible. It is likely that these phenolic acids remaining in soils after the sorghum crop had a negative impact on groundnut germination (Sène et al., 2000). Two of the major phenolic acids found in the soils (*p*-hydroxybenzoic and vanillic acid) were the same as those identified by Ben-Hammouda et al. (1995), which they found to be transmitted to the soil by root exudates, and which were reported by Rice and others to be active as phytoinhibitors (Rice, 1984; Weston et al., 1989). In the same way, synergistic inhibitory effects of vanillic and *p*-hydroxybenzoic acids (Einhellig and Rasmussen, 1978), or *p*-coumaric and ferulic acids (Einhellig et al., 1982) towards grain sorghum have been observed.

The amounts of total phenols and phenolic acids in the soil were lower in 1997, when samples were taken two weeks after the start of the rainy season, than in 1998. However, phenolics were more abundant in sorghum roots in 1996 than in 1997. Phenolics can be released from rotting roots and degraded by microorganisms (Paul et al., 1994). Burgos et al. (1980) found that *Aspergillus* spp. and *Enterobacter cloacae* quickly degraded sorghum phenolic acids in natural soils. It is likely that a great portion of the phenolics released in 1997 by the roots had been broken down, before sampling, by microbial activity following rainfall and soil imbibition. In contrast, the dry soils from 1998 were collected before the renewal of these activities. The large between-years differences in soil phenolic content might be attributed, therefore, to the differences in the sampling date and not necessarily to differences in the sorghum phenolic content. This would indicate that breakdown occurs rapidly in soil, thus leading to a decrease in phenolic compounds as soon as the rainfall season begins. This is also supported by the fact that *p*-hydroxybenzoic acid, vanillic acid, and their associated aldehydes, which were highly concentrated in the soils in 1997, are the least easily degraded phenolics in the soil (Morita, 1981; Maciack and Harms, 1986).

The total phenol contents of soil from previous sorghum rows were higher than in soil from interrows in 1997 and in 1998. The phenolic acid content of

soil samples from rows and interrows of previous sorghum were low and not significantly different in 1997, but in 1998, previous sorghum rows had four or five times higher concentrations of phenolic acids than interrows. A greater suppressive effect on succeeding crops would be expected in the prior sorghum rows than in interrows in farmers' fields, as shown by Sène et al. (2000) in certain fields. The spatial difference in the allelopathic potential of the soil from prior sorghum rows and interrows suggests that detrimental allelopathic effects from sorghum can be partly managed by cultural practices. For instance, sowing the succeeding crop in previous sorghum interrows may limit the residual allelopathic effect of sorghum; waiting for the beginning of rainy season before seeding is also a way to benefit from phenolic breakdown in the soil.

Finally, the same qualitative pattern of phenolic acids was found in vegetative parts of sorghum (var. $CE_{145\text{-}66}$) through a wide range of growing conditions, and variability in content seemed more due to climate than cropping or soil factors, as differences between years appear more important than differences between or within sites.

Acknowledgments—The field experiments were supported by the ISRA/USAID project "Natural Resource Based on Agricultural Research" (NRBAR). Plant and soil analyses were supported by the Département AGER, INA P-G, Paris. We thank Xavier Puthod for his work. The authors thank François Pellissier for help and advice and Owen Parkes for revising the English text.

REFERENCES

BEN-HAMMOUDA, M., KREMER, R. J., MONOR, H. C., and SARWAR, M. 1995. A chemical basis for differential allelopathic potential of sorghum hybrids on wheat. *J. Chem. Ecol.* 21:775–786.

BROWN, P. H., GRAHAM, R. D., and NICHOLAS, D. J. D. 1984. The effects of manganese and nitrate supply on the levels of phenolics and lignin in young wheat plants. *Plant Soil* 81:437–440.

BURGOS-LEON, W., GANRY, F., NICOU, R., CHOPART, J. L., and DOMMERGUES, Y. 1980. Etude et travaux: Un cas de fatigue des sols induite par la culture du sorgho. *Agric. Trop.* 35:319–334.

CHOPART, J. L., and NICOU, R. 1973. Effet dépressif de cultures répétéés du sorgho dans les sols sableux du Sénégal. *Afr. Soils* 17:181–188.

DELAFOND, G., and BURGOS-LEON, W. 1978. Effet du précédent cultural sorgho sur la qualité de semences d'arachide 57-442 au Sénégal. ISRA-CNRA, Bambey, Senegal.

DUSTIN, C. D., and COOPER-DRIVER, G. A. 1992. Changes in phenolic production in the hay-scented fern (*Dennstaedtia punctilobula*) in relation to resource availability. *Biochem. Syst. Ecol.* 20:99–106.

EINHELLIG, F. A., and RASMUSSEN, J. A. 1978. Synergistic inhibitory effects of vanillic and *p*-hydroxybenzoic acids on radish and grain sorghum. *J. Chem. Ecol.* 4:425–436.

EINHELLIG, F. A., and RASMUSSEN, J. A. 1989. Prior cropping with grain sorghum inhibits weeds. *J. Chem. Ecol.* 15:951–960.

EINHELLIG, F. A., and SOUZA, I. F. 1992. Phytotoxicity of sorgoleone found in grain sorghum root exudates. *J. Chem. Ecol.* 18:1–11.

EINHELLIG, F. A., SCHON, M. K., and RASMUSSEN, J. A. 1982. Synergistic effects of four cinnamaic acid compounds on grain sorghum. *J. Plant Growth Regul.* 1:251–258.

EINHELLIG, F. A., RASMUSSEN, J. A., HEJL, A. M., and SOUZA, I. F. 1993. Effects of root exudate sorgoleone on photosynthesis. *J. Chem. Ecol.* 19:369–375.

GUENZI, W. D., and MCCALLA, T. M. 1962. Inhibition of germination and seedling development by crop residues. *Soil Sci. Soc. Am. Proc.* 26:456–458.

GUENZI, W. D., and MCCALLA, T. M. 1966. Phenolic acids in oats, wheat, sorghum and corn residues and their phytotoxicity. *Agron. J.* 58:303–304.

GUPTA, R. K., and HASHLAM, E. 1978. Sorghum polyphenols. *J. Chem. Soc. Perkin I* 1978:892–896.

HORNER, J. D. 1990. Nonlinear effects of water deficits on foliar tannin concentrations. *Biochem. Syst. Ecol.* 18:211–213.

KORICHEVA, J., LARSSON, S., HAUKIOJA, E., and KEINÄNEN, M. 1998. Regulation of woody plant secondary metabolism by resource availability: Hypothesis testing by means of meta-analysis. *Oïkos* 83:212–226.

MACIAK, F., and HARMS, H. 1986. The effect of agricultural utilization on the composition and yield of phenolic acids in low peat soils. *Plant Soil* 94:171–178.

MORITA, H. 1981. Changes in phenolic composition of peat soil due to cultivation. *Soil Sci.* 131:30–33.

NICOU, R. 1980. Etude des successions culturales au Sénégal. Résultats et méthodes. *Agric. Trop.* 33:51–61.

PAUL, J. W., COVERT, J. A., and BEACHAMP, E. G. 1994. Influence of soil temperature and moisture on water-soluble phenolic compounds in manured soils. *Can. J. Soil Sci.* 74:111–114.

RICE, E. L. 1984. Allelopathy. Academic Press, New York, 422 pp.

SÈNE, M. 1999. Analyse de l'influence des systèmes de culture sur la variabilité des rendements du sorgho [*Sorghum bicolor* (L.) Moench] et sur son effet allélopathique dans le Sine-Saloum. Thèse de doctorat, INA P-G, Paris.

SÈNE, M., DORÉ, T., and PELLISSIER, F. 2000. Soil phenolic acids under rows and interrows of prior sorghum (*Sorghum bicolor*) crop inhibit germination and emergence of peanut (*Arachis hypogea*). *J. Chem. Ecol.* 26:625–637.

WESTON, L. A. 1996. Utilization of allelopathy for weed management in agroecosystems. *Agron. J.* 88:860–866.

WESTON, L. A., HARMON, R., and MOHLER, C. L. 1989. Allelopathic potential of sorghum–sudangrass hybrid (sudex). *J. Chem. Ecol.* 15:1855–1865.

WESTON, L. A., NIMBAL, C. I., and CZARNOTA, M. A. 1997. Activity and persistance of sorgoleone, a long-chain hydroquinone produced by *Sorghum bicolor*, pp. 509–516, *in* The 1997 Brighton Crop Protection Conference—Weeds.

WESTON, L. A., NIMBAL, C. I., and JEANDET, P. 1999. Allelopathic potential of grain sorghum [*Sorghum bicolor* (L.) Moench] and related species, pp. 467–477, *in* Inderjit, K. M. M. Dakshini and C. L. Foy, (eds.). Principles and Practices in Plant Ecology. Allelochemical Interactions. CRC Press, Boca Raton, Florida.

WOODHEAD, S., and BERNAYS, E. A. 1978. The chemical basis of resistance of *Sorghum bicolor* to attack by *Locusta migratoria. Entomol. Exp. Appl.* 24:123–144.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

CHEMICAL ECOLOGICAL CHARACTERISTICS OF HERBIVORY OF *Siparuna guianensis* SEEDS BY BUFFY-HEADED MARMOSETS (*Callithrix flaviceps*) IN THE ATLANTIC FOREST OF SOUTHEASTERN BRAZIL

NAOMI K. SIMAS,[1] STEPHEN F. FERRARI,[2] SONIA N. PEREIRA,[3] and GILDA G. LEITÃO[1,*]

[1]*Núcleo de Pesquisas de Produtos Naturais*
Universidade Federal do Rio de Janeiro
Bloco H, CCS, Ilha do Fundão
21.941-590, Rio de Janeiro, Brazil

[2]*Departamento de Genética*
Universidade Federal do Pará
Caixa Postal 8607, 66.075-900 Belém - PA, Brazil

[3]*Departamento de Análises Clínicas e Toxicológicas*
Fac. Farmácia, UFRJ
B1. A, 2° andar, CCS, Ilha do Fundão
21941-590, Rio de Janeiro, RJ, Brazil

(Received May 1, 2000; accepted August 30, 2000)

Abstract—The buffy-headed marmoset (*Callithrix flaviceps*) is apparently the only predispersal herbivore of the seeds of *Siparuna guianensis* at the Caratinga Biological Station in southeastern Brazil. Both the fruit receptacles and the frutioles (seeds) of *S. guianensis* are relatively rich in nutrients such as carbohydrates, proteins, and lipids, but the receptacles contain high concentrations of benzylisoquinoline alkaloids. The latter presumably act as a qualitative chemical defense, impeding the access of potential predators to the alkaloid-poor frutioles. However, on ripening, the receptacle splits open, exposing the frutioles, which enables *C. flaviceps* to avoid the plant's chemical defenses effectively. Taking care to avoid contact with the receptacle, the marmosets pluck out the frutioles and ingest the seeds. Qualitative and quantitative changes (in particular a significant reduction in daily ranging) in the marmosets' behavior during the period when *S. guianensis* frutioles were accessible indicate that this was a preferred plant resource. This is the first record of the consumption of *S. guianensis* seeds by callitrichine monkeys (which are not known to be systematic seed eaters), despite the fact that both are widely distributed in the Neotropics. It is thus possible that the

*To whom correspondence should be addressed. e-mail: ggleitao@nppn.ufrj.br

0098-0331/01/0100-0093$19.50/0

behavioral strategy observed here is a unique phenomenon resulting from a specific combination of factors, including the abundance of *S. guianensis* within the study area. The lack of other records may nevertheless be a result of insufficient sampling effort, in which case, the feeding strategies of callitrichines may have an important influence on the reproductive biology of *S. guianensis* in many areas.

Key Words—*Callithrix flaviceps*; *Siparuna guianensis*; Monimiaceae; chemical ecology; benzylisoquinoline alkaloids; seed eating; Brazilian Atlantic Forest.

INTRODUCTION

Marmosets (*Callithrix* spp., sensu Schneider and Rosenberger, 1996), the smallest-bodied extant simians (mean adult body weights 125–350 g), are widely distributed in the Neotropics south of the Amazon/Japurá river system and east of the Andes (Hershkovitz, 1977; Rylands et al., 1993). The key feature of marmoset ecology is a unique set of adaptations for the dietary exploitation of plant exudates, which these monkeys are able to obtain systematically by gouging the bark of gum-producing plants with their specialized lower anterior dentition (Coimbra-Filho and Mittermeier, 1976) and digest efficiently by fermentation in an enlarged cecum (Ferrari and Martins, 1992). Marmosets thus have access to an alternative to fruit as a source of carbohydrates that allows them to occupy, successfully, marginal and highly seasonal habitats in which most larger-bodied platyrrhines are rare or even absent.

Marmosets also exploit a variety of other plant resources, including fruit, nectar, and flowers, as well as fungi and both vertebrate and invertebrate prey (Stevenson and Rylands, 1988; Ferrari et al., 1996; Passamani, 1996; Veracini, 1997; Digby and Barreto, 1998). However, the systematic eating of seeds—as opposed to their incidental ingestion during fruit feeding—has not been recorded previously for either marmosets or other callitrichines (*Callimico*, *Leontopithecus*, and *Saguinus*), that are not specialized for gummivory. By contrast, seed eating is relatively common in medium-sized (body weight 2–4 kg) platyrrhines, the sakis and uakaris (Pitheciinae), and the capuchins (*Cebus* spp.). The former are specialized morphologically (Kinzey and Norconk, 1993) for this type of feeding behaviour, whereas capuchins rely more on their behavioral adaptability (e.g., Peres, 1991). This suggests that body size may be one important factor limiting seed-eating in callitrichines.

Chemical defense is one of the numerous mechanisms used by plants to deter mammalian herbivory, and the vast majority of deterrent substances are products of the secondary metabolism (Harborne, 1991). Most, if not all higher plants contain some kind of organic compound that is potentially toxic to animal browsers (Harborne and Turner, 1984). The feeding behavior of herbivores is

strongly influenced by secondary products, which determine the texture, taste, and flavor of plants (Luckner, 1990), and reinforce foraging strategies aimed at avoiding or minimizing their toxic effects (Leitão et al., 1999). In broad terms, plant chemical defenses can be seen as being either qualitative or quantitative. Quantitative defenses, such as essential oils, resins, and tannins, tend to reduce the ability of herbivores to digest or assimilate nutrients, whereas qualitative secondary compounds have a cumulative toxic effect (e.g., alkaloids, cyanogenic glycosides) (Luckner, 1990).

The present study focuses on the qualitative chemical defenses, in particular benzylisoquinoline alkaloids, of the Neotropical tree *Siparuna guianensis* (Monimiaceae) and their effects on the feeding behavior of buffy-headed marmosets (*Callithrix flaviceps*) in the Atlantic Forest of southeastern Brazil. The predispersal consumption of *S. guianensis* seeds by *C. flaviceps* appears to have been the result of a combination of specific factors, such as seed size, morphology, and chemical/nutrient composition, in addition to the relative abundance of the resource at the study site. While the evidence indicates that *S. guianensis* seeds were a preferred resource, the phytochemical analysis of *S. guianensis* fruit receptacles and frutioles presented here indicates that the fruit's qualitative chemical defenses dictated marmoset feeding behavior. The monkeys only ingested the lipid-rich frutioles when these were exposed by the splitting open of the mature fruits, which allowed them to avoid contact with the alkaloid defenses of the receptacles. This behavioral strategy may have a major impact on the reproductive cycle of *S. guianensis*, at least on a local scale.

METHODS AND MATERIALS

Study Site and Species

The present study took place in an area of secondary forest, dominated by *Anadenanthera peregrina* (Mimosoidae) trees, at the 880-ha Caratinga Biological Station (19°50′S 41°50′W) in Minas Gerais (Ferrari, 1991; Strier, 1992). A 50-m × 50-m trail grid was established within the study area to facilitate both the behavioral monitoring of study group members and the mapping of group movements.

The buffy-headed marmoset, *Callithrix flaviceps*, is endemic to the highland Atlantic forest of southeastern Brazil. Its geographic range, in eastern Minas Gerais and western Espírito Santo, is one of, if not the smallest of any marmoset species (Rylands et al., 1993). Like other Atlantic forest marmosets, *C. flaviceps* is a gummivore–faunivore, but (together with the closely related *Callithrix aurita*) exhibits characteristics such as relatively large home ranges (over 35 ha) that may be related to the comparatively harsh seasonal conditions it faces in the Serra da Mantiqueira mountain range (Ferrari et al., 1996).

FIG. 1. Split open fruit of *Siparuna guianensis* (1.0–1.5 cm) showing fruit receptacle with frutioles. (Photo by S. S. Ferrari.)

Siparuna guianensis (Figure 1) is found throughout most of tropical South America, northwards as far as Costa Rica (Duke, 1962). It is a monoecious shrub or tree that generally reaches a height of 5–10 m. The globose fruit receptacle is 1–1.5 cm in diameter, purple or red in color, and smells "strongly of lemon"

(Renner and Hausner, 1997). When ripe, the fruit receptacle splits open irregularly, revealing two to eight small frutioles (maximum dimension 0.5–0.8 cm), with a white, membranous epicarp (Pereira, 1991).

Behavioral Data

The behavior of a free-ranging *C. flaviceps* group, which contained 11–15 members, was monitored systematically between August 1985 and August 1986. Group members were fully habituated to the presence of human observers by the beginning of the study period. Quantitative behavioral data were collected using 1-min scan samples at 5-min intervals throughout the daily activity period (see Ferrari and Rylands, 1994) on 8–10 days each month.

During each scan, the initial behavior of each observed group member was assigned to one of five basic categories (forage, feed, travel, rest, miscellaneous) and, in the case of feeding records, food items being ingested were identified in relation to both the type of resource being exploited (i.e., fruit part, nectar, gum, etc.) and the taxon. Whenever possible, plant food sources were marked and mapped, and specimens were collected for identification.

The location of the group (central point of group dispersion) in relation to the trail grid was recorded in each scan, together with complementary information on group movements. These records were used to map group movements (daily path) and the occupation of 50-m × 50-m quadrats (day range), as defined by the trail grid.

Plant Material

Siparuna guianensis fruits were collected by José Rímoli at Caratinga Biological Station in February 1995. A voucher specimen is deposited in the herbarium of the Federal University of Minas Gerais (UFMG, Belo Horizonte, Minas Gerais, Brazil).

General Analytical Methods

^{1}H NMR spectra (CD_3OD or $CDCl_3$) were recorded on a Varian-Gemini 200 or 300 instrument. Mass spectra were recorded on a VG Autospec Mass Spectrometer (70 eV). GC-MS spectra were recorded with a Hewlett-Packard (HP) 5890 series II gas chromatograph, coupled to a MS HP mass selective detector 5972 equipped with a fused silica capillary (DB-1) column (30 m × 0.25 mm, 25-μm film thickness: Supelco, Inc.). Temperature programming was from 50°C to 270°C at 3°C/min, then maintained at 270°C for 20 min. Products were identified by comparison of their mass spectral fragmentation patterns with the National Bureau of Standards (NBS) Mass Spectra Data Base (Keller and

Milne, 1974) and mass spectra and GC retention times of standard compounds. UV spectra were obtained on a Hitashi 340 spectrometer and IR spectra on a Biorad FT-80 spectrophotometer. The HPLC apparatus comprised a Lachrom HPLC System (Merck) equipped with a D7000 interface, L-7100 pump, L-7450A diode array detector (DAD), and L-7612 solvent degasser. The injections were done manually with an injector valve (Rheodyne) equipped with a 20-μl sample loop. Data were analyzed in a Multi-Hauser Mixed Standard solution supported on a Pentium II 250-MHz (Compac) computer. A Lichrosorb RP-18 column (5 μm $\times$ 250 mm ID) N. 738342, coupled to a guard column Lichrocart 250-4 HPLC cartridge was employed. The sample elution was done with a mobile phase composed of (A) 0.2 M sodium perchlorate–60% perchloric acid (1000 : 0.2) and (B) acetonitrile, with a gradient elution of A and B changing from A : B 90 : 10 to 10 : 90 (linear gradient for 50 min); flow rate was 2.0 ml/min, monitoring was at 280 nm for peak identification.

Extraction and Isolation of Compounds

Five hundred grams of *S. guianensis* fruits were air dried and the frutioles were separated from the fruit receptacles. Each organ was ground and extracted continuously with hexane and MeOH at room temperature. The hexane and methanolic solutions were concentrated separately under reduced pressure, resulting in 15.2 g and 11.9 g of crude hexane extracts and 31.7 g and 73.27 g of crude methanolic extracts, from the frutioles and fruit receptacles, respectively. A portion of the methanolic extracts was suspended in MeOH–H_2O (9 : 1) and partitioned between hexane, dichloromethane, ethyl acetate, and butanol.

Fruit Receptacles

Part of the hexane extract (1.45 g) obtained from the partition of the methanolic extract was subjected to column chromatography over silica gel using a hexane–ethyl acetate gradient. A total of 50 fractions was collected. Fraction 5 was recrystalized from hexane to produce glucosyl sitosterol (3.5 mg) (Sakikabara et al., 1983).

The dichloromethane extract (2.1 g) was further separated into soluble portions of hexane (378 mg) and methanol (1401 mg). The hexane-soluble fraction was chromatographed over silica gel and eluted with AcOEt, with increasing amounts of MeOH, resulting in 86 fractions. Fractions 20–23 were further purified by washing with heated MeOH to afford nantenine (6.0 mg) (Guinaudeau et al., 1988).

The methanolic fraction of the dichloromethane extract was chromatographed over Si gel, eluted with AcOEt and MeOH. Three groups of eluates showed the presence of distinct spots on TLC. The AcOEt–MeOH 20%

(25.0 mg) eluate was purified twice by preparative TLC ($CHCl_3$–AcOEt–MeOH 2 : 3 : 0.5) to yield liriodenine (9.0 mg) (Guinaudeau et al., 1988). The AcOEt–MeOH 30% (40.0 mg) eluate was chromatographed over Sephadex LH-20 and eluted with MeOH 100%. Fractions 30–40 were combined and rechromatographed over the same resin to afford *N*-methyllaurotetanine (3.1 mg) (Guinaudeau et al., 1988). The third eluate (AcOEt–MeOH 40%) was rechromatographed over XAD-2 resin eluted successively with MeOH–AcOEt to give 100 fractions. Fractions A (MeOH, 10.0 mg) and B (AcOEt, 13.0 mg) were obtained from this column. Fraction A, by preparative TLC (10% $CHCl_3$/AcOEt plus 2% of NH_4OH), furnished norglaucine (3.3 mg) and asimilobine (3.3 mg) (Guinaudeau et al., 1988), while fraction B, by using the same procedure, furnished anonaine (3.5 mg) and nornantenine (2.7 mg) (Guinaudeau et al., 1988).

Analysis of Frutiole Fatty Acid Methyl Esters

The crude hexane extract was treated with MeOH–NaOH 70% for hydrolysis and the fatty acids were separated from the mixture through successive washes with ethyl ether, rendering 67.7% of fatty acid methyl esters following a derivation procedure using diazomethane. The organic phase was removed by syringe (30 μl) and analyzed within 80 min by GC-MS. The instrument and conditions are described above.

Detection of Frutiole Alkaloids and Flavonoids by HPLC

Each partition of the methanolic extract from the frutioles was analyzed by HPLC, as described above, for the qualitative identification of the alkaloids previously isolated in the fruit receptacles. The samples were diluted with methanol and analyzed within 50 min. The dichloromethane partition indicated the presence of an aporphinic alkaloid with a retention time of 10.5 min and equivalent area of 0.69%. The ethyl acetate partition showed three flavonoids with retention times of 8.0, 8.4, and 8.6 min, and equivalent areas of 5.55%, 4.9%, and 6.56%, respectively. The butanolic partition showed peaks corresponding to flavonoids, in 6.7, 6.9, and 7.3 min and 4.1%, 4.0%, and 7.7% of the equivalent area, respectively.

Glucosyl Sitosterol. Crystalline solid mp: 270–280°C. ^{1}H NMR (200 MHz, CD_5N, ppm) δ: 4.03 (m, 3H), 5.37 (m, 6H), 5,08 (d, 1H, $J = 7.5$ Hz).

Nantenine. EI-MS (70 eV) (%), m/z 339 [M^+] (87), 338 [M^+-1] (100), 324 (35), 308 (30), 296 (15), 281 (13), 265 (19), 165 (7). ^{1}H NMR (200 MHz, CD_3OD, ppm) δ: 3.65 (s, C_1-OMe), 3.87 (s, C_2-OMe), 6.59 (s, H_3), 2.55 (s, *N*-Me), 7.93 (s, H_{11}), 6.75 (s, H_8), 5.97 (dd, C_9-OCH_2O-C_{10}). UV MeOH (λ_{max}, nm): 217.0, 273.8, 283.0, 307.0, 320.0. $[\alpha]_D^{25}$: +83 ($c = 9 \times 10^{-4}$, MeOH).

Liriodenine. EI-MS (70 eV) (%), *m/z* 276 [M^+] (20), 275 [M^+-1] (100), 247 (13) 246 (10). ^{1}H NMR (200 MHz, CD_3OD, ppm) δ: 6.4 (s, C_1-OCH_2O-C_2), 7.3 (s, H_3), 7.6 (dt, H_9, J = 5 Hz), 7.8 (dt, H_{10}), 8.6 (dd, H_8), 8.65 (dd, H_{11}), 8.9 (d, H_5, J = 5 Hz). UV MeOH (λ_{max}, nm): 245.8, 268.6, 306.6, 413.0. UV MeOH/H_2SO_4 (λ_{max}, nm): 255.4, 277.0, 327.8, 450.8 IV (KBr, cm^{-1}): 565, 953, 1065, 1657.

N-*Methyl-Laurotetanine.* EI-MS (70 eV) (%), *m/z* 341 [M^+] (82), 340 [M^+-1] (100), 326 (44), 310 (20), 298 (18), 283 (14). ^{1}H NMR (200 MHz, $CDCl_3$, ppm) δ: 2.61 (s, *N*-Me), 3.64 (s, C_1-OMe), 3.88 (s, C_2-OMe), 3.89 (s, C_{10}-OMe), 6.58 (s, H_8), 8.05 (s, H_{11}). UV MeOH (λ_{max}, nm): 216.2, 281.2, 302.6. UV MeOH/NaOH (0.1N) (λ_{max}, nm): 216.2, 290.0, 316.0. $[\alpha]_D^{25}$: +78° ($c = 1.1 \times 10^{-3}$; $CHCl_3$).

Norglaucine. EI-MS (70 eV) (%), *m/z* 341 [M^+] (82), 340 [M^+-1] (100), 326 (45), 310 (20), 267 (19). ^{1}H NMR (200 MHz, CD_3OD, ppm) δ: 3.7 (s, C_1-OMe), 3.9 (s, $C_{2,9,10}$-OMe), 6.58 (s, H_3 and H_8) 8.1 (s, H_{11}). UV MeOH (λ_{max}, nm): 219.8, 282.4, 302.6. $[\alpha]_D^{25}$: +100° ($c = 2.0 \times 10^{-3}$ MeOH).

Asimilobine. EI-MS (70 eV) (%), *m/z* 267 [M^+] (61), 266 [M^+-1] (100), 251 (26), 236 (19). ^{1}H NMR (300 MHz, CD_3OD, ppm) δ: 3.6 (s, C_1-OMe), 6.78 (s, H_3), 7.4 (s, $H_{8,9,10}$), 8.4 (s, H_{11}). UV MeOH (λ_{max}, nm): 226, 271, 315. $[\alpha]_D^{25}$: −260° ($c = 11.5 \times 10^{-3}$; MeOH).

Anonain. EI-MS (70 eV) (%), *m/z* 265 [M^+] (45), 264 [M^+-1] (100), 236 (16) 206 (18). ^{1}H NMR (300 MHz, CD_3OD, ppm) δ: 5.9 and 6.1 (d and d, C_1-OCH_2O-C_2), 6.6 (s, H_3), 7.2–7.3 (m, H_8 and H_{10}), 8.1 (m, H_{11}). UV MeOH (λ_{max}, nm): 235, 271, 318. $[\alpha]_D^{25}$: −69° ($c = 2.1 \times 10^{-3}$; MeOH).

Nornantenine. EI-MS (70 eV) (%), *m/z* 325 [M^+] (70), 324 [M^+-1] (100), 310 (20), 294 (25), 281 (17), 265 (13), 250 (10) 165 (7). ^{1}H NMR (200 MHz, CD_3OD, ppm) δ: 3.6 (s, C_1-OMe), 3.85 (s, C_2-OMe), 6.79 (s, H_3), 6.81 (s, H_8), 5.9 (s, C_9-OCH_2O-C_{10}), 7.8 (s, H_{11}). U.V. MeOH (λ_{max}, nm): 234.8, 282.4, 309.0. $[\alpha]_D^{25}$: +100° ($c = 2.0 \times 10^{-3}$; $CHCl_3$).

Nutritional Content of Siparuna guianensis *Frutioles and Fruit Receptacles*

Humidity and mineral residues were estimated after desiccation (at 105°C) and incineration (in a muffle, at 550°C), respectively, according to standard procedures (Pregnolato and Pregnolato, 1985). Lipid content was estimated after extraction with diethyl ether in a Soxhlet apparatus, according to standard procedures (Pregnolato and Pregnolato, 1985). Protein content was estimated using Jones's (1941) factor: protein = total nitrogen (N) × 6.25, using a semimicro-Kjeldahl method (Horowitz et al., 1984). Carbohydrate content was estimated by difference. The caloric value was calculated using conversion factors (9.0 for lipids, 4.0 for carbohydrates, and 4.0 for proteins), and the results are expressed in kilocalories per 100 g.

RESULTS

Phytochemical Analysis of Frutioles and Fruit Receptacles of Siparuna guianensis

The hexane-soluble fraction of the methanolic extract of fruit receptacles of *S. guianensis* contained no alkaloids. Glucosyl sitosterol was isolated from this fraction. Seven aporphine alkaloids were isolated from the dichloromethane partition of the methanolic extract from the fruit receptacles (Table 1), only one of which—liriodenine—was also found in the frutioles.

Phytochemical investigation of the dichloromethane partition of the methanolic extract of the frutioles, by contrast, exhibited a very low concentration of alkaloids. In addition to liriodenine, HPLC analysis of this fraction revealed traces of other aporphinic alkaloids, more polar than those found in the fruit receptacles. Examination of the ethyl acetate and butanolic partitions revealed the absence of alkaloids, but the presence of flavonoid derivatives (Table 1).

The hexane extract from the frutioles was subjected to saponification, which revealed four different fatty acid methyl esters after methylation (Table 2). Despite other differences in the composition of fruit receptacles and frutioles, their nutritional content was relatively similar (Table 3).

Behavior of C. flaviceps

The marmosets' diet consisted primarily of plant exudates, insects, and small vertebrates. Reproductive plant parts (RPPs) only made a significant con-

TABLE 1. COMPOUNDS IDENTIFIED IN *Siparuna guianensis* FRUTIOLES AND FRUIT RECEPTACLES

	Dry matter (%)	
Compound	Fruit receptacles	Frutioles
Alkaloids		
Liriodenine	0.64	0.03
Nantenine	1.58	
N-Methyl-laurotetanine	0.21	
Norglaucine	0.23	
Asimilobine	0.23	
Anonaine	0.24	
Nornantenine	0.19	
Polar alkaloids		<0.01
Flavonoids		
		Presence detected by HPLC

TABLE 2. MASS SPECTROMETRIC DATA OF COMPOUNDS IDENTIFIED IN HEXANE EXTRACT OF *Siparuna guianensis* FRUTIOLES

Compound	*m/z* (% relative abundance)	Relative abundance (%)
Palmitic acid	270(3), 227(3), 143(8), 87(55), 74(100), 55(36), 43(58)	31.66
Linoleic acid	294(3), 263(2), 109(15), 95(34), 81(69), 67(100), 55(83), 41(93)	37.10
Oleic acid	264(4), 221(2), 180(3), 96(21), 83(31), 69(47), 55(100), 41(90)	27.09
Stearic acid	298(4), 255(2), 199(3), 143(7), 87(48), 74(100), 55(37), 43(65)	4.15

tribution to the study group's diet in the rainy season months of January and February, 1986, when they made up 64.7% and 52.7% of plant feeding records, respectively (the remainder being provided by gums). Apart from November, when fruit was the item consumed in 17.6% of plant-feeding records, RPPs contributed no more than 5% of records in any one month. This reflects the apparent lack of edible fruit within the study group's home range throughout most of the study period. Only 17% of 1329 trees marked for phenological monitoring produced fruit during the study period.

In January, the fruit of a single tree species (*Allophyllus* sp.) was consumed in 94.5% of the records of feeding on RPPs, while in February *Siparuna guianensis* provided 95.2% of the records. Both these relatively small tree species are characteristic of secondary forest and were exceptionally abundant in some parts of the group's home range. Approximately one third (32.3%) of the trees that bore fruit in the phenology sample were members of these two species.

When feeding on *Allophyllus* drupes, the marmosets consumed the sweet-tasting mesocarp surrounding the seed, which was occasionally swallowed—and defecated—intact. When feeding on *S. guianensis*, by contrast, the monkeys

TABLE 3. NUTRITIONAL CONTENT OF *Siparuna guianensis* FRUTIOLES AND FRUIT RECEPTACLES

Component	Frutioles (%)	Fruit Receptacles (%)
Humidity	7.87	10.56
Mineral residue	1.82	4.92
Proteins	8.75	9.53
Lipids	13.74	9.74
Carbohydrates	67.82	65.25
Caloric value (kcal/100 g)	429	386

ingested the seeds (frutioles) only, and ignored the fleshy receptacle. Feeding on *S. guianensis* began towards the end of January, when the ripe fruits began to split open, revealing frutioles embedded in the inner surface of the receptacle. The marmosets extracted the seeds with their mouths, apparently taking care not to touch the receptacle, and masticated them thoroughly prior to swallowing.

The seeds of *S. guianensis* constituted the single most important item (41.0% of feeding records) of the marmosets' diet in February. However, despite being a valuable source of lipids and protein (Table 3), the availability of *S. guianensis* seeds did not appear to modify significantly the foraging strategies of the study group members during February in comparison with neighboring months. The marmosets devoted a similar proportion (18.8%) of their activity time to foraging for animal prey compared with January (18.4%) and March (19.9%), the latter being a month of typically low fruit consumption. The net effect of the availability of *S. guianensis* seeds on the *C. flaviceps* diet was thus the same as that of *Allophyllus* drupes—a marked reduction in the consumption of exudates.

The principal behavioral change observed in February was a pronounced shift in ranging patterns. February was the only month in which group movements were concentrated in the southern half of the home range, within the zone of overlap with the ranges of neighboring groups. Some of the 50-m × 50-m quadrats in this area were visited eight or nine times during the 10 days of observation, whereas in other months (including July, for which the smallest monthly range was recorded), no quadrat was visited more than seven times. This shift in range use was clearly related to the distribution of *S. guianensis* within the study group's home range. Day ranging was also reduced significantly ($t = -1.77$, $P = 0.039$, one-tailed, $df = 123$) in February (mean daily path = 1052.5 ± 211.4 m, $N = 10$) in comparison with all other months (mean = 1247.6 ± 341.3 m, $N = 115$).

DISCUSSION

The exploitation of *S. guianensis* fruit by *C. flaviceps* at the Caratinga Biological Station is unusual for a number of reasons, not least because this is apparently the first record of systematic seed consumption by a callitrichine primate. In addition, the monkeys not only ignored the fruit receptacles, but actually avoided all contact with them, despite the fact that their nutritional composition was relatively similar to that of the seeds (frutioles), apart from some lipids that were more abundant in the seeds (Table 3). Saponification of this fraction revealed that palmitic and linoleic acids—common fatty acids—are the major lipid constituents (Table 2). Both frutioles and fruit receptacles were also potentially good sources of carbohydrates.

By contrast, a marked difference was found between frutioles and fruit

receptacles in terms of the content and composition of alkaloids. The fleshy receptacles contained seven different benzylisoquinoline alkaloids (Table 1), whereas the frutioles contained only one. These alkaloids, which have a variety of pharmacological effects (e.g., sedative, cytotoxic, hypotensive) on the central nervous system (Rios, 1989), appear to function as a qualitative defense against predispersal seed predation by impeding contact with the frutioles, one which is effective against all potential predators except *C. flaviceps*. It is interesting to note, in this context, that frutioles dropped on the ground were retrieved by foraging ants in a matter of seconds. The marmosets' exploitation of seeds exposed in ripe, split fruits was clearly an efficient strategy for the avoidance of chemical defenses. As *S. guianensis* fruits exhibited no external signs of ripeness, splitting may be an important behavioral cue, especially if immature seeds contain higher concentrations of alkaloids.

Plant toxins, like those of *S. guianensis*, are often bitter and can be detected easily by taste, allowing consumers to avoid the parts of the plant that contains it (Harborne and Turner, 1984). Apart from nutritional considerations, vertebrates select plant food species on the basis of taste and aroma (Harborne and Turner, 1984). Otherwise nutritious plants may thus be rejected not only because of the presence of noxious chemicals or toxins but also because of their disagreeable flavor, although this does not appear to be the case of *Siparuna guianensis*, which has a strong lemonlike scent.

In addition to their behavioral flexibility, the ability of *C. flaviceps* to exploit *S. guianensis* seeds would appear to be related to a number of other factors, such as the small body size of the marmosets. Tufted capuchins (*Cebus apella*) are also present at Caratinga, but apparently do not exploit *S. guianensis* seeds (J. Rímoli, personal communication). At least as adaptable behaviorally as marmosets, capuchins are much larger, with adult body weights of 2–4 kg. Exploitation of the small, piecemeal-ripening *S. guianensis* seeds may thus be energetically too inefficient for the capuchins. It is also possible that successful avoidance of the plant's chemical defenses depends on the small size of the seed gatherer.

What does remain unclear, however, is why the eating of *S. guianensis* seeds has not been reported in other callitrichines, despite the extensive data now available for this subfamily in terms of both species and study sites (e.g., Mittermeier et al., 1988; Rylands, 1993; Corrêa, 1995; Passamani, 1996; Veracini, 1997; Oliveira and Ferrari, 2000). Both *S. guianensis* and the Callitrichinae are widely distributed in the Neotropics, and most callitrichines exhibit a preference for disturbed or secondary habitats (Sussman and Kinzey, 1984). It is nevertheless possible that the present records were dependent on a unique or unusual combination of factors, such as the high density of *S. guianensis* at the Caratinga study site. An additional, intriguing possibility is that the behavior of *C. flaviceps* at Caratinga is a recent innovation that has yet to be transmitted "culturally"

to other populations, a situation encountered in some other nonhuman primates (e.g., Kawamura, 1965; Nishida and Uehara, 1980). Additional studies of *C. flaviceps* at other sites would help evaluate this possibility.

The changes observed in the behavior of the *C. flaviceps* study group during February 1986 indicate clearly that *S. guianensis* seeds were a preferred resource, reflecting both their abundance within the study area and their nutritional value. The significance of the reduction in daily ranging should not be underestimated, given the energetic constraints on these small-bodied primates (Goldizen et al., 1988; Ferrari, 1993). The marmosets' intensive predispersal exploitation of *S. guianensis* seeds may also have significant implications for the plant's reproductive biology at this site. It also remains to be seen how the herbivore–plant relationship (or lack thereof) influences reproductive processes in other *S. guianensis* populations.

Acknowledgments—Fieldwork on *C. flaviceps* was supported by the Brazilian National Research Council (CNPq), the Medical Research Council of Great Britain, the A. H. Schultz-Stiftung, London University Central Research Fund, the Boise Fund and the Leakey Trust. Laboratory work on the phytochemistry of *S. guianensis* was supported by CNPq. We would like to thank Bob Martin, Cida Lopes, and José Rímoli for their help with different stages of the fieldwork.

REFERENCES

COIMBRA-FILHO, A. F., and MITTERMEIER, R. A. 1976. Exudate eating and tree-gouging in marmosets. *Nature* 262:630.

CORRÊA, H. K. M. 1995. Ecologia e comportamento alimentar de um grupo de saguis-da-serra-escuros (*Callithrix aurita* E Geoffroyi 1812) no Parque Estadual da Serra do Mar, Núcleo Cunha, São Paulo, Brasil. Unpublished MSc dissertation. Universidade Federal de Minas Gerais, Minas Gerais, Brazil.

DIGBY, L. J., and BARRETO, C. E. 1998. Vertebrate predation in common marmosets. *Neotrop. Primates* 6:124–126.

DUKE, J. A. 1962. Monimiaceae, p. 230, *in* R. E. Woodson Jr. and R. W. Schery (eds.). Annals of the Missouri Botanical Garden, XLIX(3/4), Flora of Panama, Part IV.

FERRARI, S. F. 1991. Preliminary report on a field study of *Callithrix flaviceps*, pp. 159–171, *in* A. B. Rylands and A. T. Bernardes (eds.). A Primatologia no Brasil - 3. Sociedade Brasileira de Primatologia, Belo Horizonte.

FERRARI, S. F. 1993. Ecological differentiation in the Callitrichidae, pp. 314–328, *in* A. B. Rylands (ed.). Marmosets and Tamarins: Systematics, Ecology and Behaviour. Oxford University Press, Oxford.

FERRARI, S. F., and MARTINS, E. S. 1992. Gummivory and gut morphology in two sympatric callitrichids (*Callithrix emiliae* and *Saguinus fuscicollis weddelli*) from western Brazilian Amazonia. *Am. J. Phys. Anthropol.* 88:97–103.

FERRARI, S. F., and RYLANDS, A. B. 1994. Activity budgets and differential visibility in field studies of three marmosets (*Callithrix* spp.). *Folia Primatol.* 63:78–83.

FERRARI, S. F., CORRÊA, H. K. M., and COUTINHO, P. E. G. 1996. Ecology of the "southern" marmosets (*Callithrix aurita* and *Callithrix flaviceps*): How different, how similar? pp. 157–171, *in* M. A. Norconk, A. L. Rosenberger, and P. A. Garber (eds.). Adaptive Radiations of Neotropical Primates. Plenum Press, New York.

GOLDIZEN, A. W., TERBORGH, J., CORNEJO, F., PORRAS, D. T., and EVANS, R. 1988. Seasonal food shortage, weight loss, and the timing of births in saddle-back tamarins (*Saguinus fuscicollis*). *J. Anim. Ecol.* 57:893–901.

GUINAUDEAU, H., LEBOEUF, M., and CAVÉ, A. 1988. Aporphinoid alkaloids. *J. Nat. Prod.* 51:389–474.

HARBORNE, J. B. 1991. The chemical basis of plant defense, p. 46, *in* R. T. Palo and C. T. Robbins (eds.). Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

HARBORNE, J. B., and TURNER, B. L. 1984. Plant Chemosystematics. Oxford, Oxford University Press, p. 75.

HERSHKOVITZ, P. 1977. Living New World monkeys (Platyrrhini), with an Introduction to Primates, Volume 1. Chicago University Press, Chicago.

HOROWITZ, W., CHICHILO, P., and REYNOLDS, H. 1984. Official Methods of Analyses. 14th ed. Association of Official Analytical Chemists, p. 988. Virginia.

JONES, D. B. 1941. Factors for converting percentages of nitrogen in foods and feeds into percentage of protein, 22nd ed. Circ. 183. United States Department of Agriculture, Washington, D.C.

KAWAMURA, S. 1965. Sub-culture among Japanese macaques. *In* K. Imanishi, and S. A. Altmann (eds.). Monkeys and Apes: Sociological Studies. Emory University Press, Atlanta, Georgia.

KELLER, R. S., and MILNE, F. W. 1974. EPA/NIH Mass Spectra Data Base, Government Printing Office, Washington, D.C.

KINZEY, W. G., and NORCONK, M. A. 1993. Physical and chemical properties of fruit and seeds eaten by *Pithecia* and *Chiropotes* in Surinam and Venezuela. *Int. J. Primatol.* 14:207–227.

LEITÃO, G. G., MENSOR, L. L., AMARAL, L. F. G., FLORIANO, N., LIMEIRA, V. L. G., MENEZES, F. S., and LEITÃO, S. G. 1999. Phenolic content and antioxidant activity: A study on plants eaten by a group of howling monkeys (*Alouatta fusca*), pp. 883–895, *in* G. Gross, R. Hemingway, and T. Yoshida (eds.). Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology. Kluwer Academic/Plenum Publishers, New York.

LUCKNER, M. 1990. Secondary Metabolism in Microorganisms, Plants and Animals, 3rd ed. Springer-Verlag, New York.

MITTERMEIER, R. A., RYLANDS, A. B., COIMBRA-FILHO, A. F., and FONSECA, G. A. B. 1988. Ecology and behavior of Neotropical Primates, Vol. 2. World Wildlife Fund, Washington, D.C.

NISHIDA, T., and UEHARA, S. 1980. Chimpanzees, tools, and termites: Another example from Tanzania. *Curr. Anthropol.* 21:671–672.

OLIVEIRA, A. C. M., and FERRARI, S. F. (2000). Seed dispersal by black-handed tamarins, *Saguinus midas niger* (Callitrichinae, Primates): Implications for the regeneration of degraded forest habitats in eastern Amazonia. *J. Trop. Ecol.* In press.

PASSAMANI, M. 1996. Ecologia e comportamento de um grupo de sagüi-da-cara-branca (*Callithrix geoffroyi*) em um fragmento de mata atlântica no Espírito Santo. MSc dissertation. Universidade Federal de Minas Gerais, Belo Horizonte, MG.

PEREIRA, M. V. L. 1991. Taxonomia e Morfologia das Espécies do Gênero *Siparuna* Aublet (Monimiaceae) existentes no Estado do Rio de Janeiro. MSc thesis. Museu Nacional, UFRJ.

PERES, C. A. 1991. Seed predation of *Cariniana micrantha* (Lecythidaceae) by brown capuchin monkeys in central Amazonia. *Biotropica* 23:262–270.

PREGNOLATO, W., and PREGNOLATO, N. P. 1985. Normas Analíticas do Instituto Adolfo Lutz, 3rd ed. Instituto Adolfo Lutz, São Paulo, Brazil, pp. 21–27.

RENNER, S. S., and HAUSNER, G. 1997. Siparunaceae, Monimiaceae. Flora of Ecuador, 59:1–124.

RIOS, J. I., SIMON, S., and VILLAR, A. 1989. Pharmacological activity of aporphinoid alkaloids. *Fitoterapia* 60:387–412.

RYLANDS, A. B. 1993. Marmosets and Tamarins. Systematics, Behaviour, and Ecology. Oxford University Press, Oxford.

RYLANDS, A. B., COIMBRA-FILHO, A. F., and MITTERMEIER, R. A. 1993. Taxonomy, distribution and

conservation of the callitrichids, pp. 11–77, *in* A. B. Rylands (ed.). Marmosets and Tamarins. Systematics, Behaviour and Ecology. Oxford University Press, Oxford.

SAKIKABARA, J., KAIYA, T., FUKUDA, H., and OHKI, T. 1983. 6β-Hydroxyursolic acid and other terpenoids of *Enkianthus cernuus. Phytochemistry* 22:2553–2555.

SCHNEIDER, H., and ROSENBERGER, A. L. 1996. Molecules, morphology, and platyrrhine systematics, pp. 3–19, *in* M. A. Norconk, A. L. Rosenberger, and P. A. Garber (eds.). Adaptive Radiations of Neotropical Primates. Plenum Press, New York.

STEVENSON, M. F., and RYLANDS, A. B. 1988. The marmosets, genus *Callithrix*, pp. 131–222, *in* R. A. Mittermeier, A. B. Rylands, A. F. Coimbra-Filho, and G. A. B. Fonseca (eds.). Ecology and Behavior of Neotropical Primates, Vol. 2. World Wildlife Fund, Washington, D.C.

STRIER, K. B. 1992. Faces in the Forest. The Endangered Muriqui Monkeys of Brazil. Oxford University Press, New York.

SUSSMAN, R. W., and KINZEY, W. G. 1984. The ecological role of the Callitrichidae: A review. *Am. J. Phys. Anthropol.* 64:419–449.

VERACINI, C. 1997. O Comportamento alimentar de *Callithrix argentata* (Linnaeus 1771) (Primata, Callitrichinae), pp. 437–446, *in* P. L. B. Lisboa (ed.). Caxiuanã. Museu Paraense Emílio Goeldi/MCT/CNPq, Belém.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

MANDIBULAR GLAND CHEMISTRY OF GRASS-CUTTING ANTS: SPECIES, CASTE, AND COLONY VARIATION

WILLIAM O. H. HUGHES,* PHILIP E. HOWSE, and DAVE GOULSON

Biodiversity and Ecology Division, School of Biological Sciences
University of Southampton
Bassett Crescent East, Southampton, SO16 7PX, U.K.

(Received February 14, 2000; accepted August 31, 2000)

Abstract—The compositions of the alarm pheromones of two species of grass-cutting ant, *Atta bisphaerica* and *A. capiguara*, were examined, and caste and colony variations quantified. The pheromones of *A. bisphaerica* and *A. capiguara* were remarkably similar and were composed of a complex mixture of volatiles in which 4-methyl-3-heptanone and 2-heptanone were the most abundant compounds. Small but consistent intraspecific differences were found between the worker castes and between individual colonies. The results support the view that alarm pheromones are rarely species specific. The possible importance of intercolony variation is discussed.

Key Words—Leaf-cutting ants, alarm, pheromone, caste, colony, *Atta bisphaerica*, *Atta capiguara*, mandibular gland, Formicidae.

INTRODUCTION

Alarm behavior is one of the most obvious behaviors of ants, but it is also one of the hardest to define because of the wide range of responses that it can involve. The chemistry of ant alarm pheromones is equally diverse, but compounds typically have a molecular weight of between 100 and 200 and have 5–10 carbon atoms (Wilson and Bossert, 1963). The range of possible molecular weights is related to the function of alarm pheromones. An alarm response is a rapid reaction and so requires compounds of high volatility and low molecular weight in order that they evaporate rapidly (Bossert and Wilson, 1963; Wilson and Bossert, 1963). Furthermore, there is less need for the specificity shown by other pheromones, such as trail or sex pheromones, and so large, complex

*To whom correspondence should be addressed.

0098-0331/01/0100-0109$19.50/0

molecules, which are energetically more expensive to produce, are unnecessary (Blum, 1969; Hölldobler and Wilson, 1990; Vander Meer and Alonso, 1998).

Leaf-cutting ants (Hymenoptera: Formicidae: Attini: *Atta* and *Acromyrmex*) are dominant herbivores in the neotropics (Hölldobler and Wilson, 1990). They can account for as much as 50% of the total herbivory in some areas (Blanton and Ewel, 1985) and are one of the most destructive pests in many of the regions where they occur (Weber, 1972; Cherrett, 1986). *Atta* colonies, in particular, can be extremely large, with worker populations that are highly polymorphic and polyethic. When alarmed, leaf-cutting ants exhibit an aggressive response (Wilson and Regnier, 1971) and release an alarm pheromone from their mandibular glands (Butenandt et al., 1969; Blum et al., 1968; Moser et al., 1968; Riley et al., 1974; Knapp, 1995).

A number of studies have examined the chemistry of this mandibular gland secretion. In the earliest work, Butenandt et al. (1959) identified citral as the main component in *Atta sexdens rubropilosa.* The presence of this compound gives the crushed heads of *A. sexdens* workers a characteristic lemon smell. Later, Moser et al. (1968) found 4-methyl-3-heptanone and 2-heptanone at a ratio of 4 : 1 to be the principal components in major workers of *A. texana.* These ketones were also identified at species-specific ratios in the alarm pheromones of six other species of *Atta*, including *A. bisphaerica* and *A. capiguara* (Blum et al., 1968). However, subsequent research by Schildknecht (1976) and Nascimento et al. (1993) has revealed the chemistry of the mandibular gland secretion to be far more complicated than these early studies suggested, and some 56 compounds have been identified to date in *Atta* alone. However, for only very few has any behavioral activity been demonstrated. Although Butenandt et al. (1959) described citral as being the alarm-stimulating compound in *A. sexdens rubropilosa*, all other studies have concluded that 4-methyl-3-heptanone is the most important releaser of alarm behavior in *Atta* (Blum et al., 1968; Moser et al., 1968; Riley et al., 1974; Knapp, 1995; Pow, 1996).

The composition of alarm pheromones can vary between castes of ants. This was first found to be the case in the African weaver ant, *Oecophylla longinoda*, in which the glands of minor workers lack three of the main compounds found in major workers (Bradshaw et al., 1979). Similar variation between castes has been found in *Atta sexdens rubropilosa.* Nascimento et al. (1993) analyzed the mandibular gland secretion of workers of this species and found that the extracts of large workers consisted of a complex mixture of compounds dominated by citral. The secretion of small workers was 89.5% 4-methyl-3-heptanone and neither of the isomers of citral were present. A similar pattern has been found in *Atta laevigata* with 4-methyl-3-heptanone again dominating the secretion of the smaller workers (Hernández et al., 1999).

Alarm pheromones also can vary between colonies. Bradshaw et al. (1979) found that the mandibular gland chemistry of colonies of *Oecophylla longinoda*

differed significantly between geographical localities and even between colonies from the same location. Cherix (1983) also found that the alarm pheromone of *Formica lugubris* differed both qualitatively and quantitatively between colonies at the same location. This aspect has not been examined in leaf-cutting ants. Most studies (e.g., Butenandt et al., 1959; Moser et al., 1968; Schildknecht, 1976; Nascimento et al., 1993; Hernández et al., 1999) have examined ants only from a single nest. However, Whitehouse and Jaffé (1995) found that workers of *A. laevigata* respond more aggressively to the crushed heads of nonnestmates than nestmates. This suggests not only that there are intercolony differences in the alarm pheromone, but also that these differences are detectable by the ants.

The alarm pheromone chemistry of *A. bisphaerica* and *A. capiguara* has not been examined since the early study by Blum et al. (1968). Furthermore, our knowledge of caste variation in the alarm pheromone of leaf-cutting ants remains limited and that of colony variation is nonexistent. Identification of the chemistry and variation in the alarm pheromone is essential to further studies on the alarm behavior of these species. The compositions, therefore, of the alarm pheromones of *A. bisphaerica* (Forel) and *A. capiguara* (Gonçalves) were examined. Compositions for the four worker castes of each species also were compared, and intercolony differences were quantified in *A. capiguara.* The behavioral activity elicited by the compounds will be examined elsewhere (Hughes et al., 2001).

METHODS AND MATERIALS

Sample Collection. Samples of the heads of both *Atta bisphaerica* and *A. capiguara* workers were collected between March and June 1998. For both species, 10 samples of each of the four worker castes were collected, based on the estimated head width of the ants. Minors were less than 1.4 mm head width, medias 1.5–2.0 mm, foragers 2.0–3.0 mm, and soldiers had head widths >3.0 mm. The minor and media worker samples contained the heads of 20 individuals, samples of foragers comprised 10 heads, while the soldier samples contained only a single head. The *A. bisphaerica* samples were collected from a single nest near Viçosa, Minas Gerais, Brazil. The nest from which the *A. capiguara* samples were collected was located near Capinópolis, Minas Gerais. In addition, 10 samples of the heads of *A. capiguara* foragers were collected from each of four nests located on the UNESP campus in Botucatu, São Paulo, Brazil.

Nascimento et al. (1993) found that the chemical composition of the mandibular glands of *A. sexdens rubropilosa* does not differ between ants on the foraging trails and those within the nest. To reduce the disturbance to the ants, therefore, they were collected from the trails. Ants were gently removed from the trails and immediately cooled, which prevented them from becoming alarmed. They were then transported to the laboratory where their heads were

removed and placed in glass vials containing 500 μl of dichloromethane solvent. The mandibular glands are probably the only source of volatile compounds in the heads of ants (Howse and Bradshaw, 1980), and both Nascimento et al. (1993) and Knapp (1995) have found that there is no difference in the volatiles contained in crushed heads and mandibular glands of *A. sexdens rubropilosa.* Only crushed heads were examined here. Once samples contained the required number of heads, the heads were crushed thoroughly with a glass rod. After 24 hr the extract was removed and stored between −20°C and −60°C until analysis.

Chemical Analysis. The sample extracts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). The gas chromatograph used was a Hewlett Packard 5890A with a flame ionization detector. The column was DB5, 30 m × 0.25 mm, with a film thickness of 0.25 μm. Nitrogen was used as the carrier gas. The temperature program was 40°C for 2 min, then increasing at 10°C/min, with a final temperature of 280°C for 30 min. Compounds were quantified with 5-methyl-3-heptanone (97%, Aldrich) as an internal standard.

To identify the compounds, a VG-Analytical 70-250SE mass spectrometer was used, coupled to a Hewlett-Packard 5790 gas chromatograph. The column was BP1, 25 m × 0.22 mm, with a film thickness of 0.25 μm. Helium was used as the carrier gas and the temperature program was 40°C for 3 min, increasing at 10°C/min to 75°C, then increasing at 20°C/min, and ending with 5 min at 300°C. The conditions were 70 eV electron impact (EI) ionization, and an ion chamber temperature of 200°C. Compounds were provisionally identified by examination of the fragmentation pattern of their mass spectrograms. This was then confirmed by comparing the results with library mass spectrograms and with the mass spectrograms of synthetic versions.

Statistical Analysis. The GC integrator produces peak areas for each compound that are dependent upon the amount of material in the sample. To avoid this, the areas of each peak are divided by the sum of areas of all the peaks in the sample. This results in proportional data that adds up to one. However, as the data are compositional, the peak proportions cannot be used for multivariate analysis. The data, therefore, were standardized by the formula proposed by Aitchison (1986):

$$Z_{ij} = \ln [Y_{ij}/g(Y_j)]$$

where Z_{ij} is the standardized area of peak i for ant j, Y_{ij} is the observed area of the peak, and $g(Y_j)$ is the geometric mean of the areas of all the peaks of ant j included in the analysis.

The standardized data were then examined by multivariate analysis of variance (MANOVA) to determine whether the groups differed in the abundances

of compounds present. Differences between the groups were examined further by a canonical discriminant analysis. In addition, a one-way univariate analysis of variance (ANOVA) also was carried out for each peak in each analysis. Only peaks that were abundant enough to be quantified in all of the samples being examined in a particular analysis (5–10 peaks) were included. This avoided the problem of large numbers of independent variables relative to the sample size resulting in significant discriminations being found where no actual groupings exist (Panel on Discriminant Analysis, Classification, and Clustering, 1989). As a small number of samples had evaporated during storage, the analysis was weighted for the number of replicates. Only the first two discriminant functions were considered to be important, because together these always accounted for at least 90% of the variance between groups. The discriminant scores for the functions were also used to predict group membership and thus to confirm the effectiveness of the functions in discriminating between the groups.

To quantify the similarity between the groups (castes, nests, etc.), Nei's distances were calculated (Nei, 1972; Ferguson, 1980). This technique provides an index of similarity between two groups from 1 (identical) to 0 (totally different). The formula used is:

$$I = \frac{\sum x_i y_i}{\sqrt{\left(\sum x_i^2 \sum y_i^2\right)}}$$

where I is Nei's coefficient of identity, x_i is the quantity of peak i for sample x, and y_i is the quantity of peak i in sample y. After converting the data into binary values (1 = present, 0 = absent), Nei's distances were calculated with Ochiai's resemblance coefficient. This takes the same value as the cosine coefficient (Nei's distance) when binary attribute data are used (Romesburg, 1989) and allowed the trace compounds to be included in the analysis.

RESULTS

A total of 41 peaks of high volatility occurred consistently in one or more groups. Of these, nine were identified with a high degree of confidence, including almost all of the most abundant peaks. Seven of these were ketones in the C_7 to C_9 range, and the others were nonanal and the monoterpene, limonene. A provisional identification was made for a further seven peaks.

Caste Differences in Composition. In both species, there was very little differences between the castes in the composition of volatiles in their mandibular glands, as represented by the extremely high Nei's values (Figure 1). Medias

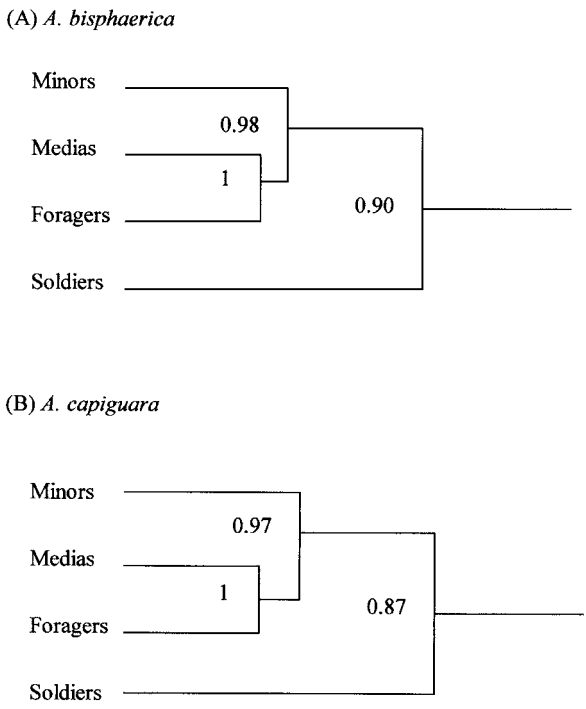


FIG. 1. Dendrograms based on Nei's distances as a measure of the degree of similarity between the composition of volatiles (presence/absence) in the extracts of the four castes (1 = identical, 0 = totally different).

and foragers contained the same compounds, and the composition of the minor worker samples was also very similar. Soldiers differed to a greater degree, and the castes differed in the proportions of the most abundant compounds (*A. bisphaerica:* $F_{40,100} = 4.66$, $P < 0.001$; *A. capiguara*; $F_{28,124} = 7.20$, $P < 0.001$) (Tables 1 and 2).

The discriminant analysis of the *A. bisphaerica* castes was based upon 10 peaks. Soldiers, and to a lesser extent minors, separated out from the other castes on function 1 (64% of the variance) (Figure 2). The function structure coefficients indicated that 4-methyl-3-heptanone contributed most to this variance. The ANOVA showed that the proportions of this ketone differed significantly between the castes ($F_{3,31} = 13.0$, $P < 0.001$), with the soldiers containing a lower proportion than the other castes (Table 1). Soldiers also had a higher proportion of 2-heptanone, although this was not significant ($F_{3,31} = 1.31$, $P = 0.29$), and they were the only caste to contain 2-nonanone and peak 29. Minor workers were distinguished from the other castes by function 2 of the discriminant anal-

TABLE 1. VOLATILES IN MANDIBULAR GLANDS OF FOUR CASTES OF *A. bisphaerica*[a]

Peak	Volatiles (%, mean ± SE)			
	Minors ($N = 9$)	Medias ($N = 8$)	Foragers ($N = 10$)	Soldiers ($N = 8$)
1	0.7 ± 0.2	trace	trace	trace
2	0	0	0	0
3	5.5 ± 2.0	1.8 ± 0.1	1.6 ± 0.1	2.2 ± 0.3
4	trace	0.3 ± 0.1	trace	trace
5	trace	trace	trace	trace
6	1.5 ± 0.3	1.0 ± 0.1	0.7 ± 0.1	1.4 ± 0.3
7 (3-hexanone)	trace	trace	trace	trace
8 (3-hexanol)	0.9 ± 0.3	0.9 ± 0.1	1.7 ± 0.2	trace
9	0	0	0	0
10	trace	trace	trace	0
11	0	0	0	0
12	0	0	0	0
13 (2-hexanol)	0	0	0	0
14	0	0	0	0
15 4-methyl-3-hexanone	7.1 ± 0.7	5.6 ± 0.4	6.8 ± 0.7	5.9 ± 0.8
16 4-methyl-2-hexanone	4.8 ± 0.6	3.2 ± 0.3	3.5 ± 0.5	4.6 ± 0.9
17 (2-pentenoate)	3.7 ± 0.7	2.4 ± 0.6	2.2 ± 0.3	3.4 ± 0.6
18	0.4 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0
19	0.4 ± 0.2	0.3 ± 0.1	1.0 ± 0.6	1.1 ± 0.5
20	2.0 ± 0.5	1.8 ± 0.5	1.8 ± 0.4	2.0 ± 0.4
21 2-heptanone	22.9 ± 2.1	27.6 ± 1.8	23.4 ± 1.2	31.6 ± 3.9
22	0	0	0	0
23	0	0	0	0
24 (2-heptanol)	1.9 ± 0.2	2.5 ± 0.2	1.9 ± 0.2	2.6 ± 0.6
25	0	0	0	0
26	0	0	0	0
27 4-methyl-3-heptanone	29.7 ± 1.5	34.9 ± 1.1	38.4 ± 1.1	21.7 ± 2.0
28	0	0	0	0
29	0	0	0	0.5 ± 0.2
30 (4-methyl-3-heptanol)	0.7 ± 0.2	2.3 ± 0.4	2.5 ± 0.2	trace
31 3-octanone	6.9 ± 0.6	6.8 ± 0.5	6.5 ± 0.3	5.5 ± 0.8
32 2-octanone	3.4 ± 0.8	2.4 ± 0.7	1.9 ± 0.5	5.2 ± 0.8
33	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.1	trace
34	0	0	0	0
35 (pinene)	0	0	0	0
36 limonene	3.7 ± 1.0	2.4 ± 1.3	2.5 ± 0.6	3.3 ± 1.0
37	0	0	0	0
38	0	0	0	0
39 2-nonanone	0	0	0	7.2 ± 1.9
40 nonanal	1.3 ± 0.3	1.8 ± 0.1	2.1 ± 0.1	1.2 ± 0.2
41	0	0	0	0
Total amount per ant (μg)	0.41 ± 0.03	0.89 ± 0.07	2.51 ± 0.29	23.8 ± 2.03

[a] Compound names in parentheses represent tentative identification only.

TABLE 2. VOLATILES IN MANDIBULAR GLANDS OF FOUR CASTES OF *A. capiguara*[a]

Peak	Volatiles (%, mean ± SE)			
	Minors ($N = 10$)	Medias ($N = 10$)	Foragers ($N = 10$)	Soldiers ($N = 8$)
1	1.2 ± 0.2	1.6 ± 0.2	1.5 ± 0.4	1.7 ± 0.3
2	0	±	0	trace
3	7.1 ± 0.9	5.8 ± 0.3	5.6 ± 0.4	7.3 ± 1.0
4	trace	trace	trace	trace
5	0	0	0	0
6	1.2 ± 0.5	trace	0.7 ± 0.2	1.1 ± 0.3
7 (3-hexanone)	1.3 ± 0.4	trace	1.0 ± 0.2	1.1 ± 0.3
8 (3-hexanol)	trace	trace	trace	0
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
13 (2-hexanol)	0	0	0	0
14	0	0	0	0
15 4-methyl-3-hexanone	10.0 ± 0.8	13.3 ± 0.6	17.8 ± 1.7	11.0 ± 2.1
16 4-methyl-2-hexanone	6.6 ± 0.4	6.7 ± 0.4	6.9 ± 0.8	6.4 ± 0.7
17 (2-pentenoate)	2.4 ± 0.7	1.1 ± 0.4	1.4 ± 0.3	2.3 ± 0.9
18	0	0	0	0
19	0.6 ± 0.2	trace	0.6 ± 0.1	0.6 ± 0.1
20	1.2 ± 0.4	trace	1.0 ± 0.3	1.4 ± 0.7
21 2-heptanone	21.0 ± 2.1	23.6 ± 1.2	20.3 ± 1.4	21.6 ± 2.7
22	0	0	0	0
23	trace	trace	trace	0.5 ± 0.1
24 (2-heptanol)	0.9 ± 0.1	trace	trace	trace
25	0	0	0	0
26	0	0	0	0
27 4-methyl-3-heptanone	29.2 ± 1.2	29.4 ± 0.9	25.9 ± 0.8	20.9 ± 1.4
28	trace	trace	0	0
29	0	0	0	trace
30 (4-methyl-3-heptanol)	1.4 ± 0.3	0.9 ± 0.2	0.6 ± 0.1	1.1 ± 0.1
31 3-octanone	12.4 ± 0.8	14.1 ± 0.6	13.4 ± 0.4	7.5 ± 0.7
32 2-octanone	0	0	0	2.7 ± 0.5
33	trace	trace	trace	trace
34	0	0	0	0
35 (pinene)	0	0	0	0
36 limonene	2.7 ± 1.2	1.1 ± 0.4	2.1 ± 0.7	2.4 ± 0.9
37	0	0	0	0
38	trace	trace	trace	trace
39 2-nonanone	0	0	0	9.2 ± 3.5
40 nonanal	0	0	0	0
41	0	0	0	0
Total amount per ant (μg)	0.46 ± 0.04	0.51 ± 0.04	0.96 ± 0.09	8.9 ± 0.08

[a] Compound names in parentheses represent tentative identification only.

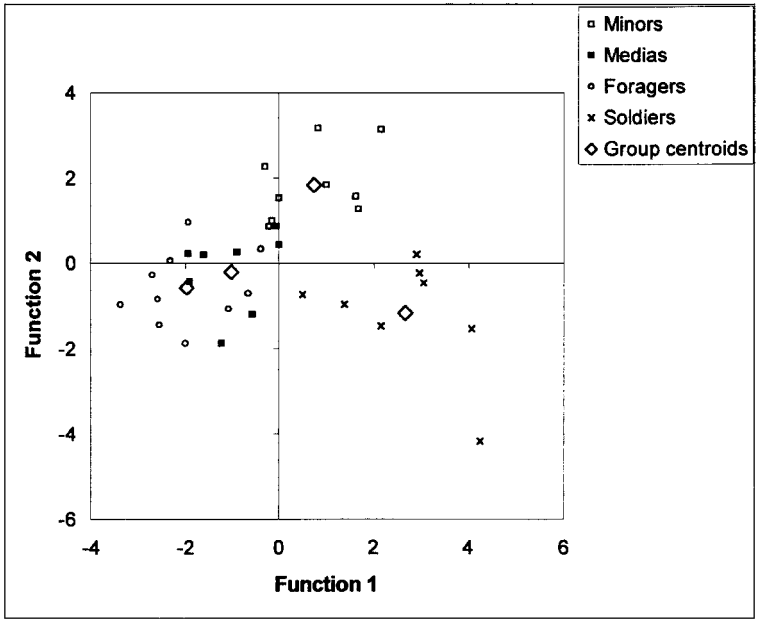


FIG. 2. Discriminant analysis of the four castes of *A. bisphaerica* [minors ($N = 9$), medias ($N = 8$), foragers ($N = 10$), and soldiers ($N = 8$)]. The proportions of 10 peaks were compared after standardization. Data points are the scores for each sample on functions 1 and 2 of the discriminant analysis, and the group centroids for each caste.

ysis (27% of variance) (Figure 2), which was mainly due to peak 3. This was higher in minors than in the other castes ($F_{3,31} = 5.30$, $P = 0.005$) (Table 1). Medias and foragers were not differentiated by either function (Figure 2), and their chemical profiles were virtually identical. The predicted classification based on the discriminant scores misclassified only 14% of samples (0/9 minors, 3/8 medias, 1/10 foragers, and 1/8 solliders).

Separation of the castes of *A. capiguara* by the discriminant analysis followed a similar pattern, with the analysis being based upon seven peaks. Both function 1 (55% of variance), and function 2 (36% of variance) separated soldiers and minors from the other two castes, with medias and foragers being very similar (Figure 3). Four peaks contributed to function 1. Peak 3 was higher ($F_{3,34} = 6.16$, $P = 0.002$), and 4-methyl-3-hexanone lower ($F_{3,34} = 3.99$, $P = 0.015$), in minors and soldiers compared with the other two castes (Table 2). There was also less 3-octanone in the soldiers samples ($F_{3,34} = 3.14$, $P = 0.038$), and the proportion of 4-methyl-3-heptanone decreased from minors to soldiers, although this was not significant ($F_{3,34} = 2.32$, $P = 0.093$). Function 2 was due

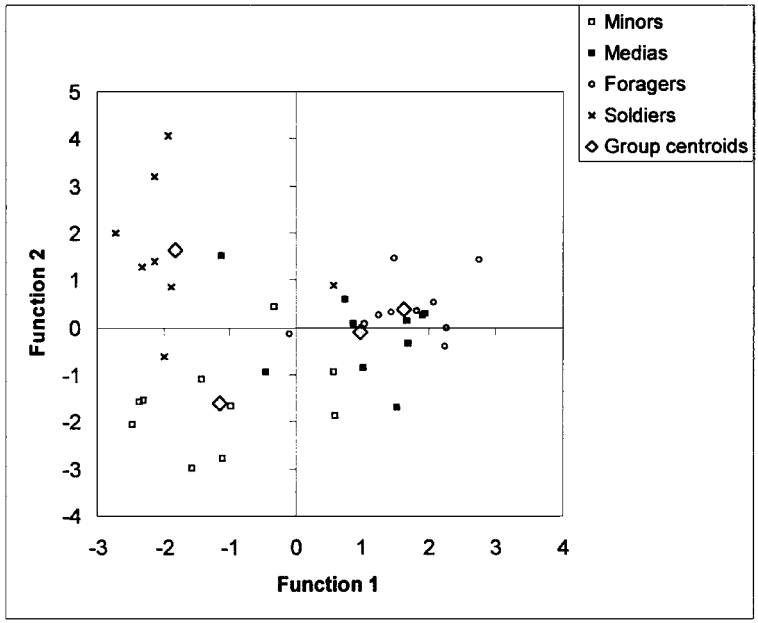


FIG. 3. Discriminant analysis of the four castes of *A. capiguara* [minors ($N = 10$), medias ($N = 10$), foragers ($N = 10$), and soldiers ($N = 8$)]. The proportions of seven peaks were compared after standardization. Data points are the scores for each sample on functions 1 and 2 of the discriminant analysis, and the group centroids for each caste.

mainly to 2-heptanone, although this did not differ between the castes ($F_{3,34} = 0.84$, $P = 0.481$). In addition, 2-octanone and 2-nonanone were found only in the soldiers (Table 2). Misclassification based on the discriminant scores was greater than with *A. bisphaerica*, with 26% being misclassified (2/10 minors, 4/10 medias, 2/10 foragers, 2/8 soldiers).

Species Differences in Composition. There was surprisingly little difference between *A. bisphaerica* and *A. capiguara* in the compounds present in their profiles (Nei's distance of 0.86), although they did differ in the proportions of the most abundant compounds ($F_{12,134} = 49.24$, $P < 0.001$). They were separated with a single function in the discriminant analysis, which used six peaks. This was contributed to mainly by 4-methyl-3-heptanone and 3-octanone. There was more 4-methyl-3-heptanone ($F_{1,71} = 28.44$, $P < 0.001$), and less 3-octanone ($F_{1,71} = 5.95$, $P = 0.017$) in *A. bisphaeric* (Tables 1 and 2). Overall, the proportion of 2-heptanone was also greater in *A. bisphaerica* ($F_{1,71} = 24.9$, $P < 0.001$), while there was more 4-methyl-3-hexanone ($F_{1,71} = 9.03$, $P = 0.004$) and peak 3 ($F_{1,71} = 44.5$, $P < 0.001$) in *A. capiguara.* Nonanal, peak 19, and peak 10

were found only in *A. bisphaerica*, and while 2-octanone was present in all the *A. bisphaerica* castes, it was found only in soldiers of *A. capiguara.* Prediction from the discriminant scores misclassified only 4.1% of the samples (3/35 *A. bisphaerica*, 0/38 *A. capiguara*).

Nest Differences in Composition. The nests at the Botucatu site differed from one another in the compounds present in their profiles (average Nei's distance of 0.76), and the proportions of the most abundant compounds also differed between the nests ($F_{12,124} = 9.85$, $P < 0.001$) (Table 3). In the discriminant analysis, with six peaks, nest B was clearly separated from the others on function 2 (26% of variance) (Figure 4). This was due mainly to 4-methyl-3-heptanone, of which there was more in nest B ($F_{3,33} = 19.84$, $P < 0.001$), and to 4-methyl-3-hexanone, of which there was less ($F_{3,33} = 12.33$, $P < 0.001$) (Table 3). 2-Heptanone was also more abundant in nests A, B, and C, than in nest D. All nests were separated by function 1 (73% of variance) (Figure 4). This was due to peak 7, which varied between an average of 18.1 ± 0.8% in nest D and only 1 ± 0.1% in nest A, with nests B and C being intermediate. The nests also differed considerably in the minor peaks that were present in their profiles (Table 3). Only 8.1% of samples were misclassified by predictions based on the discriminant scores (0/10 nest A, 2/9 nest B, 1/9 nest C, 0/9 nest D).

DISCUSSION

The volatile compounds identified in *A. bisphaerica* and *A. capiguara* were all within the 100–200 molecular weight range that is characteristic of alarm pheromone compounds (Wilson and Bossert, 1963). The main compounds were mostly methyl and ethyl ketones, which are the groups that dominate the alarm pheromones of myrmicine ants (Blum and Brand, 1972; Parry and Morgan, 1979). 4-Methyl-3-heptanone, 2-heptanone, 3-octanone, and 4-methyl-3-hexanone have previously been identified in the alarm pheromones of *Atta* (Schildknecht, 1976; Blum et al., 1968; Moser et al., 1968; Riley et al., 1974; Nascimento et al., 1993; Knapp, 1995; Hernández et al., 1999). In fact, 4-methyl-3-heptanone and 2-heptanone are extremely common alarm compounds and are found in a number of other ant species (Hölldobler and Wilson, 1990). The small quantities present of minor compounds prevented accurate mass spectrometry, and so their identity remains uncertain. However, at least some appeared to be low molecular weight aliphatic ketones and alcohols of the types commonly seen in the alarm pheromones of other leaf-cutting ants.

There were small but consistent differences between the castes in both species, although the profiles of medias and foragers were virtually indistinguishable. The similarity contrasts with studies on *A. sexdens rubropilosa* (Nascimento et al., 1993; Knapp, 1995) and *A. laevigata* (Hernández et al., 1999),

TABLE 3. VOLATILES IN MANDIBULAR GLANDS OF FORAGERS FROM FOUR *A. capiguara* NESTS[a]

Peak	Volatiles (%, mean ± SE)			
	Nest A (N = 10)	Nest B (N = 9)	Nest C (N = 9)	Nest D (N = 9)
1	1.7 ± 0.3	1.1 ± 0.3	1.5 ± 0.3	2.9 ± 0.2
2	0	trace	0	0
3	7.6 ± 0.7	4.6 ± 0.4	6.8 ± 0.9	3.1 ± 0.3
4	0	0	0	0
5	0	0	0	0
6	0.6 ± 0.2	0	0	0
7 (3-hexanone)	1.0 ± 0.1	1.8 ± 0.3	6.1 ± 1.7	18.1 ± 0.8
8 (3-hexanol)	trace	2.2 ± 0.4	trace	0.6 ± 0.2
9	trace	0	trace	0
10	0	trace	0	trace
11	0	0.5 ± 0.2	0	0.7 ± 0.2
12	0	0	0	trace
13 (2-hexanol)	0	trace	0	0.7 ± 0.1
14	0	0.8 ± 0.2	0	0
15 4-methyl-3-hexanone	15.3 ± 0.8	5.9 ± 1.9	18.0 ± 1.3	11.0 ± 0.6
16 4-methyl-2-hexanone	3.6 ± 0.4	2.8 ± 0.6	2.2 ± 0.6	2.3 ± 0.5
17 (2-pentenoate)	1.6 ± 0.8	0.1 ± 0.1	0	0
18	1.6 ± 0.2	2.0 ± 0.2	2.9 ± 0.5	trace
19	trace	0.8 ± 0.2	trace	8.7 ± 0.5
20	1.5 ± 0.5	0	0	0
21 2-heptanone	19.7 ± 1.3	20.2 ± 0.9	17.2 ± 1.2	11.0 ± 1.1
22	0	trace	0	0
23	1.0 ± 0.2	trace	trace	trace
24 (2-heptanol)	trace	2.0 ± 0.3	trace	trace
25	trace	trace	0	0
26	trace	0	0	0
27 4-methyl-3-heptanone	18.2 ± 0.7	34.6 ± 1.9	18.5 ± 1.2	13.8 ± 1.4
28	0	0	0	1.1 ± 0.2
29	0	0	0	0.9 ± 0.2
30 (4-methyl-3-heptanol)	1.1 ± 0.2	4.4 ± 0.8	2.0 ± 0.5	5.4 ± 0.3
31 3-octanone	17.2 ± 0.7	9.9 ± 1.6	18.3 ± 1.1	10.9 ± 0.3
32 2-octanone	0	0	0	0
33	1.8 ± 0.2	0.8 ± 0.2	trace	1.6 ± 0.3
34	0	0	0	trace
35 (pinene)	0	0	trace	2.4 ± 0.4
36 limonene	2.0 ± 1.0	trace	trace	0
37	trace	trace	0	0
38	0	0	0.2 ± 0.2	1.4 ± 0.2
39 2-nonanone	trace	trace	trace	0
40 nonanal	1.6 ± 0.1	3.4 ± 0.5	1.2 ± 0.3	2.2 ± 0.1
41	trace	trace	0	0
Total amount per ant (μg)	0.74 ± 0.06	0.98 ± 0.07	0.65 ± 0.07	0.94 ± 0.05

[a] Compound names in parentheses represent tentative identification only.

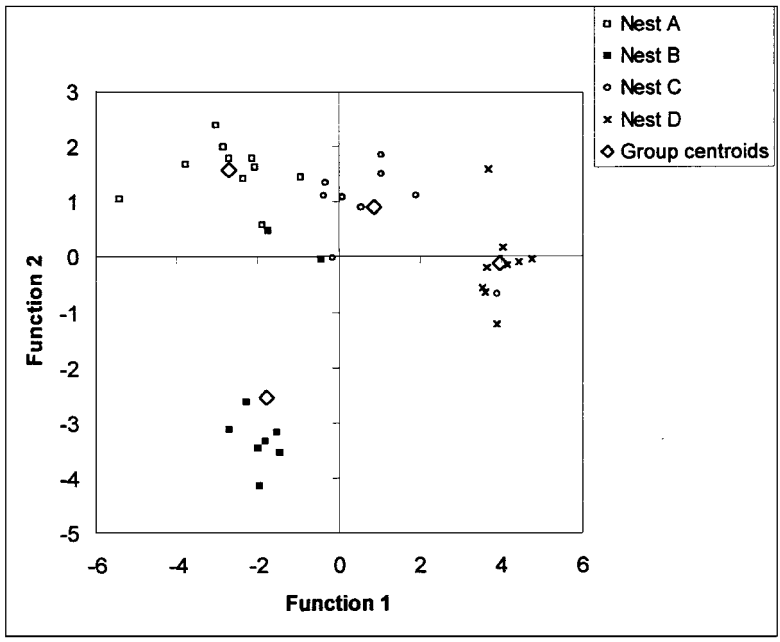


FIG. 4. Discriminant analysis of foragers from the four Botucatu nests of *A. capiguara* [nest A ($N = 10$), nest B ($N = 9$), nest C ($N = 9$), and nest D ($N = 9$)]. The proportions of six peaks were compared after standardization. Data points are the scores for each sample on functions 1 and 2 of the discriminant analysis, and the group centroids for each nest.

in which the alarm pheromone of the larger ants was a complex mixture of compounds while that of the smallest workers was almost pure 4-methyl-3-heptanone. Although the soldiers of both, *A. bisphaerica* and *A. capiguara* did have proportionally less 4-methyl-3-heptanone than the other castes, such a strong division between small and large workers was clearly not present in these species. In part, this may be ascribed to the size of the ants in the samples. Nascimento et al. (1993) and Hernández et al. (1999) reported that the alarm pheromones of ants <1.8 mm and <2.5 mm in head width, respectively, did not differ in composition. However, Knapp (1995) found that it was only the pheromones of workers with a head width of <1 mm that were dominated by 4-methyl-3-heptanone. The minors of *A. bisphaerica* and *A. capiguara* analyzed here had a head width of up to 1.4 mm, and so included individuals that were larger than the size range which Knapp (1995) found to be dominated by 4-methyl-3-heptanone. It would be interesting to examine in more detail the mandibular gland chemistry of the minor workers of *A. bisphaerica*,

A. capiguara, and indeed other species, to see if the caste differences described in *A. sexdens rubropilosa* and *A. laevigata* are a widespread phenomenon. The reason for caste variation is another area that deserves further research. Nascimento et al. (1993) and Bradshaw et al. (1979) have suggested that differences in chemical composition between castes may be due to living in different environments. However, individuals from every leaf-cutting ant worker caste may be found both inside and outside the nest, and no direct evidence for this theory has been found. The reason for caste variation, therefore, is still unclear.

There was also very little difference between *A. bisphaerica* and *A. capiguara* in the composition of the mandibular glands, although they could be consistently distinguished by the proportions of their major components. They were both very different from *A. sexdens rubropilosa*, and none of the larger molecular weight compounds, such as citral, were found. The most behavioral active compound in the alarm pheromone of *A. sexdens rubropilosa*, as well as *A. laevigata*, *A. cephalotes*, and *A. texana*, is 4-methyl-3-heptanone (Blum et al., 1968; Moser et al., 1968; Riley et al., 1974; Knapp, 1995; Pow, 1996). This was the most abundant compound in all the worker castes of both *A. bisphaerica* and *A. capiguara*, apart from the soldiers. It also has been identified in the mandibular glands of *A. colombica* and *A. robusta* (Blum et al., 1968). The lack of species specificity of alarm pheromones has been recognized by a number of authors (Wilson, 1965; Blum, 1969; Parry and Morgan, 1979; Vander Meer and Alonso, 1998). Unlike many other pheromones, discrimination between species is not essential to the function of alarm pheromones (Law and Regnier, 1971; Hölldobler and Wilson, 1990). Indeed, there could even be benefits to being able to recognize and respond to alarm pheromones produced by other species (Blum, 1969; Vander Meer and Alonso, 1998).

One explanation for the discrepancies between studies on the same species is colony-specific differences. Both the presence of minor compounds and the proportions of major compounds differ significantly among the nests of *A. capiguara.* In fact, the relatively high number of trace compounds that varied between colonies caused average Nei's distances between colonies to be lower than that between the two species. Similar intercolony differences in both the major and trace components have been found in weaver ants, *Oecophylla longinoda* (Bradshaw et al., 1975, 1979). Why colonies should differ to such a degree is unclear. Possibly the subtle differences in the composition of the alarm pheromone allow the recognition of nestmates (Howse, 1979; Bradshaw and Howse, 1984). Whitehouse and Jaffé (1995) have shown that the ability of leaf-cutting ants to distinguish between nestmates and nonnestmates is dependent upon a cue present in the head of the target ant. We present here the first evidence that the chemistry of leaf-cutting ant heads differs among colonies, supporting the hypothesis that alarm pheromones play an important role in nestmate recognition.

Acknowledgments—We are grateful to Malcolm Cherrett and John Bradshaw for comments on an earlier version of this work; to Evaldo Vilela, the Universidade Federal de Viçosa, Luiz Forti, and UNESP for providing supporting and facilities in Brazil; and to Kathy Ballard, John Langley, Dave Coreener, and the late Ian Stevens for technical assistance. The study was funded by Griffin LLC (Georgia, U.S.A.)

REFERENCES

AITCHISON, J. 1986. The Statistical Analysis of Compositional Data. Chapman and Hall, London.

BLANTON, C. M., and EWEL, J. J. 1985. Leaf-cutting ant herbivory in successional and agricultural tropical ecosystems. *Ecology* 66:861–869.

BLUM, M. S. 1969. Alarm pheromones. *Annu. Rev. Entomol.* 14:57–80.

BLUM, M. S., and BRAND, J. M. 1972. Social insect pheromones: Their chemistry and function. *Am. Zool.* 12:553–576.

BLUM, M. S., PADOVANI, F., and AMANTE, E. 1968. Alkanones and terpenes in the mandibular glands of *Atta* species (Hymenoptera: Formicidae). *Comp. Biochem. Physiol.* 26:291–299.

BOSSERT, W. H., and WILSON, E. O. 1963. The analysis of olfactory communication among animals. *J. Theor. Biol.* 5:443–469.

BRADSHAW, J. W. S., and HOWSE, P. E. 1984. Sociochemicals of ants, pp. 429–473, *in* W. J. Bell and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

BRADSHAW, J. W. S., BAKER, R., and HOWSE, P. E. 1975. Multicomponent alarm pheromones of the weaver ant. *Nature* 258:230–231.

BRADSHAW, J. W. S., BAKER, R., HOWSE, P. E., and HIGGS, M. D. 1979. Caste and colony variations in the chemical composition of the cephalic secretions of the African weaver ant, *Oecophylla longinoda. Physiol. Entomol.* 4:27–38.

BUTENANDT, A., LINZEN, B., and LINDAUER, M. 1959. Uber einen Duftsoff aus der Mandibeldrüse des Blattshneiderameise *Atta sexdens rubropilosa* Forel. *Arch. Anat. Microsc. Morphol. Exp.* 48:13–19.

CHERIX, D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera: Formicidae). *Bull. Soc. Entomol. Suisse* 56:57–65.

CHERRETT, J. M. 1986. The economic importance and control of leaf-cutting ants, pp. 165–192, *in* S. B. Vinson (ed.). Economic Impact and Control of Social Insects. Praeger, New York.

FERGUSON, A. 1980. Biochemical Systematics and Evolution. Blackie and Son Ltd., London.

HERNÁNDEZ, J. V., CABRERA, A., and JAFFÉ, K. 1999. Mandibular gland secretion in different castes of the leaf-cutter ant *Atta laevigata. J. Chem. Ecol.* 25:2433–2444.

HÖLLDOBLER, B., and WILSON, E. O. 1990. The Ants. Belknap Press of Harvard, Cambridge, Massachusetts.

HOWSE, P. E. 1979. The uniqueness of insect societies: aspects of defence and integration, pp. 345–374, *in* G. Larwood and B. R. Rosen (eds.). Biology and Systematics of Colonial Organisms. Academic Press, London.

HOWSE, P. E., and BRADSHAW, J. W. S. 1980. Chemical systematics of social insects with particular reference to ants and termites, pp. 71–90, *in* F. A. Bisby, J. G. Vaughan, and C. A. Wright (eds.). Chemosystematics: Principles and Practice. Academic Press, London.

HUGHES, W. O. H., HOWSE, P. E., VILELA, E. F., and GOULSON, D. 2001. The response of grass-cutting ants to natural and synthetic versions of their alarm pheromone. *Physiol. Entomol.* In press.

KNAPP, J. J. 1995. Chemical aspects of communication and defence in leaf-cutting ants. Unpublished PhD thesis. University of Southampton, U.K.

LAW, J. H., and REGNIER, F. E. 1971. Pheromones. *Annu. Rev. Biochem.* 40:533–548.

MOSER, J. C., BROWNLEE, R. C., and SILVERSTEIN, R. 1968. Alarm pheromones of the ant *Atta texana. J. Insect Physiol.* 14:529–535.

NASCIMENTO, R. R., MORGAN, E. D., BILLEN, J., SCHOETERS, E. DELLA LUCIA, T. M. C., and BENTO, J. M. 1993. Variation with caste of the mandibular gland secretion in the leaf-cutting ant *Atta sexdens rubropilosa. J. Chem. Ecol.* 19:907–918.

NEI, M. 1972. Genetic distance between populations. *Am. Nat.* 106:283–292.

PANEL ON DISCRIMINANT ANALYSIS, CLASSIFICATION, AND CLUSTERING. 1989. Discriminant analysis and clustering. *Stat. Sci.* 4:34–69.

PARRY, K., and MORGAN, E. D. 1979. Pheromones of ants: A review. *Physiol. Entomol.* 4:161–189.

POW, E. M. 1996. The responses of workers of the leaf-cutting ant *Atta sexdens rubropilosa* (Forel) to their alarm pheromone. Unpublished PhD thesis. University of Southamptom, U.K.

RILEY, R. G., SILVERSTEIN, R. M., and MOSER, J. C. 1974. Isolation, identification, synthesis and biological activity of volatile compounds from the heads of *Atta. J. Insect Physiol.* 20:1629–1637.

ROMESBURG, H. C. 1989. Cluster Analysis for Researchers. Krieger Publishing, Melbourne, Florida.

SCHILDKNECHT, H. 1976. Chemical ecology—a chapter of modern natural products chemistry. *Angew. Chem. Int. Ed.* 15:214–222.

VANDER MEER, R. K., and ALONSO, L. E. 1998. Pheromone directed behavior in ants, pp. 159–192, *in* R. K. Vander Meer, M. D. Breed, K. E. Espelie, and M. L. Winston (eds.). Pheromone Communication in Social Insects. Westview Press, Boulder, Colorado.

WEBER, N. A. 1972. Gardening ants. The attines. *Mem. Am. Phil. Soc.* 92:1–146.

WHITEHOUSE, M. E. A., and JAFFÉ, K. 1995. Nestmate recognition in the leaf-cutting ant *Atta laevigata. Insectes Soc.* 42:157–166.

WILSON, E. O. 1965. Chemical communication in the social insects. *Science* 149:1064–1071.

WILSON, E. O., and BOSSERT, W. H. 1963. Chemical communication among animals. *Recent Prog. Horm. Res.* 17:673–716.

WILSON, E. O., and REGNIER, F. E. 1971. The evolution of the alarm-defense system in the formicine ants. *Am. Nat.* 105:279–289.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

ALLELOCHEMICALS IN WHEAT (*Triticum aestivum* L.): VARIATION OF PHENOLIC ACIDS IN SHOOT TISSUES

HANWEN WU,[1,5] TERRY HAIG,[2,5] JAMES PRATLEY,[1,5] DEIRDRE LEMERLE[4,5] and MIN AN[3]

[1] *Farrer Centre for Conservation Farming*

[2] *School of Science and Technology*

[3] *Environmental and Analytical Laboratories*
Charles Sturt University
PO Box 588, Wagga Wagga, NSW 2678, Australia

[4] *NSW Agriculture, Wagga Agricultural Institute*
Wagga Wagga, NSW 2650, Australia

[5] *Cooperative Research Centre for Weed Management Systems*

(Received May 11, 2000; accepted September 1, 2000)

Abstract—Seven known phenolic acids implicated in wheat allelopathy were analyzed in a worldwide collection of 58 wheat accessions by gas chromatography and tandem mass spectrometry (GC-MS-MS). Chemical analysis showed that accessions differed significantly in the production of *p*-hydroxybenzoic, vanillic, syringic, *trans*-*p*-coumaric, *cis*-*p*-coumaric, *trans*-ferulic, and *cis*-ferulic acids in the shoots of 17-day-old wheat seedlings. The concentrations of *p*-hydroxybenzoic, vanillic, *cis*-*p*-coumaric, and *cis*-ferulic acids were normally distributed in the 58 accessions. A binormal distribution was found for syringic and *trans*-ferulic acids and a skewed normal distribution for *trans*-*p*-coumaric acid. The concentration of each compound also varied with phenolic acids. The relative abundance of each phenolic acid was ordered decreasingly as *trans*-ferulic, vanillic, *trans*-*p*-coumaric, *p*-hydroxybenzoic, syringic, *cis*-ferulic, and *cis*-*p*-coumaric acids. The concentration of total identified phenolic acids varied from 93.2 to 453.8 mg/kg in the shoots of 58 accessions. The content of each phenolic acid or group was highly associated with others in the shoots of wheat seedlings. Wheat accessions with high levels of total identified phenolic acids in the shoots are generally strongly allelopathic to the growth of annual ryegrass.

Key Words—Wheat, *Triticum aestivum* L., allelopathy, allelochemicals, phenolic acids, shoots, weed suppression.

INTRODUCTION

Phenolic compounds are among the most abundant groups of secondary metabolites in plants. Phenolics bear hydroxylated aromatic rings, and include simple phenols, phenolic acids, phenylpropanoids, coumarins, quinones, flavonoids, tannins, and other miscellaneous phenols (Harborne, 1980). They are known to be of significance in insect and disease resistance (Harborne, 1985) and in allelopathy (Inderjit, 1996). Many phenolic compounds are inhibitory to germinating seeds and growing plants (Rice, 1984). *p*-Hydroxybenzoic, vanillic, *p*-coumaric, syringic, and ferulic acids are the predominant phenolic acids identified as allelopathic agents in various natural and agroecosystems (Guenzi and McCalla, 1966; Blum et al., 1991; Chou et al., 1991; Ben-Hammouda et al., 1995).

Wheat (*Triticum aestivum* L.) possesses allelopathic potential for weed suppression. Aqueous extracts of straws were allelopathic to the germination and growth of a number of weed species (Steinsiek et al., 1982; Liebl and Worsham, 1983). Allelopathic ability of aqueous extracts differ significantly between cultivars (Guenzi et al., 1967; Wu et al., 1998). The allelopathic activity of wheat straws has been demonstrated under field conditions (Shilling et al., 1985). The results show that phytotoxins in wheat straws are water soluble and are prone to leach into the soil to influence the growth of weeds in the vicinity.

The chemical basis for the allelopathic potential of wheat straw has been investigated. Phenolic acids are a main category of allelochemicals implicated in wheat allelopathy with *p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, and ferulic acids being most frequently reported (Guenzi and McCalla, 1966; Lodhi et al., 1987). Ferulic and *p*-coumaric acids inhibit the radicle growth of radish (*Raphanus sativa*) at concentrations of 10^{-4}–10^{-3} M (Lodhi et al., 1987). Liebl and Worsham (1983) found that ferulic acid reduced germination and root length of *Ipomoea lacunosa*, *I. purpurea*, *Ambrosia artemisiifolia*, and *Sida spinosa*, at 5×10^{-3} M. Ferulic acid was further decarboxylated by a bacterium living on the carpels of *S. spinosa* seed to a more toxic styrene derivative 4-hydroxy-3-methoxystyrene (Shilling et al., 1985). Blum et al. (1991) reported that a mixture of phenolic acids similar to that obtained from the wheat soil under no-tillage systems reduced radicle and hypocotyl length of *Trifolium incarnatum.*

Although allelopathic activity varies considerably among accessions in a number of field crops, including wheat (Putnam and Duke, 1974; Fay and Duke, 1977; Spruell, 1984; Dilday et al., 1994; Wu et al., 1991a, 2000), little is known of the chemical basis for the varied activity. Several studies have screened a single active compound of plant tissues in a diverse crop collection in relation to plant natural defense against pests and diseases (Nicol et al., 1992; Lovett and Hoult, 1992), but screening for allelochemical content in plant tissues has been limited (Chou et al., 1991). Moreover, the allelopathic activity does not appear to be due to the effects of a single compound, but is more likely the result

of the combination and interaction of many allelochemicals (Einhellig, 1995). Simultaneous screening of multiple compounds responsible for weed suppression will be more informative.

Chemical analysis has shown that 39 wheat accessions differed significantly in total phenolic contents and that the allelopathic activity of wheat residues was highly associated with the total phenolic contents contained in each extract (Wu et al., 1998). The aim of the present study was to screen simultaneously seven known phenolic acids in the shoots of 17-day-old wheat seedlings in a diverse collection of 58 wheat accessions by gas chromatography and tandem mass spectrometry (GC-MS-MS).

METHODS AND MATERIALS

Wheat Growth. Based on previous experiments, a worldwide collection of 58 wheat accessions (*T. aestivum* L.) from the Australian Winter Cereals Collection was selected and grown according to the procedure described previously (Wu et al., 2000). Briefly, 12 pregerminated wheat seeds (surface-sterilized) of each accession were uniformly selected and aseptically sown on the agar surface with the embryo up, in three rows on one half of a glass beaker (500 ml) prefilled with 30 ml of 0.3% water agar. The beaker was wrapped with a piece of parafilm and placed in a controlled growth cabinet with a daily light/dark cycle of 13 hr/11 hr and a temperature cycle at 25°C/13°C. After the growth of seedlings for seven days, a piece of preautoclaved white paperboard was inserted across the center and down the middle of the beaker with the lower edge of the paperboard kept 1 cm above the agar surface. The beaker was again wrapped with parafilm and placed back in the growth cabinet for continuous growth of 10 more days.

Preparation of Shoot Extracts. The procedure previously described for the preparation of shoot extracts was used (Wu et al., 1999b). Briefly, shoots of 17-day-old seedlings were harvested for each accession and immediately freeze-dried. Then 0.100 g of shoots was cut into 2-mm lengths, ground into powder, macerated with 3 ml of 0.001 M HCl, sonicated at 5°C for 15 min, and centrifuged at 20,000 rpm and 10°C for 15 min. The supernatant was then collected and extracted three times with 10-ml portions of diethyl ether. The ether was evaporated under reduced pressure at 35°.

Derivatization and Quantitation. The derivatization and quantitation of samples were identical to those described previously (Wu et al., 1999b). Briefly, the silylation of shoot samples was accomplished by the addition of 1.0 ml of MSTFA (Alltech Australia) at 60°C for 30 min. The silylated samples were directly analyzed by GC-MS-MS. Each phenolic acid was identified and quantified by comparing retention time and product ion spectrum with that in the

user library created from the standard compound. Quantitative analysis was performed by the internal standard method (Wu et al., 1999b) and is reported in units of milligrams per kilogram of dry matter.

GC-MS-MS Analysis. GC-MS-MS analysis was carried out on a Varian 3400 CX gas chromatograph coupled with a Varian Saturn 2000 ion trap mass spectrometer. Parahydroxybenzoic acid (PHB), vanillic acid (VAN), syringic acid (SYR), paracoumaric acid (COU), ferulic acid (FER), and the internal standard (parachlorobenzoic acid) were obtained from Sigma-Aldrich Chemical Co. Silylated samples or standard compounds were introduced via a DB-5MS fused-silica capillary column of 30 m $\times$ 0.25 mm $\times$ 0.25 μm (J & W Scientific, Alltech Australia). The gas chromatographic and tandem mass spectrometric conditions for the analysis of wheat samples were identified to those reported previously (Wu et al., 1999b). Mass spectral scan time from m/z 50 to 450 was 1.0 sec (using three microscans). Nonresonant collision-induced dissociation (CID) was used for MS-MS. All samples were run in triplicate.

Data Analysis. All experimental data were subjected to analysis of variance with Genstat 5 (Release 3.2), and the treatment means were tested separately for least significant difference (LSD) at a 5% level of probability.

RESULTS

Variations in Levels of Phenolic Acids in Wheat Shoots. All 58 wheat accessions contained the seven phenolic acids, *p*-hydroxybenzoic, vanillic, syringic, *trans-p*-coumaric, *cis-p*-coumaric, *trans*-ferulic, and *cis*-ferulic acids analyzed in the shoots, and the concentrations of each phenolic acid differed significantly among accessions (Table 1, Figures 1–3). The content of *p*-hydroxybenzoic acid in shoots ranged from 9.8 to 49.3 mg/kg shoot dry weight, vanillic acid from 12.9 to 68.8 mg/kg, *cis-p*-coumaric acid from 0.8 to 11.2

TABLE 1. HIGHEST AND LOWEST CONCENTRATIONS OF PHENOLIC ACIDS IN WHEAT SHOOTS[a]

Phenolic acid	Lowest		Highest	
PHB	Federation	9.8 ± 3.8	Sunco	49.3 ± 1.4
VAN	Emika	12.9 ± 0.1	SST 6	68.8 ± 5.2
cis-COU	Federation	0.8 ± 0.1	Sunstar	11.2 ± 1.0
SYR	Sunco	1.9 ± 0.0	Sunstar	61.5 ± 3.4
cis-FER	Sunco	0.2 ± 0.0	Bernina	17.0 ± 1.4
trans-COU	Federation	11.4 ± 0.4	Sunstar	117.7 ± 4.8
trans-FER	Sunco	3.2 ± 0.2	Sunstar	149.3 ± 6.6

[a] Data presented as the means ± SD, mg/kg of dry matter.

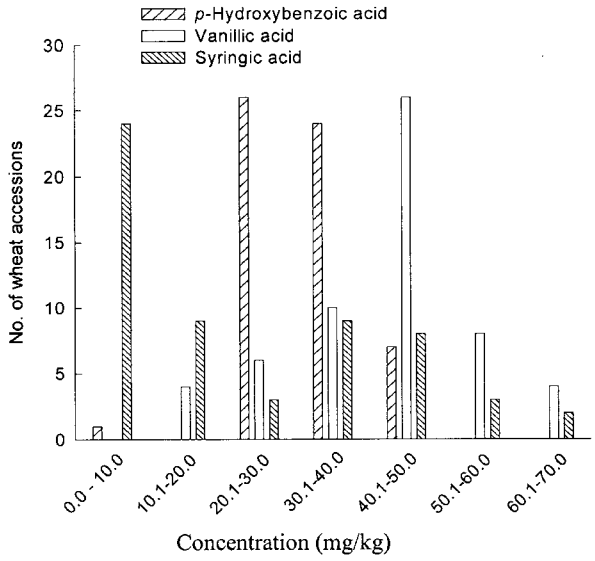


FIG. 1. Frequency distribution of the benzoic derivatives in 58 wheat accessions.

mg/kg, syringic acid from 1.9 to 61.5 mg/kg, *cis*-ferulic acid from 0.2 to 17.0 mg/kg, *trans*-*p*-coumaric acid from 11.4 to 1117.7 mg/kg, and *trans*-ferulic acid from 3.2 to 149.3 mg/kg.

Distribution of Phenolic Acids Among Wheat Accessions. The distribution of the level of each phenolic acid is presented in Figures 1–3. The levels of *p*-hydroxybenzoic and vanillic acids were normally distributed in the 58 wheat accessions, while a binormal distribution was found for the syringic acid (Figure 1). Only one accession produced *p*-hydroxybenzoic acid at a level less than 10.0 mg/kg, and seven accessions at a level of more than 40.0 mg/kg. Four accessions produced vanillic acid at less than 20.0 mg/kg and 12 accessions at more than 50.0 mg/kg; 24 accessions produced syringic acid at less than 10.0 mg/kg and 5 accessions at more than 50.0 mg/kg dry weight.

A skewed normal distribution was found for the *trans*-*p*-coumaric acid and a binormal distribution for the *trans*-ferulic acid (Figure 2). There were 10 accessions that produced a level of *trans*-ferulic acid at less than 25.0 mg/kg and six accessions at more than 130.0 mg/kg. Eleven accessions produced *trans*-*p*-coumaric acid at less than 25.0 mg/kg and six accessions at more than 55.0 mg/kg.

The levels of *cis*-*p*-coumaric and *cis*-ferulic acids were also normally distributed in the 58 wheat accessions (Figure 3). There was one accession that produced a level of *cis*-*p*-coumaric acid at less than 1.0 mg/kg and six acces-

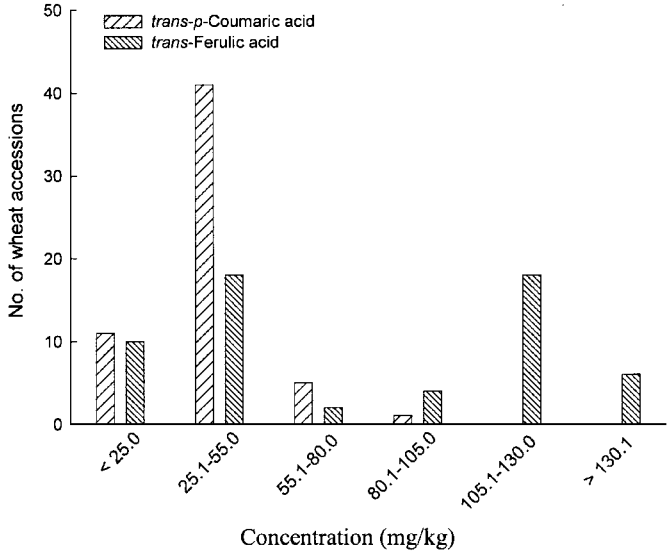


FIG. 2. Frequency distribution of the *trans*-cinnamic derivatives in 58 wheat accessions.

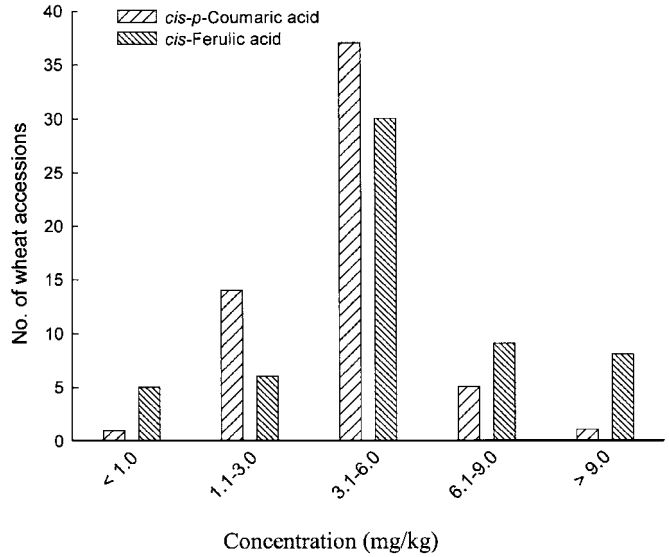


FIG. 3. Frequency distribution of the *cis*-cinnamic derivatives in 58 wheat accessions.

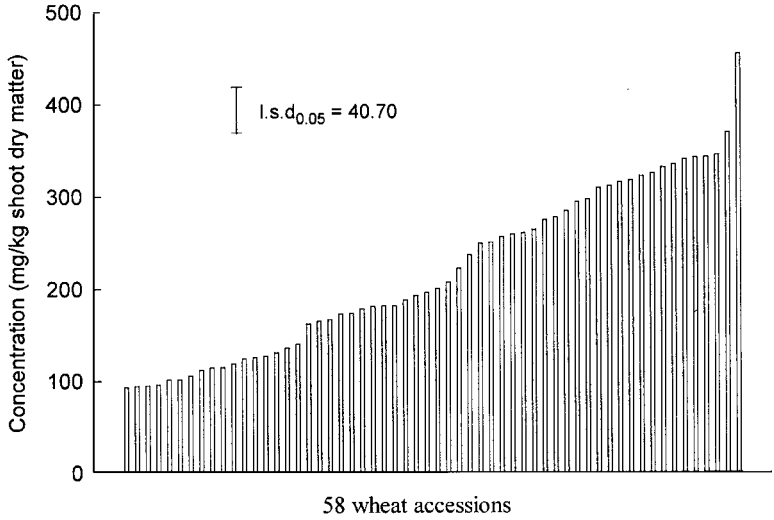


FIG. 4. Variation of total identified phenolic acids in wheat shoots.

sions at more than 6.0 mg/kg. Five accessions produced *cis*-ferulic acid at less than 1.0 mg/kg and 17 accessions at more than 6.0 mg/kg.

The concentration of total identified phenolic acids varied from 93.2 to 453.8 mg/kg in the shoots of 58 accessions (Figure 4). There were seven accessions that produced a level of total identified phenolic acids at less than 110.0 mg/kg: Emika, Canada 4125, Sunco, RAC 710, Hartog, Federation, and Eretria. Correspondingly, these accessions were often found to contain lower levels of individual phenolic acids in the shoot tissues. Eight accessions contained total identified phenolic acids at more than 330.0 mg/kg: Tasman, Bernina, Wattines, Insignia, Khapli, RAC 820, SST 6, and Sunstar. Correspondingly, higher concentrations of individual phenolic acids were often found in these accessions. Forty-three accessions produced total identified phenolic acids in a range of 110.0–330.0 mg/kg.

Different phenolic acids occurred in varied amounts in the shoots of the same accession. Of the seven phenolic acids analyzed, *trans*-ferulic acid was the most predominant with an average level of 74.1 ± 48.1 mg/kg in the shoots. The *cis* isomers of *p*-coumaric and ferulic acids were produced at the lowest level, occurring at 4.2 ± 1.6 and 5.2 ± 3.6 mg/kg, respectively. The following are listed in decreasing order of the concentration in the shoots of 17-day-old seedlings: *trans*-ferulic acid > vanillic acid (42.0 ± 12.1 mg/kg) > *trans*-*p*-coumaric acid (38.5 ± 18.3 mg/kg) > *p*-hydroxybenzoic acid (31.1 ± 7.3 mg/kg) > syringic acid (22.9 ± 18.2 mg/kg) > *cis*-ferulic acid > *cis*-*p*-coumaric acid.

TABLE 2. CORRELATION AMONG PHENOLIC ACIDS EXTRACTED FROM WHEAT SHOOTS[a]

Phenolic acid	PHB	VAN	*cis*-COU	SYR	*cis*-FER	*trans*-COU	*trans*-FER
PHB	1						
VAN	0.204	1					
cis-COU	0.274[b]	0.408[c]	1				
SYR	0.231	0.645[c]	0.384[c]	1			
cis-FER	0.004	0.527[c]	0.485[c]	0.456[c]	1		
trans-COU	0.265[b]	0.534[c]	0.734[c]	0.519[c]	0.478[c]	1	
trans-FER	0.152	0.664[c]	0.389[c]	0.920[c]	0.613[c]	0.532[c]	1

[a] Data expressed as the correlation coefficient between phenolic acids.
[b] Significant correlation at $P < 0.05$.
[c] Significant correlation at $P < 0.01$.

Correlation Between Phenolic Acids in Wheat Shoots. The metabolism of many phenolic compounds is known to follow the shikimate pathway (Harborne, 1989). There were significant correlations between contents of phenolic acids in wheat shoots, except for *p*-hydroxybenzoic acid (Table 2). The content of *p*-hydrozybenzoic acid did not significantly correlate to that of vanilic, syringic, *cis*-ferulic, and *trans*-ferulic acids. After grouping the phenolic acids, it was found that there were significant correlations among benzoic acid derivatives, cinnamic acid derivatives total coumaric acid, and total ferulic acid (Table 3).

DISCUSSION

The contents of phenolic acids in wheat shoot tissues varied with accessions and compounds. *trans*-Ferulic acid was the predominant phenolic com-

TABLE 3. CORRELATION AMONG GROUPS OF PHENOLIC ACIDS EXTRACTED FROM WHEAT SHOOTS[a]

Phenolic groups[b]	Benzoics	Cinnamics	Total COU	Total FER
Benzoics	1			
Cinnamics	0.864[c]	1		
Total COU	0.592[c]	0.742[c]	1	
Total FER	0.853[c]	0.966[c]	0.542[c]	1

[a] Data expressed as the correlation coefficient between phenolic groups.
[b] Benzoics refers to the benzoic acid derivatives, including *p*-hydroxybenzoic, syringic, and vanillic acids; cinnamics refers to the cinnamic acid derivatives, including *cis*- and *trans*-*p*-coumaric, *cis*- and *trans*-ferulic acids; total COU includes the *cis*- and *trans*-*p*-coumaric acids; total FER includes the *cis*- and *trans*-ferulic acids.
[c] Significant correlation at $P < 0.01$.

pound identified. The abundance of *trans*-ferulic and *trans*-*p*-coumaric acids in wheat tissues has been reported previously. Salomonsson et al. (1978) found that *trans*-ferulic and *trans*-*p*-coumaric acids were the dominant acids in the aqueous alkaline extracts of barley, oat, wheat, rye, and rice straws, each at 510–3100 and 440–3100 mg/kg of straw, respectively. *cis*-*p*-Coumaric acid and *cis*-ferulic acid were at smaller levels of 130–530 and 100–560 mg/kg, respectively. Lodhi et al. (1987) found that ferulic and *p*-coumaric acids in the soil were present in higher concentrations than *p*-hydroxybenzoic, syringic, vanillic, and *p*-coumaric acids, and the amount of ferulic acid was higher than that of *p*-coumaric acid. However, Ben-Hammouda et al. (1995) reported that the concentrations of *p*-hydroxybenzoic, syringic, and vanillic acids in various sorghum plant parts were consistently higher than those of ferulic acid and *p*-coumaric acids, with ferulic acid being most frequently not detectable. It is seen, therefore, that the concentration of each phenolic acid varies greatly depending on biotic and abiotic factors, such as crop types and accessions, plant ages and parts, extraction methods, and the analytical techniques (EI-Basyouni and Towers, 1964; Guenzi and McCalla, 1996; Kaminsky and Muller, 1978; Lodhi et al., 1987; Ben-Hammouda et al., 1995).

Phenolic compounds have been implicated in residue allelopathy of a number of crops, including rice (Chou and Lin, 1976), sorghum (Ben-Hammouda et al., 1995), and wheat (Guenzi and McCalla, 1966; Salomonsson et al., 1978; Lodhi et al., 1987). The differential level of each phenolic acid identified in the present study could contribute in part to the variability of allelopathic potential of wheat accessions. Our research has shown that wheat seedling allelopathy on the growth of annual ryegrass (*Lolium rigidum* Gaud.) differed in a worldwide collection of 453 wheat accessions (Wu et al., 1999a). The eight accessions with the highest levels of total identified phenolic acids reported in the present study, with the exception of Insignia, which was only weakly allelopathic, were strongly allelopathic to the growth of *L. rigidum.* Similarly, the seven accessions with the lowest levels of total identified phenolics, with the exception of Sunco, in which an intermediate level of allelopathic potential was found, were weakly allelopathic to the growth of *L. rigidum.*

Allelochemicals present in plant shoot tissues may greatly contribute to residue allelopathy. Nevertheless, amounts of allelochemicals present in plant roots may be more critical to the actual exudation of such chemicals by the living roots into the growth environment. Exudation of allelochemicals is one of the processes whereby crops exert allelopathic activity. Further research is necessary to investigate the concentrations of phenolic acids in root tissues of wheat seedlings so that the allelochemical relationship between the contents of roots and shoots, and the involvement of root allelochemicals in wheat allelopathy can be determined.

Acknowledgments—This research is funded by both Charles Sturt University (CSU) and the Australian Cooperative Research Centre for Weed Management Systems. The authors are grateful for the gifts of wheat accessions from the Australian Winter Cereals Collection, and the provision of GC-MS-MS instrumentation from the Environmental and Analytical Laboratories at CSU.

REFERENCES

BEN-HAMMOUDA, M., KREMER, R. J., MINOR, H. C., and SARWAR, M. 1995. A chemical basis for the differential allelopathic potential of sorghum hybrids on wheat. *J. Chem. Ecol.* 21:775–786.

BLUM, U., WENTWORTH, T. R., KLEIN, K., WORSHAM, A. D., KING, L. D., GERIG, T. M., and LYU, S. W. 1991. Phenolic acid content of soils from wheat-no till, wheat-conventional till, and fallow-conventional till soybean cropping systems. *J. Chem. Ecol.* 17:1045–1068.

CHOU, C. H., and LIN, H. J. 1976. Autointoxication mechanism of *Oryza sativa.* I. Phytotoxic effects of decomposing rice residues in soil. *J. Chem. Ecol.* 2:353–367.

CHOU, C. H., CHANG, F. J., and OKA, H. I. 1991. Allelopathic potential of wild rice *Oryza perennis. Taiwania* 36:201–210.

DILDAY, R. H., LIN, J., and YAN, W. 1994. Identification of allelopathy in the USDA-ARS rice germplasm collection. *Aust. J. Exp. Agric.* 34:907–910.

EI-BASYOUNI, S., and TOWERS, G. H. N. 1964. The phenolic acids in wheat I. Changes during growth and development. *Can. J. Biochem.* 42:203–210.

EINHELLIG, F. A. 1995. Allelopathy: current status and future goals, pp. 1–24, *in* Inderjit, K. M. M. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications. American Chemical Society Symposium Series No. 582, American Chemical Society, Washington, D.C.

FAY, P. K., and DUKE, W. B. 1977. An assessment of allelopathic potential in *Avena* germplasm. *Weed Sci.* 25:224–228.

GUENZI, W. D., and MCCALLA, T. M. 1966. Phenolic acids in oats, wheat, sorghum, and corn residues and their phytotoxicity. *Agron. J.* 58:303–304.

GUENZI, W. D., MCCALLA, T. M., and NORSTADT, F. A. 1967. Presence and persistence of phytotoxic subtances in wheat, oat, corn and sorghum residues. *Agron. J.* 59:163–165.

HARBORNE, J. B. 1980. Plant phenolics, pp. 329–402, *in* E. A. Bell and B. V. Charlwood (eds.). Secondary Plant Products. Encyclopedia of Plant Physiology. New Series, Volume 8, Springer-Verlag, New York.

HARBORNE, J. B. 1985. Phenolics and plant defence, pp. 393–408, *in* C. F. Sumere and P. J. Lea (eds.). Biochemistry of Plant Phenolics, Annual Proceedings of the Phytochemical Society of Europe. Clarendon Press, Oxford, U.K.

HARBORNE, J. B. 1989. General procedures and measurement of total phenolics, pp. 1–28, *in* J. B. Harborne (ed.). Methods in Plant Biochemistry: Volume 1 Plant Phenolics. Academic Press, London.

INDERJIT. 1996. Plant phenolics in allelopathy. *Bot. Rev.* 62:186–202.

KAMINSKY, R., and MULLER, W. H. 1978. A recommendation against the use of alkaline soil extractions in the study of allelopathy. *Plant Soil* 49:641–645.

LIEBL, R. A., and WORSHAM, A. D. 1983. Inhibition of pitted morning glory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J. Chem. Ecol.* 9:1027–1043.

LODHI, M. A. K., BILAL, R., and MALIK, K. A. 1987. Allelopathy in agroecosystems: Wheat phytotoxicity and its possible roles in crop rotation. *J. Chem. Ecol.* 13:1881–1891.

LOVETT, J. V., and HOULT, A. H. C. 1992. Gramine: the occurrence of a self-defence chemical in

barley, *Hordeum vulgare* L., pp. 426–429. Proceedings, 6th Australian Agronomy Conference, Armidale, Australia.

NICOL, D., COPAJA, S. V., WRATTEN, S. D., and NIEMEYER, H. M. 1992. A screen of worldwide wheat cultivars for hydroxamic acid levels and aphid antixenosis. *Ann. Appl. Biol.* 121:11–18.

PUTNAM, A. R., and DUKE, W. B. 1974. Biological suppression of weeds: Evidence for allelopathy in accessions of cucumber. *Science* 185:370–372.

RICE, E. L. 1984. Allelopathy, 2nd ed. Academic Press, Orlando, Florida.

SALOMONSSON, A. C., THEANDER, O., and AMAN, P. 1978. Quantitative determination by GLC of phenolic acids as ethyl derivatives in cereal straw. *J. Agric. Food Chem.* 26:830–835.

SHILLING, D. G., LIEBL, R. A., and WORSHAM, A. D. 1985. Rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) mulch: The suppression of certain broadleaved weeds and the isolation and identification of phytotoxins, pp. 243–271, *in* A. C. Thompson (ed.). The Chemistry of Allelopathy: Biological Interaction among Plants. American Chemical Society Symposium Series No. 268, American Chemical Society, Washington, D.C.

SPRUELL, J. A. 1984. Allelopathic potential of wheat accessions. *Diss. Abstr. Int. B* 45:1102B.

STEINSIEK, J. W., OLIVER, L. R., and COLLINS, F. C. 1982. Allelopathic potential of wheat (*Triticum aestivum*) straw on selected weed species. *Weed Sci.* 30:495–497.

WU, H., PRATLEY, J., LEMERLE, D., HAIG, T., and VERBEEK, B. 1998. Differential allelopathic potential among wheat accessions to annual ryegrass, pp. 567–571, *in* D. L. Michalk and J. E. Pratley (eds.). Proceedings, 9th Australian Agronomy Conference, Wagga Wagga, Australia.

WU, H., PRATLEY, J., LEMERLE, D., and HAIG, T. 1999a. Crop cultivars with allelopathic capability. *Weed Res.* 39:171–180.

WU, H., HAIG, T., PRATLEY, J., LEMERLE, D., and AN, M. 1999b. Simultaneous determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one by GC/MS/MS in wheat (*Triticum aestivum* L.). *J. Chromatogr. A.* 864:315–321.

WU, H., PRATLEY, J., LEMERLE, D., and HAIG, T. 2000. Laboratory screening for allelopathic potential of wheat (*Triticum aestivum*) accessions against annual ryegrass (*Lolium rigidum*). *Aust. J. Agric. Res.* 51:259–266.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

PLANT-GROWTH INHIBITORY ACTIVITY OF CEDRELANOLIDE FROM *Cedrela salvadorensis*

CARLOS L. CÉSPEDES,[1,*] JOSÉ S. CALDERÓN,[1] JUAN R. SALAZAR,[1] BLAS LOTINA-HENNSEN,[2] and ROSABEL SEGURA[3]

[1]*Instituto de Química*
[2]*Facultad de Química*
UNAM, Ciudad Universitaria
Coyoacán 04510, México D.F., México
[3]*Facultad de Ciencias*
Universidad Nacional de Colombia
Santa Fé de Bogotá, Colombia

(Received November 29, 2000; accepted September 2, 2000)

Abstract—The effect of cedrelanolide, the most abundant limonoid isolated from *Cedrela salvadorensis* (Meliaceae), was assayed as a plant-growth inhibitory compound against monocotyledonous and dicotyledonous seeds. This compound inhibited germination, seed respiration, and seedling dry weights of some plant species (*Lolium multiflorum*, var. Hercules, *Triticum vulgare*, var. Salamanca, *Physalis ixocarpa*, and *Trifolium alexandrinum*). Our results indicate that cedrelanolide interferes with monocot preemergence properties, mainly energy metabolism of the seeds at the level of respiration. In addition, the compound inhibits photophosphorylation, H^+ uptake, and noncyclic electron flow. This behavior might be responsible for its plant-growth inhibitory properties and its possible role as an allelopathic agent.

Key Words: *Cedrela salvadorensis*, cedrelanolide, Meliaceae, phytogrowth inhibition, seed respiration, Hill reaction inhibitor, limonoid, nortriterpenoid.

INTRODUCTION

Limonoids from the family Meliaceae are attracting considerable interest because of their insect antifeedant properties, with special focus on the ring-cleaved example, azadirachtin (Kraus, 1995). In contrast, less is known about the allelopathic activity of compounds in the azadirone, gedunin, mexicanolide, and sen-

*To whom correspondence should be addressed. e-mail: ccespede@servidor.unam.mx

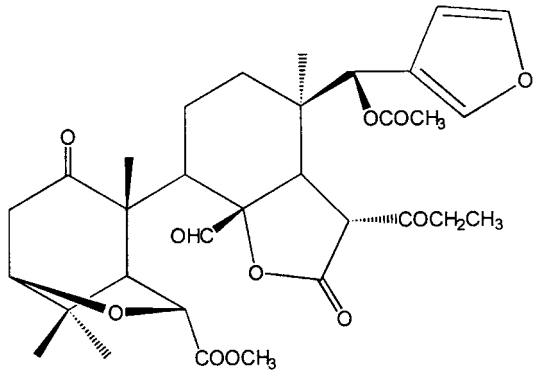


FIG. 1. Chemical structure of cedrelanolide.

danin classes. In a limited range of structures, they are also found in the families Rutaceae and Cneoraceae (Champagne et al., 1992). These natural products possess a wide range of biological activities including insect antifeedant activity (Isman et al., 1996; Mata R., 1996; Jiménez et al., 1997; Zhou et al., 1998; Veitch et al., 1999; Céspedes et al., 2000), plant-growth regulating properties (Céspedes et al., 1998, 1999a,b), medicinal effects in animals and humans (MacKinnon et al., 1997; Matsuda et al., 1998), and fungicidal (Govindachari et al., 1999; Weimin et al., 1998), bactericidal (Tada et al., 1999), and antiviral (Lowery et al., 1997) activities. Among the many limonoids isolated from these families, cedrelanolide (Figure 1) was found for the first time in *Cedrela salvadorensis* (Meliaceae), a Mexican specimen (Segura et al., 1994).

We found that some limonoids obtained from *Cedrela* species have different mechanisms of action on photosynthesis (Céspedes et al., 1998; Lotina-Hennsen et al., 1998). For example, gedunin and 7-oxo-7-deacetoxygedunin, isolated from *C. odorata* and *C. salvadorensis*, act as energy-transfer inhibitors interacting with CF_0 (Achnine et al., 1999) while odoratol has impairment effects on the donor side of photosystem II (Achnine et al., 1998). However, α-photogedunin and β-photogedunin isolated from *C. salvadorensis* and *C. ciliolata* (Syn *dugesii*) inhibit germination and respiration of monocot and dicot weed seeds, and the epimeric mixture partially inhibits photophosphorylation, H^+ uptake, and noncyclic electron flow (Céspedes et al., 1999a). In similar form, α- and β-photogedunin acetates inhibit seed germination, seedling growth, and seed respiration. In addition, these derivatives are Hill reaction inhibitors with two different targets of inhibition in the photosynthesis electron transport rate (Céspedes et al., 1998, 1999b).

The insecticidal behavior of cedrelanolide on *Ostrinia nubilalis*, the European corn borer, has been reported (Jiménez et al., 1997), as has the antifeedant

activity on *Spodoptera frugiperda* (Céspedes et al., 2000). Cedrelanolide may increase plant resistance to insect herbivores; however, its mode of action is unknown.

We are continuing investigations for biologically active limonoids with potential herbicidal properties (Lotina-Hennsen et al., 1998) and now report the phytotoxic properties of cedrelanolide on germination, root and shoot development, and respiration of seeds of two monocots (*Lolium multiflorum* and *Triticum vulgare*) and two dicots (*Physalis ixocarpa* and *Trifolium alexandrinum*). In addition to these inhibitory activities, the effects of this compound on the photosynthesis system are presented.

METHODS AND MATERIALS

Chemicals and Solvents. All reagents used were commercially available in either AR or chromatographic grade. Tricine, sorbitol, and methyl viologen (MV) were purchased from Sigma Chemical Co. Methanol, $CHCl_3$, CH_2Cl_2, KCl, $CuSO_4$, NH_4Cl, $MgCl_2$, pyridine, acetic anhydride, silica gel GF_{254} analytical chromatoplates, silica gel grade 60, 70–230, and 60 Å for column chromatography, hexane, and ethyl acetate were purchased from Merck.

Apparatus. ^1H NMR spectra were recorded at 300 and 500 MHz, and ^{13}C NMR spectra at 75 and 125 MHz, on Varian VXR-300S and VXR-500S spectrometers, respectively. Chemical shifts (parts per million) are related to $(CH_3)_4Si$ as internal reference. $CDCl_3$ and acetone-d_6 from Aldrich Chemical Co. were used as solvents. Coupling constants are quoted in hertz. IR spectra were obtained in KBr on Perkin-Elmer 283-B and FT-IR Nicolet Magna 750 spectrophotometers. Electron impact mass spectra were taken on a Jeol JMS-SX102A (70 eV). UV spectra were determined on a Shimadzu UV-160 spectrophotometer. Optical rotation was measured on a Jasco DIP-360 spectropolarimeter. Melting points were obtained on a Fisher-Johns hot-plate apparatus and are uncorrected. Electron transport and oxygen evolution were determined with a Clark-type electrode connected to YSI oxygraph model 5300.

Plant Material. The heartwood of *Cedrela salvadorensis* was collected in Morelia, State of Michoacán, in February 1997. A voucher has been deposited at the ethnobotanical collection of the National Herbarium (MEXU), Instituto de Biología, UNAM (Voucher: M. T. German and P. Tenorio No. 2.174. Register number: 800.193).

Isolation of Cedrelanolide. Cedrelanolide was obtained from a $CHCl_3$ extract of the stem bark of *C. salvadorensis* as colorless crystals (CH_2Cl_2–MeOH), mp = 280°, $[\alpha]_D^{20}$ – 10.24° (c 2.56, $CHCl_3$). The molecular formula $C_{32}H_{40}O_{11}$ was determined by mass spectrometry (HR-FAB-MS), *m/z* 601.2626 [M^++1], EI-MS, *m/z* (relative intensity) 541 (M^+–59, 1), 511 (1.4), 455 (25),

431 (20), 370 (50), 137 (100), 95 (41), 57 (37). Signals in the IR spectrum, in addition to the furan absorptions (1502 and 874 cm^{-1}), included strong carbonyl bands at 1790 (γ lactone), 1747 (ester), 1727 (aldehyde), and 1701 cm^{-1} (ketone). UV (EtOH) 208 nm. The shifts of ^{1}H NMR and ^{13}C NMR spectral data were identical with those published in the literature (Segura et al., 1994).

Seed Germination Bioassays. Monocot seeds of *Lolium multiflorum* (Poaceae) ryegrass var. Hercules were purchased from Barenbreig. *Triticum vulgare* (Graminae) wheat var. Salamanca, and two dicotyledons seeds—*Trifolium alexandrinum* (Leguminoseae) red clover var. Andino and *Physalis ixocarpa* (Solanaceae) green tomato—were purchased from Semillas Berentsen, S. A. de C. V. Celaya, Guanajuato, Mexico. Germination tests were run in triplicate with 100 *L. multiflorum* seeds, 40 *T. vulgare* seeds, 100 *T. alexandrinum* seeds, and 100 *P. ixocarpa* seeds for each concentration of sample for five days (three for germination and two for root and shoot growth). The number of seeds used for each experiment was selected so that an appreciable change in O_2 uptake could be detected by the oxygraph. The test seeds were held in the dark at 25°C in 9.0-cm Petri dishes containing an 8.5-cm sheet of Whatman No. 1 filter paper and 10.0 ml of test or control solution. The dishes were wrapped with Parafilm foil and incubated at 28°C in the dark. The number of germinated seeds was determined according to the criterion of 1 mm extrusion of the radicle. Germination rates were counted at 72 and 120 hr later for root and shoot growth measurements. Cedrelanolide was initially dissolved in methanol and diluted with water. The maximum final concentration of methanol was 0.1% and the same methanol concentration was used in the control solution. The pH of the solution was adjusted to 6.5 before the bioassay. Seeds were selected for uniformity of size, and damaged ones were discarded (Li et al., 1992).

Growth Bioassays. Coleoptyle or hypocotyle and root lengths for all germinated seeds were measured after 120 hr, in three replicates, after which the germinated plants were dried to constant weight at 40°C.

Seed Respiration. Seed respiration was measured polarographically as oxygen uptake during the germination process with a YSI oxygraph model 5300. The oxygen uptake, in the presence of different concentrations of cedrelanolide, was evaluated over 5 and 10 min, in a nonilluminated cell. The requirement for oxygen was plotted as a percentage of the control at 100%.

The data were analyzed by ANOVA ($P < 0.05$), and GI_{50}, RI_{50}, and I_{50} values for each activity were calculated by probit analysis on the basis of the percentage of inhibition obtained at each concentration of cedrelanolide. I_{50} is the concentration producing 50% inhibition, and GI_{50} is the concentration inhibiting 50% germination, and RI_{50} is the concentration that inhibits 50% of respiration.

Chloroplast Isolation and Chlorophyll Determination. Isolated chloroplasts were prepared from market spinach leaves (*Spinacia oleracea*) as previously reported (Mills et al., 1980; Saha et al., 1971; Jiménez et al., 1996) and the pellet

was resuspended, unless indicated, in a solution containing 400 mM sucrose, 5 mM $MgCl_2$, and 10 mM KCl buffered with 30 mM Na^+-tricine at pH 8.0. Chlorophyll concentration was determined according to Strain et al. (1971).

Measurement of Proton Uptake and ATP Synthesis. Proton uptake was measured as the rise in pH from 8.0 to 8.1 (Dilley, 1972). A combination microelectrode connected to a Corning potentiometer (model 12 Research pH-meter) with expanded scale and registered on a Gilson recorder was used. The reaction medium was 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, and 1 mM Na^+-tricine at pH 8. ATP synthesis was measured titrimetrically according to the procedure of Dilley (1972), with 50 μM methyl viologen (MV) added as an electron acceptor for the Hill reaction.

Measurement of Electron Transport. Photosynthesis noncyclic electron transport rates from water to MV were monitored with a YSI (Yellow Spring Instrument) model 5300 oxygen monitor connected to a Clark-type electrode. The reaction medium was the same as that used in the H^+-uptake assay except for the tricine concentration (15 mM), and in the case of the uncoupled electron transport measurement 6 mM NH_4Cl was added. All reaction mixtures were illuminated with light from a projector lamp (GAF 2660) filtered through 5 cm of 1% $CuSO_4$ solution at 20°C (Calera et al., 1995; Van Gorkom and Gast, 1996).

RESULTS AND DISCUSSION

Seed Germination. Cedrelanolide inhibited seed germination for the four species tested (*L. multiflorum*, *T. vulgare*, *P. ixocarpa*, and *T. alexandrinum*) as concentration increased to 500 μM (Figure 2). The GI_{50} (concentration inhibiting 50% germination) values indicate that the monocot *L. multiflorum* is the most susceptible to cedrelanolide, with GI_{50} = 120.6 μM, and causing 100% at 300 μM (Table 1). The data suggest slow permeation of the compound through the seed membranes. In our test the preemergence assays were done before emergence of seeds, according to Hatfield and Karlen (1994) and Mohr and Schopfer (1995). These results suggest either that the oxygenated functions in the molecule play a role in inhibition because of lipophilicity or that the hydrophilicity of the oxygenated function of cedrelanolide (at physiological pH) makes it difficult for cedrelanolide to reach the target site.

Root and Shoot Elongation. Radicle and shoot development was partially inhibited by cedrelanolide. The monocots *T. vulgare* and *L. multiflorum* were more sensitive to cedrelanolide (I_{50} varying from 140 to 300 μM, Table 1) than were dicot seedlings (Figure 3), with the exception of *T. vulgare* roots, which were resistant to cedrelanolide inhibition (Figure 4).

Growth inhibition was concentration-dependent. As in germination, cedrelanolide had the least inhibitory effects on growth of *T. vulgare* (100%)

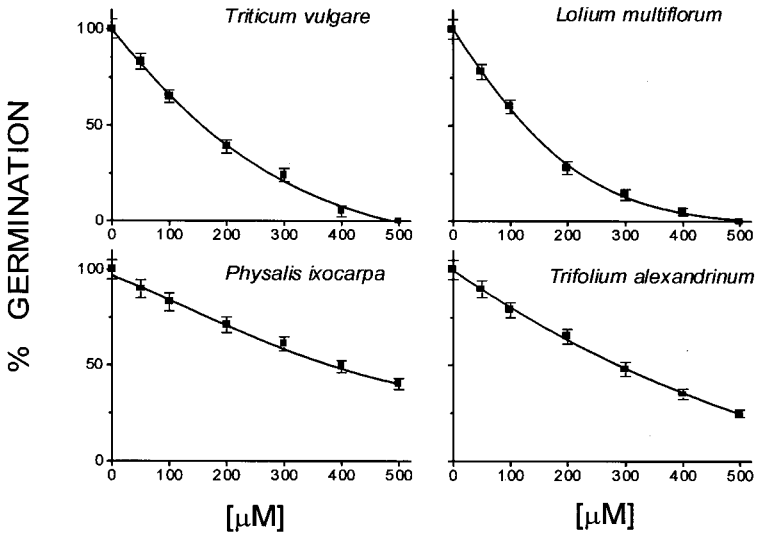


FIG. 2. Concentration-dependent inhibition of cedrelanolide on seed germination expressed as percent of control germination. Each value represents mean ± SE ($N = 5$) in error bars.

at 500 μM, and the least inhibitory effect on *L. multiflorum* at 300 μM. Here values indicate that the mechanisms of action of cedrelanolide may differ for growth and germination (Einhellig, 1986).

TABLE 1. EFFECT OF CEDRELANOLIDE ON SEED GROWTH (ELONGATION, GERMINATION, RESPIRATION, AND DRY WEIGHT)

	Cedrelanolide (μM)[a]				
	Elongation (EI_{50})		Germination	Respiration	Dry Weight
Species	Stems	Roots	(GI_{50})	(RI_{50})[b]	(DWI_{50})
T. vulgare	297.0	—	157.0	100	155.0
L. multiflorum	142.0	114.0	125.0	100	351.0
T. alexandrinum	452.0	393.0	289.0	400[c]	
P. ixocarpa	500.0	466.0	370.0	300	

[a] Means of three experiments. The EI_{50}, GI_{50}, RI_{50}, and DWI_{50} are the inhibitory concentrations that give 50% inhibition of seedling length (stem and roots), seed germination, respiration, and dry weight, respectively. Calculated by ANOVA ($P < 0.05$).
[b] Values giving 50% inhibition at 24 hr.
[c] Value at 30 hr.

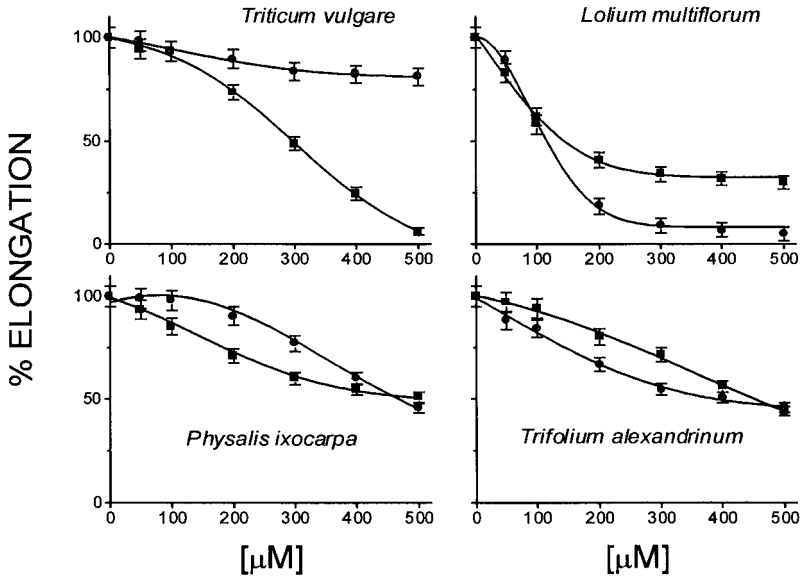


FIG. 3. Effects of solutions of cedrelanolide on seedling growth. (●, roots, ■, stems) of mono- and dicot seeds. Each value represents mean ± SE ($N = 5$) in error bars. The inhibition efficacy was expressed as a percentage of cedrelanolide activity inhibited compared with the control value (100%).

Dry Weight. In general, development of monocot species was more sensitive to cedrelanolide than dicot species. Dry weight of dicot species was not affected by cedrelanolide (Figure 4). Seedling biomass (dry weight) of monocots diminishes with concentration of cedrelanolide in similar form to germination.

Seed Respiration. Seed respiration was more sensitive to cedrelanolide than seed germination for all species tested. Inhibition of seed respiration by cedrelanolide was a function of time and concentration of the natural product. Thus, as incubation time or concentration increased, inhibition increased (Figure 5). The RI_{50} values (concentration inhibiting 50% respiration) show that monocot (*T. vulgare* and *L. multiflorum*) respiration was more sensitive to cedrelanolide than that of dicots (Table 2). These values suggest that cedrelanolide acts as an uncoupler to phosphorylation at low concentration, but at higher concentrations it either inhibits energy transduction or the respiration redox enzymes.

Effect of Cedrelanolide on ATP Formation, Proton Uptake, and Electron Transport Rate. To assess the effects of cedrelanolide on photosynthesis, we evaluated the effects on different photosynthetic reactions. The assay concentration ranged from 0 to 150 μM in 10 μM increments. Figure 6 shows that photosynthesis photophosphorylation from water to methyl viologen was inhibited by

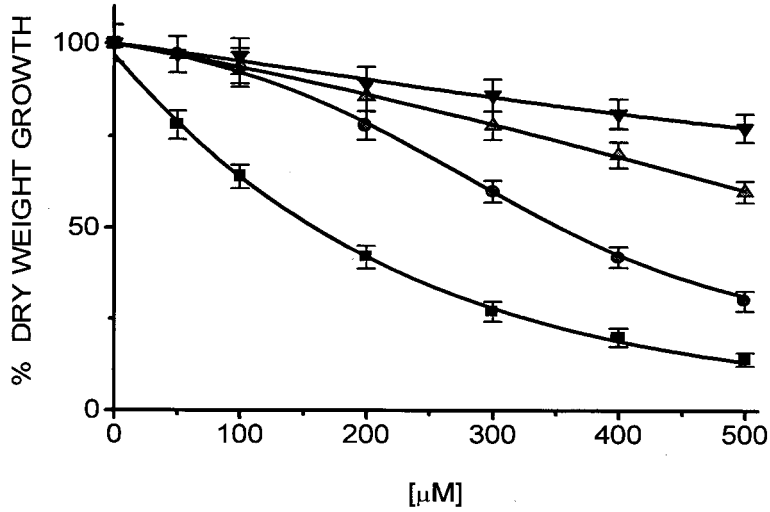


FIG. 4. Inhibition by solutions of cedrelanolide on dry weight of seedlings (■, *T. vulgare*; ●, *L. multiflorum*; ▲, *P. ixocarpa*; ▼, *T. alexandrinum*). Each value represents mean ± SE ($N = 5$) in error bars. The inhibition efficacy was expressed as a percentage of cedrelanolide activity inhibited compared with the control value (100%).

cedrelanolide at 100 μM with an IC_{50} of 29.5 μM (Table 2). In addition, Figure 6 shows that cedrelanolide inhibits proton uptake partially at 34.9 μM (IC_{50}) and completely at 100 μM. Based on Figure 7, we suggest that cedrelanolide inhibits the whole electron transport chain from water to MV at 60 μM, measured as oxygen uptake under photophosphorylating conditions. These effects are similar to that observed with the photogedunin acetate derivatives (Céspedes et al., 1998). The photogedunin acetates show these effects at concentrations of 10–30 μM, while cedrelanolide shows similar effects between 20 and 90 μM. According to pI_{50} values, cedrelanolide has similar potency as a Hill reaction inhibitor to some commercial herbicides that affect chloroplast electron flow. These results suggest that the presence of oxygenated functions significantly enhances the inhibitory potency of these compounds as electron transport inhibitors. Our group has demonstrated the effects of gedunin and 7-oxo-7-deacetoxygedunin (which are oxygenated limonoids) on the electron transport chain in the 300 and 400 μM range (Achnine et al., 1999). The pI_{50} values (Table 2) also indicate that this compound is a potent Hill reaction inhibitor, and its abundance in the plant *Cedrela salvadorensis* suggests a role as a defensive allelochemical.

Inhibition of germination, seed respiration, and radicle or shoot elongation by cedrelanolide was similar to effects of concentration ranges of phenolic acid derivatives, i.e., 100–1000 μM (Einhellig, 1986, 1995). Cedrelanolide

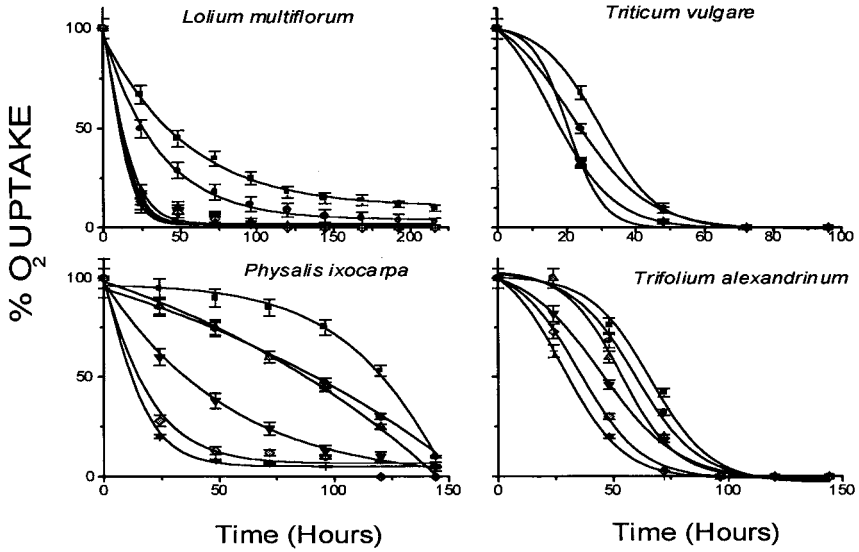


FIG. 5. Inhibition of respiration of *T. vulgare*, *L. multiflorum*, *P. ixocarpa*, and *T. alexandrinum* seeds by solutions of cedrelanolide, expressed as percent of O_2 uptake rate as a function of control seed respiration (■, 50 μM; ●, 100 μM; ▲, 200 μM; ▼, 300 μM; ♦, 400 μM; +, 500 μM. Each value represents mean ± SE ($N = 5$) in error bars.

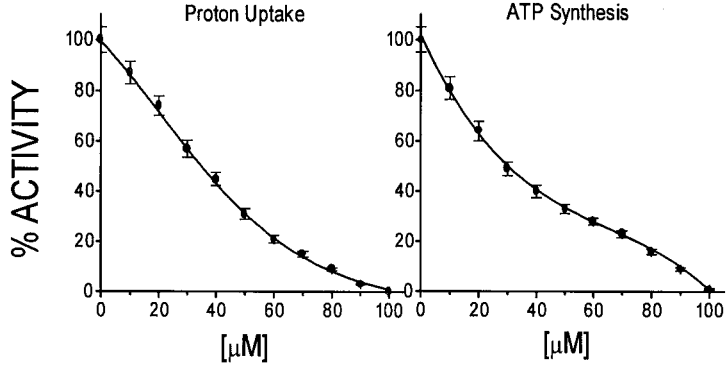


FIG. 6. Rate of ATP synthesis and proton uptake with increasing concentration of cedrelanolide in chloroplasts isolated from fresh spinach leaves. Each value represents mean ± SE ($N = 5$) in error bars. The inhibition efficacy was expressed as a percentage of cedrelanolide activity inhibited compared with the control value (100%). Control values for ATP synthesis and H^+ uptake were 350 and 280 μmol/mg chlorophyll/hr, respectively.

TABLE 2. EFFECT OF CEDRELANOLIDE (μM) ON PHOTOSYNTHESIS ELECTRON TRANSPORT RATE MEASUREMENTS IN INTACT FRESHLY LYSED SPINACH CHLOROPLASTS[a]

Activity	pI_{50}	IC_{50}
H^+-uptake	4.457	34.9
ATP synthesis	4.530	29.5
Basal	4.376	42.0
Uncoupled	4.468	34.0
Phosphorylating	4.610	24.5

[a] Means of three experiments. Values are expressed as the dose (μM concentration) that gives 50% inhibition. Calculated by ANOVA ($P < 0.05$).

inhibited respiration, germination, shoot and root elongation, and dry weight in a concentration-dependent manner. In general, radicle and shoot elongation were less sensitive to cedrelanolide inhibition than were germination and seed respiration (see I_{50}, Table 1).

Humilinolides A, B, C, and D (tetranortriterpenoids of the mexicanolide group) and the MeOH extract of the seeds from *Swietenia humilis* (Meliaceae), displayed marked phytoxicity towards radicle growth of *Amaranthus hyponcondriacus* and *Echinochloa crusgalli.* The IC_{50} of the extract was 275.95 and

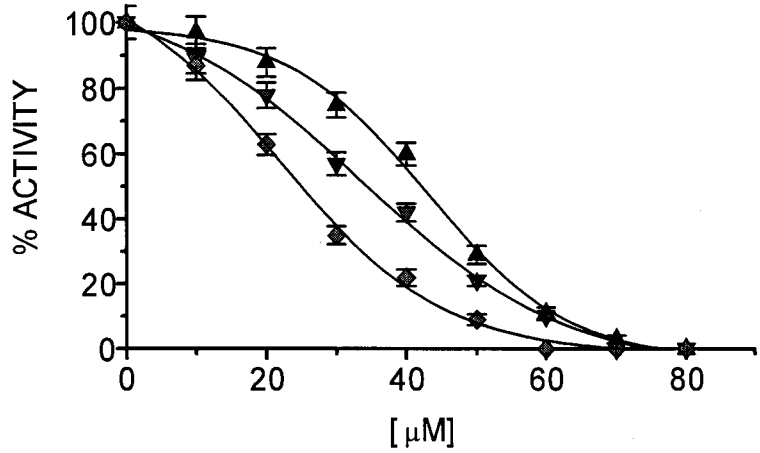


FIG. 7. Effects of cedrelanolide on electron transport rate (tridown, uncoupled; ♦, phosphorylating; and ▲, basal) in chloroplasts isolated from fresh spinach leaves. Control rate values (in μequiv e^-/hr/mg chlorophyll) are 300, 600, and 1050, respectively. Each value represents mean ± SE ($N = 5$) in error bars. The inhibition efficacy was expressed as a percentage of activity inhibited compared with the control value (100%).

171.54 ppm on radicle growth of *A. hyponcondriacus* and *E. crusgalli*, respectively. The humilinolides A and C showed the same effect at 99.06 and 163.0 ppm for *E. crusgalli*, respectively; and at 199.0 and 215.08 ppm for *A. hypocondriacus*, respectively. Humilinolides B and D were not inhibitory (Segura-Correa et al., 1993). These results show that cedrelanolide is a more potent inhibitor than the humilinolides to mono- and dicot seeds.

Achnine et al. (1999) investigated the effect of the limonoids gedunin, 7-oxogedunin, 7-deacetoxigedunin, and odoratol on photosystem I and photosystem II and reported that these natural products were inhibitory at different sites on the electron transport carrier of the photosynthesis process. However, the effect of other limonoids on seed germination, root and seedling growth, and seed respiration was unclear. Cedrelanolide is unique among limonoids reported to date (Segura et al., 1994) that should be biogenetically derived from a methylangolensate type of precusor. The unusual C-15 acyl group could be produced by an actyl transfer from a suitable ester, probably at C-30 (Taylor, 1979). A second modification would be the opening of the δ-lactone ring to give a free acid functionality at C-16 and the acyloxy group at C-17, and the C-16 acid could relactonize with a free hydroxyl group at C-8 to yield the γ-lactone portion.

This compound has certain similarities with sendanin and toosendanin and their derivatives (Isman et al., 1996; Schmutterer, 1995). We have recently reported that other limonoids cause different types of inhibition and characteristic effects on seed germination and respiration (Céspedes et al., 1998, 1999a,b). We know of no other reports of the effects of these kind of compounds on growth or germination.

Acknowledgments—This work was supported by CONACyT-Mexico grants 27975-N and DGAPA-UNAM grants IN216698 and IN215799. We thank Beatriz King-Diaz, MSc (Facultad de Química, UNAM) for help in the determination of allelopathic activities; Isabel Chávez, Wilber Matus, Rocío Patiño, Javier Pérez Flores, and Luis Velasco (Instituto de Química) for technical assistance; and Professor Teresa German (Instituto de Biología, UNAM), for identifying the plant material.

REFERENCES

ACHNINE, L., MATA, R., IGLESIAS-PRIETO, R., and LOTINA-HENNSEN, B. 1998. Impairment of photosystem II donor side by the natural product odoratol. *J. Agric. Food Chem.* 46:5313–5317.

ACHNINE, L., MATA, R., and LOTINA-HENNSEN, B. 1999. Interference of the natural product 7-oxo-7-deacetoxygedunin with CF_0 of H^+-ATPase on spinach chloroplasts. *Pestic. Biochem. Physiol.* 63:139–149.

CALERA, M. R., SOTO, F., SANCHEZ, P., BYE, R., HERNÁNDEZ, B., ANAYA, A. L., LOTINA-HENNSEN, B., and MATA, R. 1995. Biochemically active sesquiterpene lactones from *Ratibida mexicana. Phytochemistry* 40:419–425.

CÉSPEDES, C. L., CALDERÓN, J. S., KING-DIAZ, B., and LOTINA-HENNSEN, B. 1998. Phytochemical and biochemical characterization of epimeric photogedunin derivatives. Their different sites of

interaction on the redox electron transport carrier of *Spinacea oleracea* L. *J. Agric. Food Chem.* 46:2810–2816.

CÉSPEDES, C. L., CALDERÓN, J. S., SALAZAR, J. R., KING-DIAZ, B., and LOTINA-HENNSEN, B. 1999a. Allelopathic activity of photogedunins from *Cedrela ciliolata* (Meliaceae). *Bol. Soc. Chil. Quím.* 44:173–183.

CÉSPEDES, C. L., CALDERON, J. S., GÓMEZ-GARIBAY, F., SEGURA, R., KING-DIAZ, B., and LOTINA-HENNSEN, B. 1999b. Phytogrowth properties of limonoids isolated from *Cedrela ciliolata. J. Chem. Ecol.* 25:2665–2676.

CÉSPEDES, C. L., CALDERÓN, J. S., LINA, L., and ARANDA, E. 2000. Growth inhibitory effects on fall armyworm. *Spodoptera frugiperda* of some limonoids isolated from *Cedrela* spp. (Meliaceae). *J. Agric. Food Chem.* 48:1903–1908.

CHAMPAGNE, D. E., KOUL, O., ISMAN, M. B., SCUDDER, G. G. E., and TOWERS, G. H. N. 1992. Biological activity of limonoids from the rutales. *Phytochemistry* 31:377–394.

DILLEY, R. A. 1972. Ion transport (H^+, K^+, Mg^{2+} exchange phenomena). *Methods Enzymol.* 24:68–74.

EINHELLIG, F. A. 1986. Mechanisms and modes of action of allelochemicals, pp. 171–188, *in* R. A. Putnam and Ch-Sh. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

EINHELLIG, F. A. 1995. Mechanism of action of allelochemicals in a allelopathy, pp. 96–116, *in* Inderjit, K. M. M. Dakshini, and F. A. Einhellig (eds.). Allelopathy Organisms, Processes, and Applications, ACS Series 582, American Chemical Society, Washington, D.C.

GOVINDACHARI, T. R., SURESH, G., BANUMATHY, B., MASILAMANI, S., GEETHA GOPALAKRISHNAN, and KUMARI, G. N. K. 1999. Antifungal activity of some B,D-seco limonoids from two meliaceous plants. *J. Chem. Ecol.* 25:923–933.

HATFIELD, J. L., and KARLEN, D. L. 1994. Sustainable Agriculture Systems. Lewis Publishers, Boca Raton, Florida.

ISMAN, M. B., MATSUURA, H., MACKINNON, S., DURST, T., TOWERS, G. H. N., and ARNASON, J. T. 1996. Phytochemistry of the Meliaceae: So many terpenoids, so few insecticides, pp. 155–178, *in* J. T. Romeo, J. A. Saunders, and P. Barbosa (eds.). Phytochemical Diversity and Redundancy in Ecological interactions. Plenum Press, New York.

JIMÉNEZ, A., MATA, R., LOTINA-HENNSEN, B., ANAYA, A. L., and VELASCO, L. 1996. Phytogrowth-inhibitory compounds from *Malmea depressa. J. Nat. Prod.* 59:202–204.

JIMÉNEZ, A., MATA, R., PEREDA-MIRANDA, R., CALDERON, J., ISMAN, M. B., NICOL, R., and ARNASON, J. T. 1997. Insecticidal limonoids from *Swietenia humilis* and *Cedrela salvadorensis. J. Chem. Ecol.* 23:1225–1234.

KRAUS, W. 1995. Biologically active ingredients, pp. 35–88, *in* H. Schmutterer (ed.). The Neem Tree *Azadirachta indica*, A. Juss and Other Meliaceous Plants. Sources of Unique Natural Products from Integrated Pest Management, Medicine, Industry and Other Purposes XXIII. VCH, Weinheim.

LI, H. H., NISHIMURA, H., HASEGAWA, K., and MIZUTANI, J. 1992. Allelopathy of *Sasa cernua. J. Chem. Ecol.* 18:1785.

LOTINA-HENNSEN, B., MATA, R., CALDERÓN, J. S., CÉSPEDES, C. L., and JIMENEZ-ESTRADA, M. 1998. Secondary metabolites isolated from Mexican plants: Target and mechanism of action on photosynthesis, pp. 731–749, *in* S. G. Pandalai and A. Gayathri. (eds.). Recent Research Developments in Agricultural & Food Chemistry, Vol. 2. Research Signpost, Trivandrum, India.

LOWERY, D. T., EASTWELL, K. C., SMIRLE, M. J. 1997. Neem seed oil inhibits aphid transmission of potato virus Y to pepper. *Ann. Appl. Biol.* 130:217–225.

MACKINNON, S., DURST, T., ARNASON, J. T., ANGERHOFER, C., PEZZUTO, J., SÁNCHEZ-VINDAS, P. E., POVEDA, L. J., and GBEASSOR, M. 1997. Antimarial activity of tropical Meliaceae extracts and gedunin derivatives. *J. Nat. Prod.* 60:336–341.

MATA, R. 1996. Bioactive compounds of medicinal and agrochemical interest from Mexican plants. *Rev. Latinoam. Quím.* 24:76–83.

MATSUDA, H., YOSHIKAWA, M., and KUBO, M. 1998. Antinociceptive and antiinflammatory activities of limonin isolated from the fruits of *Evodia rutaecarpa* var bodinieri. *Planta Med.* 64:339–342.

MILLS, J. D., MITCHELL, P., and SCHURMANN, P. 1980. Modulation of coupling ATPase activity in intact chloroplasts. *FEBS Lett.* 112:173–177.

MOHR, H., and SCHOPFER, P. 1995. Plant Physiology. Springer-Verlag, Berlin. 629 pp.

SAHA, S., OUITRAKUL, R., IZAWA, S., and GOOD, N. 1971. Electron transport and phosphorylation in chloroplasts in function of the electron acceptor. *J. Biol. Chem.* 246:3204–3209.

SCHMUTTERER, H. 1995. The Neem Tree *Azadirachta indica*, A. Juss and Other Meliaceous Plants: Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. VCH, Weinheim.

SEGURA, R., CALDERÓN, J., TOSCANO, R., GUTIÉRREZ, A., and MATA, R. 1994. Cedrelanolide I, a new limonoid from *Cedrela salvadorensis. Tetrahedron Lett.* 35:3437–3440.

SEGURA-CORREA, R., MATA, R., ANAYA, A. L., HERNANDEZ-BAUTISTA, B., VILLENA, R., SORIANO-GARCIA, M., BYE, R., and LINARES, E. 1993. New tetranortriterpenoids from *Swietenia humilis. J. Nat. Prod.* 56:1567–1574.

STRAIN, H. H., COPPE, B. T., and SVEC, W. A. 1971. Analytical procedures for the isolation, identification, estimation and investigation of the chlorophylls. *Methods Enzymol.* 23:452–466.

TADA, K., TAKIDO, M., and KITANAKA, S. 1999. Limonoids from fruit of *Melia toosendan* and their cytotoxic activity. *Phytochemistry* 51:787–791.

TAYLOR, D. A. H. 1979. A limonoid, Pseudrelone B, from *Pseudocedrela kotschyii. Phytochemistry* 18:1574–1576.

VAN GORKOM, H. J., and GAST, P. 1996. Measurement of photosynthetic oxygen evolution, pp. 391–405, *in* J. Amesz and A. J. Hoff (eds). Biophysical Techniques in Photosynthesis. Advances in Photosynthesis. Kluwer Academic, Dordrecht, The Netherlands.

VEITCH, N. C., WRIGHT, G. A., and STEVENSON, P. C. 1999. Four new tetranortriterpenoids from *Cedrela odorata* associated with leaf rejection by *Exopthalmus jekelianus. J. Nat. Prod.* 62:1260–1263.

WEIMIN, Z., WOLFENDER, J. L., HOSTETTMANN, K., RENSHENG, X., and GUOWEI, Q. 1998. Antifungal alkaloids and limonoids derivatives from *Dictamnus dasycarpus. Phytochemistry* 47:7–11.

ZHOU, J-B., TADERA, K., MINAMI, Y., YAGI, F., KURAWAKI, J., TAKEZAKI, K., and NAKATANI, M. 1998. New limonoids from *Melia toosendan. Biosci. Biotechnol. Biochem.* 62:496–500.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

INFLUENCE OF DEFOLIATION ON TOXIC ALKALOID CONCENTRATION AND ALKALOID POOLS IN TALL LARKSPUR

M. H. RALPHS* and D. R. GARDNER

USDA/ARS Poisonous Plant Laboratory
1150 E. 1400 North, Logan, Utah 84341

(Received May 1, 2000; accepted September 2, 2000)

Abstract—This study was replicated at two locations in the mountains of central Utah. In 1997, ten uniform plants of tall larkspur (*Delphinium barbeyi*) in the early bud stage (40 cm in height) were selected at each site and clipped at 5 cm above soil level. In 1998, one stalk from each plant was harvested on a weekly basis; in 1999, one stalk was harvested at four times during its phenological development. Toxic and total alkaloid concentrations were measured and alkaloid pools in the entire stalk were calculated. Clipping reduced stalk height to less than 50 cm in 1998 and 65 cm in 1999, compared to over 100 cm in unclipped control plants. Alkaloid concentration was similar to control plants, but toxic alkaloid pools were 70% lower than control plants, because of the reduction in biomass of the stalks. Clipping reduced subsequent vigor and the amount of toxic and total alkaloids in tall larkspur.

Key Words—Tall larkspur, *Delphinium barbeyi*, norditerpenoid alkaloids, defoliation, cattle poisoning.

INTRODUCTION

Larkspurs (*Delphinium* spp.) are an important group of poisonous plants on mountain rangelands. They are relatively palatable to all livestock (Pfister et al., 1988) but acutely toxic to cattle (Olsen, 1978), causing widespread deaths throughout the mountains in the western United States (Nielsen and Ralphs, 1988).

*To whom correspondence should be addressed. e-mail: mralphs@cc.usu.edu

0098-0331/01/0100-0151$19.50/0

The toxic compounds in larkspur are norditerpenoid alkaloids. Alkaloids containing the *N*-(methylsuccinimido)-anthranilic ester group (referred to as MSAL alkaloids) are the most toxic (Manners et al., 1995), with methyllycaconitine (MLA) being the most prominent toxic alkaloid. We have differentiated the class of toxic alkaloids (MSAL) in larkspurs from the other alkaloids (Gardner et al., 1997) and quantified them as a measure of potential toxicity to cattle (Ralphs et al., 1997).

Environmental stresses on tall larkspur (*D. barbeyi*) have little effect on toxic alkaloid levels. Light stress and photosynthesis inhibition (Ralphs et al., 1998a) and the herbicide glyphosate (Ralphs et al., 1998b) reduced the dry weight of the plants, thus increasing alkaloid concentration proportionately, but the absolute amount of alkaloids, or alkaloid pools, were not affected. Damage from the larkspur mirid (*Hopplomachus affiguratus*) actually reduced toxic alkaloid concentration (Ralphs et al., 1998c), rather than inducing alkaloid synthesis, as suggested by the plant defense theory (Tallamy and Raupp, 1991).

Laycock (1975) clipped Duncecap larkspur (*D. occidentale*) in the vegetative stage for two successive years and reported that the concentration of total alkaloids declined by 50%, compared with unclipped plants. If defoliation reduced the toxicity of larkspur, clipping or mowing larkspur patches might reduce the risk of poisoning cattle. However, the generalized plant defense theory (Rhoades, 1979, 1985) predicts that some plants induce synthesis of toxic defense compounds in response to defoliation as a self-defense mechanism. Sometimes the response is delayed to the next season's growth (Tuomi et al., 1990). This delayed resistance may be an active response induced by defoliation (Haukioja, 1980) or an indirect response resulting from nutrient stress (Tuomi et al., 1990; Bryant et al., 1993). The carbon/nutrient balance theory predicts that if either carbon or nitrogen limits growth, the other nutrient becomes excessive and can be shunted to defense compounds (Bryant et al., 1983). The objective of this study was to determine the effects of clipping tall larkspur on subsequent vigor, alkaloid concentration, and alkaloid pools.

METHODS AND MATERIALS

The study was replicated at two locations in the mountains of central Utah. The Ferron Reservoir site is 46 km west of Ferron at an elevation of 3150 m. It is in a subalpine zone and the vegetation consisted of scattered subalpine fir stands interspersed in the tall forb plant community dominated by tall larkspur, western cone flower (*Rudbeckia occidentalis*), sweet cicely (*Osmorhiza occidentalis*), and mountain brome (*Bromus carinatus*).

The Mt. Terrell site is 40 km east of Salina at 3230 m in the subalpine zone. Tall larkspur dominated the tall forb community with violet (*Viola purpurea*),

sedge (*Carex* spp.), and mountain brome as understory species. Both sites were fenced to prevent any grazing interference during the experiment.

Ten uniform plants (20–30 stalks/plant) were selected at each site and marked with an orange tent stake. The plants were clipped 5 cm above the soil on July 16, at Salina and July 17, 1997 at Ferron. The plants were in the early bud stage, prior to elongation of the inflorescence. In 1998, one stalk was harvested from each plant, on a weekly basis, to evaluate seasonal trends in toxic and total alkaloid responses to clipping the previous year. In 1999, one stalk was harvested in the late vegetative, early flower, late flower, and pod stages of development. The height of the stalk was measured when harvested. It was placed in an airtight plastic bag and immediately frozen on Dry Ice. The samples were freeze dried and then weighed to obtain dry weight. After grinding through a cyclone grinder, the alkaloids were extracted and analyzed by Fourier-transformed infrared spectroscopy (FTIR) (Gardner et al., 1997) to determine the concentration of the toxic MSAL alkaloids and total alkaloids. Alkaloid pools in each stalk were calculated by multiplying alkaloid concentration by the dry weight of the stalk (Ralphs et al., 2000). Pools are a better estimate of secondary compound synthesis because concentration can change by being diluted in a large plant, or concentrated in a small plant (Bryant et al., 1993).

The data were analyzed in two parts. The first analysis compared vigor measurements (number of stalks and dry weight per stalk) in the same plant when it was clipped in 1997 and at equivalent growth stages in 1998 and 1999. Data were analyzed in a random mixed analysis of variance in a split-plot design comparing locations and year, with plants within location as a random factor. The data were transformed by the log transformation prior to analysis, but actual means are presented. There was a location × year interaction ($P < 0.01$), so the model was reduced to a one-way analysis of variance comparing years within each location. Means were separated by least significance difference pairwise comparisons.

The second analysis compared seasonal trends of stalk height and alkaloid concentrations and pools between the plants that had been defoliated in 1997, and 10 undefoliated control plants at each location in 1998 and 1999. These variables were compared in a repeated measures mixed analysis of variance using compound symmetry covariance in a split plot design. Treatment (control, clipped), location, and year were the main effects, and plants within treatment and location was the factor repeated over weeks. The data were transformed by log transformation prior to analysis, but actual means are presented in the graphs.

RESULTS

Vigor. There was an initial difference in the number of stalks per plant between the two locations ($P < 0.001$). The Salina site had an average of 31

TABLE 1. VIGOR OF TALL LARKSPUR WHEN CLIPPED IN 1997 IN EARLY BUD STAGE AND RESPONSE IN 1998 AND 1999[a]

Location	Year	Plants (N)	Stalks/plant (N, mean ± SE)	Stalk weight (g, mean ± SE)
Ferron	1997	10	18 ± 2.3 a	5.3 ± 1.1 a
	1998	8	7 ± 1.4 b	1.8 ± 0.4 b
	1999	8	9 ± 3.4 b	2.2 ± 0.4 b
Salina	1997	10	31 ± 1.7 a	3.6 ± 0.3 a
	1998	9	16 ± 2.5 b	1.7 ± 0.2 b
	1999	8	32 ± 4.9 a	1.5 ± 0.1 b

[a] Means within locations not followed by the same letter differ ($P < 0.05$).

stalks/plant, compared with 18 stalks/plant at Ferron. Clipping greatly reduced the vigor of tall larkspur plants, in both number of stalks per plant and stalk weight (Table 1). The number of stalks per plant declined by 50% in 1998 and remained low in 1999 at Ferron. Stalks per plant increased, however, to pretreatment levels in 1999 at Salina. Stalk weight declined at both locations and remained low in both years.

Figure 1 illustrates the growth pattern of larkspur plants through the 1998 growing season. The control plants exceed 100 cm in height when mature. The plants clipped in 1997 reached a height of only 50 cm in 1998 and 65 cm in 1999 ($P < 0.001$).

Alkaloids. The concentrations of toxic and total alkaloids in defoliated plants were similar to the control plants and steadily declined through the growing season in both years (Figure 2). There was a year difference ($P < 0.001$) with both toxic and total alkaloids slightly lower in 1999.

There was considerable variability in concentration of toxic alkaloids in defoliated plants in 1998 (i.e., wide standard error bars; Figure 2). Some of the defoliated plants were severely stunted. Their stalks were small and spindly, and they remained in the vegetative stage throughout the growing season. In some but not all of these stunted plants, toxic and total alkaloids remained high throughout the growing season, in contrast with the other plants in which alkaloid concentrations declined with maturity. Perhaps the prolonged juvenile stage of the stunted plants accounted for the elevated and retained alkaloid concentrations.

The total amount of alkaloid in a stalk, or alkaloid pool, regardless of its size, was estimated by multiplying the dry weight of the stalk by its alkaloid concentration. Pools of both toxic and total alkaloids in clipped plants were only one third of the undefoliated control plants (Figure 3). Clipping suppressed both the growth and total amount of alkaloids in tall larkspur in the subsequent growing season.

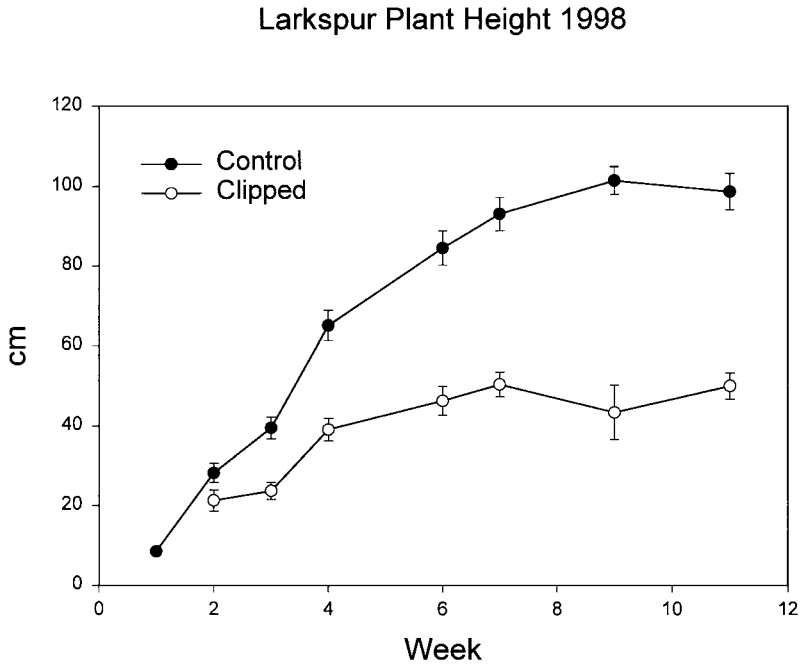


FIG. 1. Tall larkspur plant height through the 1998 growing season of undefoliated control plants and plants that were clipped in 1997.

DISCUSSION

Clipping tall larkspur in the early bud stage in 1997 greatly reduced the vigor of the same plants in 1998 and 1999. This agrees with the results of Laycock's (1975) experiments in which clipping duncecap larkspur in the early vegetative stage for two years was most detrimental to vigor when measured in the third year. Cronin (1971), however, reported that clipping tall larkspur at various frequencies during the growing season was not detrimental to the growth of the same plants the following year, but they failed to produce flowers. Vigor of other forbs in the tall forb community is also reduced by clipping: bluebell (*Mertensia arizonica* var. *leonardi*) (Laycock and Conrad, 1969), white polemonium (*Polemonium foliosissimum*) (McDonough and Laycock, 1975), Porter ligusticum (*Ligusticum porteri*), edible valeriam (*Valeriana edulis*), and Richardson geranium (*Geranium richardsoni*) (Julander, 1968).

Laycock (1975) further reported that the concentration of total alkaloids declined by 50% following two years of clipping. Earlier extraction methods for the titration analytical technique (Williams and Cronin, 1963) did not retrieve

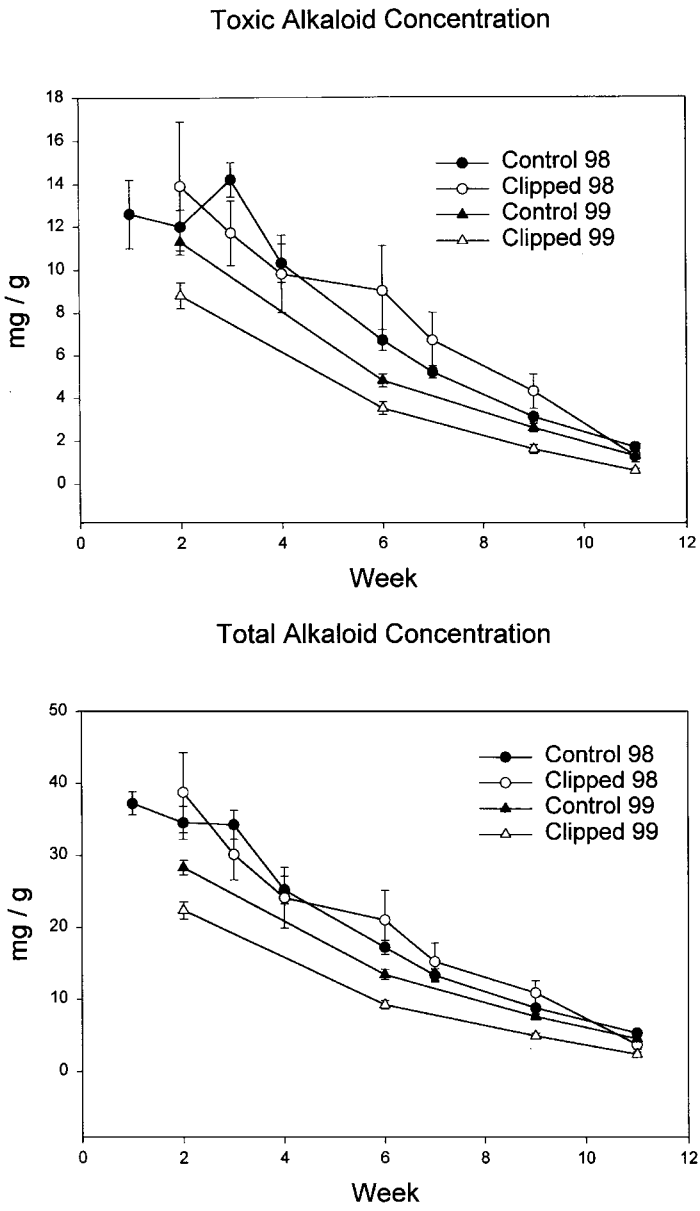


FIG. 2. Trends in toxic and total alkaloid concentration through the 1998 and 1999 growing season of control plants and plants that were clipped in 1997.

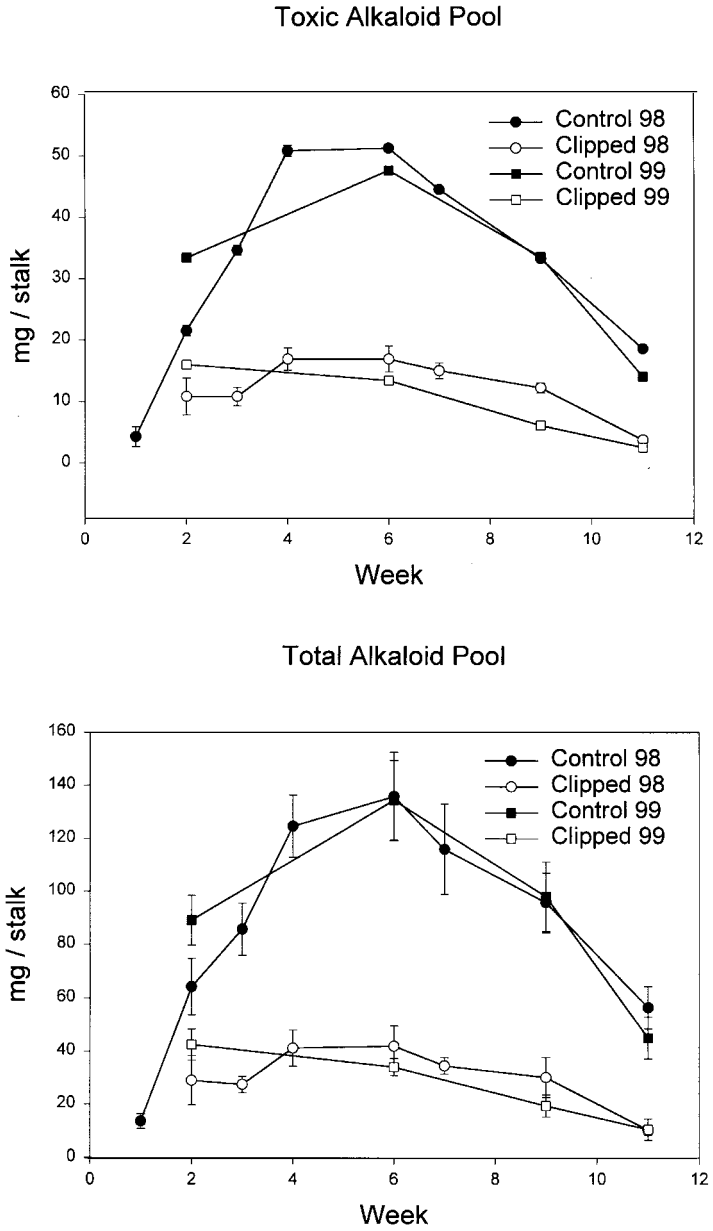


FIG. 3. Pools of toxic and total alkaloids in control plants and clipped plants in 1998 and 1999.

the toxic class of alkaloids (G. D. Manners, personal communication) and would subsequently underestimate the total alkaloid concentration. However, this does not explain the relative decline in total alkaloid concentration in Laycock's (1975) study and the lack of decline in alkaloid concentration in our study.

There was very little regrowth of clipped plants in 1997, and they were substantially smaller in 1998, both in height and stalk weight. Clearly their photosynthetic capacity was reduced. The carbon–nutrient balance theory (Bryant et al., 1983, 1992; Toumi et al., 1990) predicts that since carbon gain would be limited, nitrogen in excess of that used for growth could be used to increase synthesis of alkaloids. However, alkaloid synthesis apparently did not increase (alkaloid concentration was similar to controls and alkaloid pools were lower). In contrast, wild tobacco shifts its allocation of nitrogen to nicotine synthesis following clipping, at the expense of growth and reproduction (Ohnmeis and Baldwin, 1994; Baldwin et al., 1990).

Larkspur grows from buds on the crown of the tap root. These buds start to swell in the late summer and early fall, remain active and grow slowly beneath the snowpack over winter, and initiate rapid growth even before the snow melts in the spring (Kreps, 1969). Perhaps the removal of photosynthetic material during the 1997 growing season greatly restricted the energy flow to the crown and developing buds, resulting in the significant reduction in the number and vigor of stalks in 1998 and 1999.

Benn and May (1964) reported that alkaloids in *Delphinium brownii* are synthesized in roots and probably follow the mevalonate pathway of terpene biosynthesis or the recently proposed pathway starting from pyruvate and glyceraldehyde-3-phosphate (Lichtenthaler et al., 1997). Since energy and carbon were apparently limited in the roots of our clipped plants, alkaloid biogenesis may also have been limited.

Tall larkspur is not palatable to cattle in its early growth stages, but becomes palatable as it matures into the flower and pod reproductive stages (Pfister et al., 1997). Since clipping reduced the number and size of larkspur stalks, and prevented them from maturing into the reproductive stages, it may reduce the likelihood of cattle grazing clipped plants, as well as reducing the total larkspur biomass available to grazing animals. Alkaloid pools were also lower in the shorter stalks, although concentration of alkaloids remained unchanged. The reduced vigor of clipped plants may shift the competitive advantage to associated species in the plant community, which could further suppress larkspur growth. Thus, clipping may reduce the overall risk of poisoning in cattle.

REFERENCES

BALDWIN, I. T., SIMS, C. L., and KEAN, S. E. 1990. The reproductive consequences associated with inductible alkaloid response in wild tobacco. *Ecology* 71:252–262.

BENN, M. H., and MAY, J. 1964. The biosynthesis of diterpenoid alkaloids. *Experientia* 29(5):252–253.

BRYANT, J. P., REICHARDT, P. B., and CLAWSEN, T. P. 1992. Chemically mediated interactions between woody plants and browsing mammals. *J. Range Manage.* 45:18–24.

BRYANT, J. P., REICHARDT, P. B., CLAWSEN, T. P., and WERNER, R. H. 1993. Effects of mineral nutrition on delayed inducible resistance in Alaska paper birch. *Ecology* 74:2072–2084.

CRONIN, E. H. 1971. Tall larkspur: Some reasons for its continuing preeminence as a poisonous plant. *J. Range Manage.* 24:258–263.

GARDNER, D. R., MANNERS, G. D., RALPHS, M. H., and PFISTER, J. A. 1997. Quantitative analysis of norditerpenoid alkaloids in larkspur (*Delphinium* spp.). *Phytochem. Anal.* 8:55–62.

HAUKIOJA, E. 1980. On the role of plant defenses in the fluctuation of herbivore populations. *Oikos* 35:202–213.

JULANDER, O. 1968. Effect of clipping on herbage and flower stalk production of three summer range forbs. *J. Range Manage.* 21:74–79.

KREPS, L. B. 1969. The alkaloids of *Delphinium occidentale* S. Wats. PhD dissertation. Utah State University, Logan, Utah.

LAYCOCK, W. A. 1975. Alkaloid content of duncecap larkspur after two years of clipping. *J. Range Manage.* 28:257–259.

LAYCOCK, W. A., and CONRAD, P. W. 1969. How time and intensity of clipping affect tall bluebell. *J. Range Manage.* 22:299–303.

LICHTENTHALER, H. K., ROHMER, M., and SCHWENDER, J. 1997. Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiol. Plant.* 101:643–652.

MANNERS, G. D., PANTER, K. E., and PELLETIER, S. W. 1995. Structure–activity relationships of norditerpenoid alkaloids occurring in toxic larkspur (*Delphinium*) species. *J. Nat. Prod.* 58:863–869.

MCDONOUGH, W. T., and LAYCOCK, W. A. 1975. Growth and development of *Polemonium foliosissimum* on high elevation rangelands in Utah. *Am. Midl. Nat.* 94:27–37.

NIELSEN, D. B., and RALPHS, M. H. 1998. Larkspur: Economic considerations, pp. 119–130, *in* L. F. James, M. H. Ralphs, and D. B. Nielsen (eds.). The Ecology and Economic Impact of Poisonous Plants on Livestock Production. Westview Press, Boulder, Colorado.

OHNMEIS, T. E., and BALDWIN, I. T. 1994. The allometry of nitrogen allocation to growth and an inducible defense under nitrogen limited growth. *Ecology* 75:995–1002.

OLSEN, J. D. 1978. Tall larkspur poisoning in cattle and sheep. *J. Am. Vet. Med. Assoc.* 173:762–765.

PFISTER, J. A., RALPHS, M. H., and MANNERS, G. D. 1988. Cattle grazing tall larkspur on Utah mountain rangeland. *J. Range Manage.* 41:118–122.

PFISTER, J. A., RALPHS, M. H., MANNERS, G. D., GARDNER, D. R., PRICE, K. W., and JAMES, L. F. 1997. Early season grazing by cattle of tall larkspur (*Delphinium* spp.) infested rangeland. *J. Range Manage.* 50:391–398.

RALPHS, M. H., MANNERS, G. D., PFISTER, J. A., GARDNER, D. R., and JAMES, L. F. 1997. Toxic alkaloid concentration in tall larkspur species in the western U.S. *J. Range Manage.* 50:497–502.

RALPHS, M. H., MANNERS, G. D., and GARDNER, D. R. 1998a. Influence of light and photosynthesis on alkaloid concentration in larkspur. *J. Chem. Ecol.* 24:167–182.

RALPHS, M. H., MANNERS, G. D., and GARDNER, D. R. 1998b. Toxic alkaloid response to herbicides used to control tall larkspur. *Weed Sci.* 46:116–119.

RALPHS, M. H., GARDNER, D. R., JONES, W. A., and MANNERS, G. D. 1998c. Diterpenoid alkaloid concentration in tall larkspur plants damaged by the larkspur mirid. *J. Chem. Ecol.* 24:829–840.

RALPHS, M. H., GARDNER, D. R., and PFISTER, J. A. 2000. A functional explanation for pat-

terns of norditerpenoid alkaloid levels in tall larkspur (*Delphinium barbeyi*). *J. Chem. Ecol.* 26:1595–1607.

RHOADES, D. F. 1979. Evolution of plant chemical defense against herbivores. pp. 3–54, *in* G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

RHOADES, D. F. 1985. Offensive–defensive interactions between herbivores and plants: Their relevance in herbivore population dynamics and ecological theory. *Am. Nat.* 125:205–238.

TALLAMY, D. W., and RAUPP, M. J. 1991. Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

TUOMI, J. P., NIEMEDA, P., and SIREN, S. 1990. The Panglossian paradigm and delayed inducible accumulation of foliar phenolics in mountain birch. *Oikos* 59:399-410.

WILLIAMS, M. C., and CRONIN, E. H. 1963. Effects of silvex and 2,4,5-T on alkaloid content of tall larkspur. *Weeds* 11:317–319.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

INVOLVEMENT OF LIGNINLIKE COMPOUNDS IN TOXICITY OF DIETARY ALDER LEAF LITTER AGAINST MOSQUITO LARVAE

JEAN-PHILIPPE DAVID, DELPHINE REY, JEAN-CLAUDE MEYRAN,* and GERARD MARIGO

Laboratoire "Ecosystèmes et Changements Environnementaux"
Centre de Biologie Alpine
Université Joseph Fourier
BP 53, 38041 Grenoble Cedex 9, France

(Received March 7, 2000; accepted September 3, 2000)

Abstract—The toxicological characteristics of dietary decomposed alder leaf litter against mosquito larvae were further investigated through enzymatic and chemical purification of a phenoliclike cell-wall fraction isolated from crude litter. The toxicity of the subfractions obtained was controlled by standard bioassays on third instars of *Aedes aegypti* chosen as a reference target species. Enzymatic hydrolyses of the cell-wall fraction were performed with caylase, pectolyase, esterase, and β-glycosidase, in order to release, respectively, cellulose material and phenolic compounds bound to lignins. These treatments did not affect the larvicidal activity and the phenolic activity of the cell-wall fraction. Chemical alkaline and acid hydrolyses were carried out to break ester and glycosidic bonds of the cell-wall fraction. Comparison of HPLC profiles of the hydrolysates from both toxic and nontoxic fractions did not reveal differences between the phenolic acids released. Aluminum chloride, known for its phenolic complexing activity, counteracted the larvicidal activity of the cell-wall fraction. Altogether, these results suggest the involvement of ligninlike compounds in the toxicity of dietary alder leaf litter against larval mosquitoes. The toxicity of this fraction, which was very sensitive to drastic and smooth oxidations, seemed to be associated with a strong oxidative potential. These results are discussed in relation to a possible mode of action of lignins in the plant–mosquito interactions.

Key Words—Alder leaf litter, larvicidal activity, ligninlike compounds, HPLC, bioassays, *Aedes aegypti*.

*To whom correspondence should be addressed. e-mail: jcmeyran@ujf-grenoble.fr

0098-0331/01/0100-0161$19.50/0

INTRODUCTION

Because of their various biological effects (reviewed in Appel, 1993), polyphenols are among the most important secondary compounds involved in plant–insect dietary interactions (Ali et al., 1999). Whereas the influence of polyphenols from green leaves on the ecotoxicology of terrestrial herbivorous insects has received much attention (Steinly and Berenbaum, 1985; Scriber et al., 1989), their dietary interaction with aquatic detritivorous Arthropoda is far less documented (Newman, 1991).

The ecotoxicological consequences of such an interaction are particularly important in mosquito breeding sites from Alpine hydrosystems (Rey et al., 1996). The polyphenols of decomposed leaf litter from riparian vegetation appear to be involved in the habitat segregation of larval Culicidae through their differential dietary toxicity (Rey et al., 1998a, 2000a). Such toxicity, mainly an interaction between alder [*Alnus glutinosa* (L.) Gaertn.] and larval *Aedes* taxa, appeared to be exerted against the midgut epithelium of the detritivorous larvae (Rey et al., 1999a; David et al., 2000a). The histopathological effects of dietary polyphenols observed in this target organ are stronger than those obtained with the bacterioinsecticide *Bacillus thuringiensis* var. *israelensis*, suggesting the possible use of those vegetable polyphenols in mosquito control (Rey et al., 1998b).

The detritivorous arthropodan fauna from Alpine mosquito breeding sites shows differential tolerance to those ingested polyphenols (Rey et al., 2000a). Different levels of cytochrome P-450 monooxygenase and esterase activity were associated with differential detoxifying capacities among species and ecotypes (Rey et al., 1999b, 2000b).

Preliminary toxicological characterization of the alder leaf litter indicated that the settling of the larvicidal effect required a 10-month decaying process in the mosquito breeding site, under complex seasonal biotic, climatic, and hydrologic factors (David et al., 2000b). The toxicity of the decomposed leaf litter seems to be intrinsic and associated with the high activity of insoluble phenolics of an extractible cell-wall fraction. Both toxicity and phenolic activity did not appear to be affected by a sequential extraction procedure with water, organic solvents, and a detergent (David et al., 2000b). At this stage of the purification procedure, this fraction included cellulose compounds (cellulose, hemicelluloses), pectic compounds, phenolic polymers of a lignin-type, and other insoluble polyphenols. Among those components, lignins, which are the most prominent polyphenols usually bound to plant cell-wall (Wallace and Fry, 1994), appear to be candidates for toxicity.

Further chemical transformation and fractionation of this cell-wall fraction are necessary to isolate the source of toxicity. For this purpose, we used gentle methods such as enzymatic hydrolyses (Faulds et al., 1999), or more drastic

methods such as alkaline and acid hydrolyses (Cvikrovà et al., 1993) before phenolic characterization and toxicological checking of the subfractions.

The toxicity of dietary vegetable phenolics is generally exerted on the midgut of herbivorous insects through oxidative mechanisms (reviewed by Summer and Felton, 1994). The sensitivity of the toxic cell-wall fraction to several chemical oxidants was tested in order to examine the involvement of oxidative stress in the larvicidal effects of the decomposed alder leaf litter.

METHODS AND MATERIALS

Plant and Insect Materials. The toxicity of decomposed alder leaf litter on larval mosquitoes was investigated on a purified cell-wall fraction extracted from crude 10-month-old litter. Samples were collected in August 1997 and 1998 in a woodland subjected to periodic flooding in the Rhône-Alpes region of France (Pautou and Girel, 2000), and they were deep-frozen until use. Extraction of the cell-wall fraction was performed according to the sequential fractionation procedure of Alibert et al. (1969), modified by David et al. (2000b), with water, absolute ethanol, acetone, and benzene, before Triton X-100 treatment. The cell-wall fraction was air-dried and stored at 4°C before subsequent enzymatic and chemical fractionation. Phenolic characterization and toxicity controls were performed on the subfractions.

A cell-wall fraction was extracted as above from samples of nontoxic 10-month-old litter for comparison of HPLC profiles of hydrolysates between toxic and nontoxic alder leaf litter. This nontoxic litter originated from a woodland near the site of collection of the toxic litter, but was subject to sparse flooding.

A. aegypti L. larvae, originating from a Bora-Bora strain raised in our laboratory (Rey et al., 1999b), were used as a standard for toxicity controls. This species was chosen because of its known sensitivity to crude alder leaf litter and cell-wall fraction (David et al., 2000a) and its continuous availability for experimentation.

Enzymatic Hydrolyses. Enzyme hydrolysis is helpful in the elucidation of the composition of the cell-wall fraction (Faulds et al., 1999). Information on the constituents of the cell-wall fraction involved in the toxicity factor may be provided by comparative larval toxicity controls of cell-wall fractions treated by differential enzyme hydrolysis. Several enzymes were used for this comparative toxicological analysis.

Caylase (caylase 345, Cayla S.A. Toulouse, France; a complex of extracellular enzymes with high cellulase and hemicellulase activities) and pectolyase (Sigma) were used separately or mixed together, in order to solubilize the polysaccharide and pectin compounds. In all cases, 100 mg of purified toxic cell-wall fraction, suspended in 20 ml of water adjusted to pH 5.5, was treated for 60 min at 36°C

with 200 mg and 20 mg of caylase and pectolyase, respectively. After filtration, the treated material was water rinsed and centrifuged (10,000*g* for 5 min) three times. Evaluation of the sugar quantity released in the supernatant, with glucose as a reference, was performed following the colorimetric method of Arnold (1965), by using the 3,5-dinitrosalicylic acid reagent. Absorbance was measured at 530 nm with a Perkin-Elmer 551 spectrophotometer.

β-Glycosidase (20 mg of 2.5 units/mg, Sigma) and esterase (30 μl of 4.16 units/ml, Sigma) were used to break down glycosidic and ester linkages, respectively. The procedure was identical to the above. For esterase, the pH was adjusted to 7.5 to obtain maximum enzymatic activity.

In all cases, toxicological checking and evaluation of total levels of phenolics (= phenolic activity) were performed on air-dried pellets. Total phenolics released after β-glycosidase and esterase digestion also were measured.

Alkaline and Acid Hydrolyses. For alkaline hydrolysis, 130 mg of purified toxic cell-wall fraction was treated with 30 ml 2 N NaOH under a nitrogen atmosphere for 2 hr at 20°C. After neutralization, the slurry was centrifuged at 20,000*g* for 10 min, and the pellet was rinsed with water and centrifuged as above.

For acid hydrolysis, 300 mg of purified toxic cell-wall fraction was treated with 20 ml N H_2SO_4 under agitation for 2 hr at 100°C. The neutralized slurry was filtered, water-rinsed, and centrifuged as mentioned above.

After hydrolysis, the resulting pellets were air-dried and stored at 4°C before determination of phenolic activity and larval toxicity control. The supernatants were evaporated to dryness, and the residue was dissolved in methanol to discard the sodium salts. The hydrolysates, solubilized in methanol, were chemically characterized through HPLC analysis.

HPLC Analysis. A reversed-phase system, including a Waters Spherisorb 5-μm ODS-2 column (250 × 4.6 mm ID; Alltech, Deerfield, Illinois) was used for the characterization of the phenolic acids released by acid/alkaline hydrolyses of the cell-wall fraction. The column was equipped with a 5-mm precolumn, a Dionex 4500i gradient pump, a Shimadzu SIL-9A autoinjector, a Shimadzu SPD 6A UV detector, and Shimadzu Workstation Class CR-10 integration software. The mobile phase consisted of ultrapure water with 1% orthophosphoric acid, and methanol (HPLC grade). The column was eluted with a 60-min water–methanol gradient at a flow rate of 0.5 ml/min. The gradient comprised 5 min isocratic 80 : 20, 40 min linear to 45 : 55, followed by 10 min linear to 80 : 20. Sample size was 150 μl and phenolic acids were detected at 280 and 320 nm.

The identification of phenolic acids eluted was performed by comparison with retention times of appropriate standards (gallic acid, ellagic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, and ferulic acid) (Sigma). HPLC profile comparisons were made between toxic and nontoxic cell-wall frac-

tions in order to elucidate possible compounds released from the hydrolyzed cell-wall fraction that may be involved in the toxic factor.

Chemical Oxidations. Chemical oxidations were performed with aqueous solutions of $KMnO_4$ (0.02 N), $FeCl_3$–$FeK_3(CN)_6$ (1%, v/v) and NaOH (0.001 N to 0.2 N: see Figure 3 below). A 300-mg toxic cell-wall fraction was mixed in 30 ml of oxidative solutions for 2 hr under agitation at 20°C. Treated powders were water rinsed and centrifuged (10,000*g* for 5 min) three times, then air-dried before determination of phenolic activity and toxicity control.

Chelation with Aluminum Chloride. A purified toxic cell-wall fraction (10 mg) was suspended into 50 ml of 0.05% $AlCl_3$ aqueous solution at pH 7, and incubated 1 hr at 20°C.

Determination of Phenolic Activity. Determination of the phenolic compounds within the subfractions resulting from the enzymatic and chemical treatments was done in triplicate, with 5–10 mg (dry weight) of powder. Measurements were done with the colorimetric method by the Folin-Ciocalteau reagent and 20% Na_2CO_3, following the method of Marigo (1973) modified by David et al. (2000b), with gallic acid used as a standard. Absorbance was measured at 760 nm with a Perkin-Elmer 551 spectrophotometer.

Toxicity Controls. Toxicity of the subfractions obtained after the different enzyme activities and chemical treatments was checked with standard bioassays (WHO, 1981) on third-instar *A. aegypti*. Triplicate bioassays were conducted at 25°C, on samples including 20 specimens of the same size, in plastic vials containing 50-ml water suspensions of the different subfractions (10 mg/50 ml), with pH adjusted to 7.5. Controls were reared in tap water. Mortality was checked 2 hr after the beginning of the treatment. Results were corrected by Abbott's formula (Abbott, 1925).

RESULTS

Purification of Toxic Cell-Wall Fraction by Enzymatic Digestion. Enzymatic hydrolysis of the toxic cell-wall fraction with caylase and pectolyase alone or mixed together showed no obvious decrease of toxicity within this fraction (Table 1). Nevertheless, a significant amount of polysaccharide and pectin compounds were released by this treatment (representing 150, 30, and 210 μg glucose/mg for caylase, pectolyase, and both mixed together, respectively), indicating the efficiency of the treatments. The residual phenolic activity was not affected by these enzymatic digestions (Table 1).

These results suggest that the polysaccharidic compounds included in the cell-wall fraction and released by the enzyme activities may not be involved in the larvicidal effect. Moreover, the same relation previously mentioned by David et al. (2000b) between toxicity and total phenolic activity was observed here.

TABLE 1. EFFECTS OF ELIMINATION OF CELLULOSE AND PECTIN COMPOUNDS BY ENZYMATIC HYDROLYSIS ON TOXIC CELL-WALL FRACTION

Toxic cell-wall fraction	Toxicity (% mortality ± SE)	Residual phenolic activity (μg equiv gallic acid/mg powder)	Released sugars (μg glucose/mg powder)
Untreated	89 ± 6.6	36 ± 1.6	—
Treated with pectolyase	95 ± 5.4	31.2 ± 2.1	30 ± 5
Treated with caylase	94.8 ± 3.3	35.8 ± 2.4	150 ± 7.5
Treated with both pectolyase and caylase	84 ± 5.2	31.9 ± 0.9	210 ± 12

Hydrolysis of ester and glycosidic linkages within the cell-wall fraction, with esterase and β-glycosidase, released small amounts of phenolic compounds (1.4 and 3.2 μg equiv gallic acid/mg powder, respectively) (Table 2). There was no obvious decrease of toxicity within the subfractions obtained. Thus, the polysaccharidic compounds and phenolic acids linked to the insoluble cell-wall fraction did not seem to be involved in the larvicidal effect of the litter.

These results confirm our knowledge of the composition of the cell-wall fraction, showing that its main components involved in toxicity are supposed to be related to the ligninlike compounds. They constitute the main polyphenolic constituents remaining within the cell-wall fraction after enzymatic treatment.

Chemical Treatments. Both acid and alkaline hydrolysis of the toxic cell-wall fraction led to a strong loss of toxicity (Table 3). In the same way, an important decrease of phenolic activity within this fraction was observed after hydrolysis (i.e., from 48% for acid hydrolysis up to 55% for alkaline hydrolysis).

However, these drastic hydrolyses did not release any significant toxic compounds, as shown by the comparison of HPLC profiles of the supernatants resulting from hydrolyses of toxic versus nontoxic cell-wall fractions (Figure 1). In both cases, among the few solubilized compounds, four of them were identi-

TABLE 2. EFFECTS OF ELIMINATION OF CELL-WALL LINKED PHENOLIC COMPOUNDS BY ENZYMATIC HYDROLYSIS ON TOXIC CELL-WALL FRACTION

Toxic cell-wall fraction	Toxicity (% mortality ± SE)	Residual phenolic activity (μg equiv gallic acid/mg powder)	Released phenolic compounds (μg equiv gallic acid/mg powder)
Untreated	92.5 ± 3.6	37 ± 1.2	
Treated with β-glucosidase	99 ± 1.2	34.1 ± 1.6	1.4 ± 0.1
Treated with esterase	97.3 ± 3.3	33 ± 1.9	3.2 ± 0.2

TABLE 3. EFFECTS OF ACID AND ALKALINE HYDROLYSIS ON TOXIC CELL-WALL FRACTION

Toxic cell-wall fraction	Toxicity (% mortality ± SE)	Phenolic activity (μg equiv gallic acid/mg powder)
Untreated	98.5 ± 1.6	35.8 ± 1.2
Treated with H_2SO_4	2.5 ± 1.3	18.7 ± 2.6
Treated with NaOH	0	16.2 ± 4.2

fied by comparison with commercial compounds: gallic acid (R_t = 11.5 min), *p*-hydroxybenzoic acid (R_t = 26.7 min), vanillic acid (R_t = 29.8 min), and *p*-coumaric acid (R_t = 37 min). The most abundant compounds present in both profiles were *p*-hydroxybenzoic acid and gallic acid. None of them appeared to be more represented in the hydrolysate from the toxic cell-wall fraction than in that from the nontoxic one. There was no significant difference between alkaline (not shown) and acid (Figure 1) hydrolysis at both 280 (Figure 1) and 320 nm (not shown). HPLC results suggested that drastic hydrolyses did not solubilize any toxic component from the cell-wall fraction but may break them down or change their configuration into nontoxic forms. Thus, the phenolic compounds from the cell-wall fraction involved in the toxicity may be associated with the ligninlike compounds themselves rather than with the phenolic acids bound to lignins.

In the same way, strong chemical oxidations of purified toxic cell-wall fraction induced considerable decrease of both toxicity and phenolic activity (Figure 2). Nevertheless, in our experiment, $KMnO_4$ seemed to be more efficient than $FeCl_3$–$K_3(CN)_6$ among the oxidants used. Similar results were found after gentler oxidations with NaOH at low concentrations (Figure 3). These results indicated that the toxicity of the cell-wall fraction was rapidly canceled for NaOH concentrations under a threshold of 0.2 N and that a phenolic activity up to 15 μg equiv gallic acid/mg powder was necessary for a visible larvicidal effect.

The involvement of phenolic compounds within the toxic factor was also confirmed by treatment of the purified toxic cell-wall fraction with $AlCl_3$, known for its phenolic complexing activity. $AlCl_3$-treated samples only induced 1.5% of larval mortality, while untreated samples induced 97.5% of mortality, and $AlCl_3$ controls did not show any effect on larval survival (data not shown).

DISCUSSION

A preliminary characterization has associated the larvicidal properties of the decomposed alder leaf litter from Alpine mosquito breeding sites to an insoluble cell-wall fraction with a strong phenolic activity (David et al., 2000a). The current enzymatic and chemical purification of this cell-wall fraction revealed a

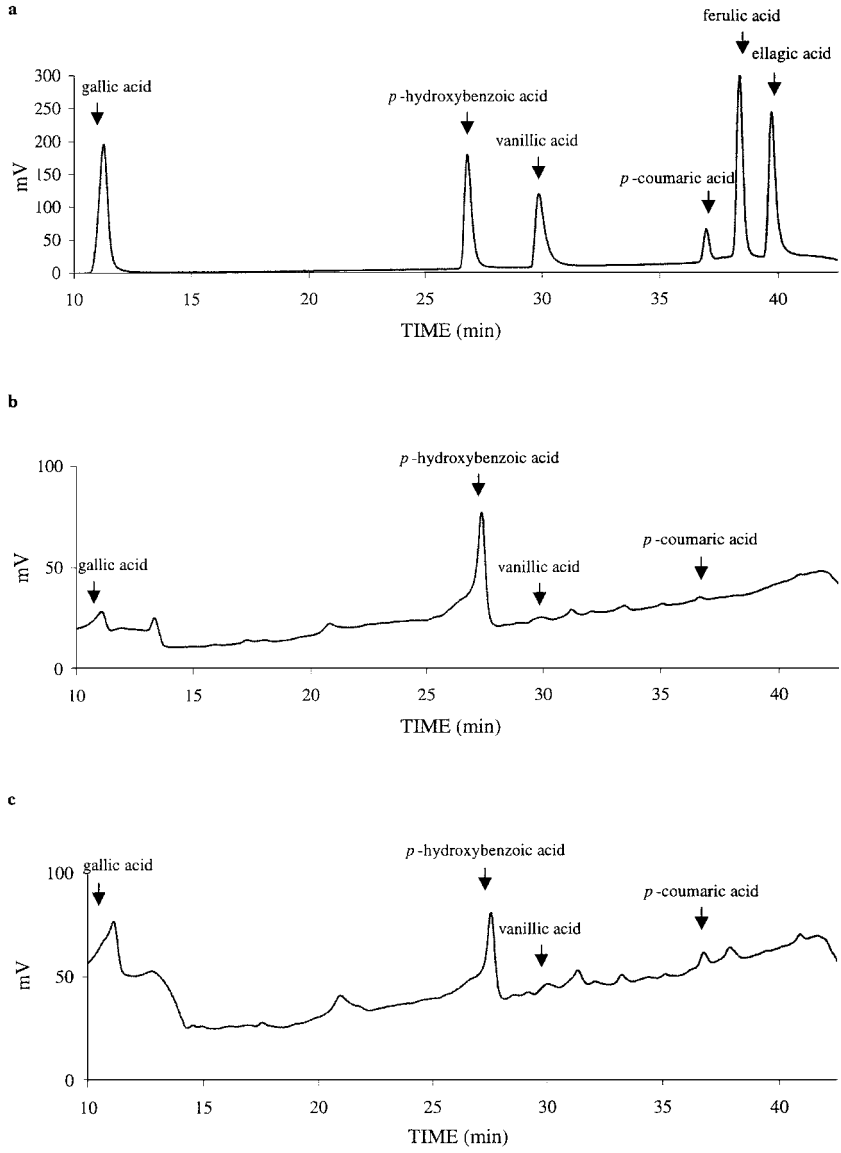


FIG. 1. Comparative HPLC profiles of standard compounds (a), toxic cell-wall fraction after drastic acid hydrolysis (b), and nontoxic cell-wall fraction after drastic acid hydrolysis (c). Extracts were run on reversed-phase HPLC, and phenolics were detected by absorbance at 280 nm. Peaks identified are indicated on the chromatogram.

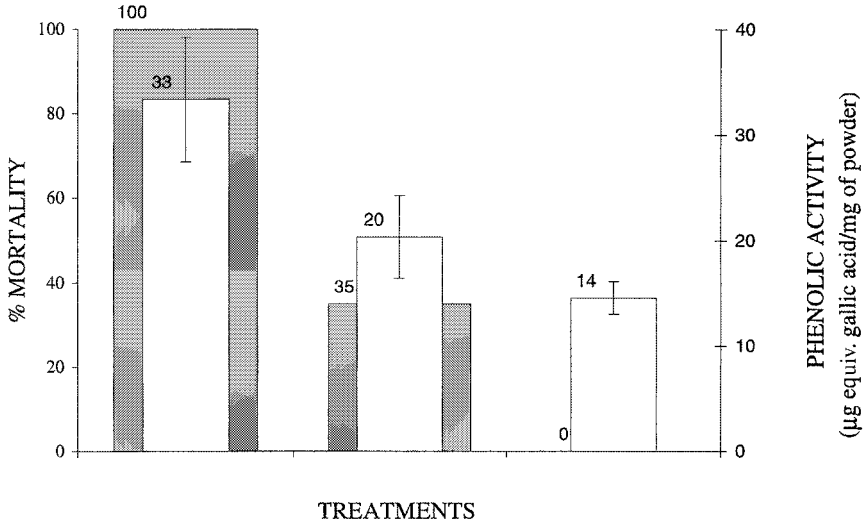


FIG. 2. Effects of chemical oxidation on toxicity (dark bars) and phenolic activity (white bars) of the cell-wall fraction.

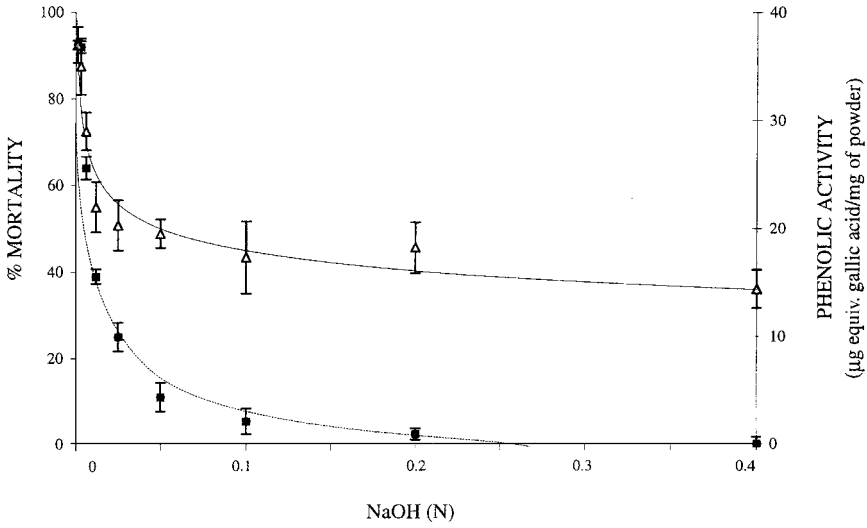


FIG. 3. Effects of gentle NaOH oxidation on toxicity and phenolic activity of the cell-wall fraction (■, % mortality; △, phenolic activity).

toxic subfraction, including polyphenols, which may be characterized as lignin-like compounds. The sensitivity of the toxic fraction to both drastic and gentle oxidations suggested that the oxidation potential of these compounds within the larval midgut epithelium may be an important component of toxicity of alder leaf litter.

Characterization of Ligninlike Compounds as Toxic Elements from Cell-Wall Fraction. All the toxicological results of our fractionation and HPLC design are congruent and suggest the involvement of ligninlike compounds in the toxicity of the cell-wall fraction from decomposed alder leaf litter.

Lignins are phenolic polymers (Lewis and Yamamoto, 1990) that are built by oxidative coupling of three major C_6–C_3 phenylpropanoid units together with amounts of C_6–C_1 units such as *p*-hydroxybenzoic acid, which may be linked via ester and ether bonds to the rest of the lignin molecule (Wallace and Fry, 1994). However, as lignins were insoluble, there is no analytical method currently available to directly characterize them, and we have used several indirect characterization traits.

Our enzymatic design for digestion of the cell-wall fraction with hydrolases, alone or mixed, which may have synergistic interactions (Faulds et al., 1999), led to a purified subfraction with a high phenolic content. This subfraction may correspond to a purified ligninlike fraction, according to Wallace and Fry (1994), in the phenolic content of the vegetable cell wall.

Lignins were present within the plant cell wall in covalent attachment to structural polysaccharides, and in ester linkages to phenolic acids (Wallace et al., 1991). Esterified phenolic acids were easily liberated from cell walls by 1 N NaOH at room temperature (Iiyama et al., 1990). Hydrolysis of these complexes may lead to ferulic, *p*-coumaric, vanillic, sinapic, and *p*-hydroxybenzoic acid (Wallace and Fry, 1994). As most of these compounds have been found in hydrolysates from the toxic cell-wall fraction without any larvicidal effect, this was another indication of the involvement of the lignin cores themselves in the toxicity factor, and not of the bound phenolic acids.

In the same way, oxidations released phenols from reactive sites of the lignin macromolecules, and the analysis of the oxidation products has been used as a relative measure of the total lignin concentration in plants or leaf litter (see references in Amelung et al., 1999). We successfully used the oxidation of the toxic subfraction by potassium permanganate (Gellerstedt, 1992), and, to a lesser extent, by $FeCl_3$–$FeK_3(CN)_6$ and dilute NaOH. In all cases, there was a correlation between degradative oxidation and loss of toxicity together with phenolic activity. Results were obvious when using serial doses of NaOH under the threshold of 0.2 N. In the same way, the ability of numerous structural groups of the phenolics from the subtoxic fraction to form complexes with metals, including $AlCl_3$ (Jurd, 1962), was another qualitative trait suggesting the involvement of lignins in the toxicity factor.

Possible Involvement of Ligninlike Compounds in Toxicity of Cell-Wall Fraction. The possible involvement of ligninlike compounds in the larvicidal properties of decomposed alder leaf litter was expected as lignins are known to be one of the predominant phenolic components in leaf litter (Molloy et al., 1977). As soon as the more labile components have been degraded, litter decomposition curves are dominated by lignins (Minderman, 1968). Lignins are among the most important defensive chemicals in plants, providing not only physical but also chemical defense against insects (Wainhouse et al., 1998). As suggested from an evolutionary standpoint, lignins and ligninlike compounds may have been originally defense-related (Boudet et al., 1996). Besides the well-known role of lignins in decreasing the digestibility of plant cell walls by phytophagous consumers indirectly through their deleterious effect on rumen flora (see references in Bennett and Wallsgrove, 1994), direct larvicidal properties of lignins were suggested for some xylophagous Coleoptera (Wainhouse et al., 1990). In a toxicological investigation of integumental lignin against Coleoptera larvae, antibiosis phenomena were observed by Stamopoulos (1988), who used artificial diet with lignin.

Toxicological Effect of Ligninlike Compounds in Larval Midgut. The ligninlike products currently characterized in the cell-wall fraction of decomposed alder leaf litter may act as toxic elements through their phenolic activity. This was strongly suggested by our preliminary data (David et al., 2000a,b) and our current fractionation experiments, both indicating a strict association between toxicity and phenolic activity. The loss of toxicity currently observed within the oxidized cell-wall fraction suggests a correlation between oxidative and toxic power.

The involvement of oxidative processes in litter toxicity is not surprising because, generally, oxidative activation is the most common biochemical mode of action of phenolics (Appel, 1993). Oxidation may be an important component of phenolic toxicity in mosquito larval gut, as also proposed for lepidopteran larvae (Barbehenn and Martin, 1994). The autooxidation of phenolics may result in the formation of superoxide radicals and other reactive oxygen species (see references in Summer and Felton, 1994), leading to a direct oxidative stress of the digestive system of actively feeding larvae. This process may be enhanced by the alkaline pH of the larval gut (Clements, 1992) or by oxidative enzymes such as peroxidases and polyphenol oxidases (Appel, 1993). When the oxidative stress exceeds the antioxidant resources of the midgut tissues, protein oxidation and lipid peroxidation could ensue. Such redox cycling may be directly related to the histopathological effects of the ingested leaf litter on the midgut epithelium (David et al., 2000a). The midgut epithelium seemed to be the privileged target for the deleterious effect of phenolics due to the high relative reactivity of free radicals and reactive oxygen species. The major damages may be observed on tissues close to the site where the oxidants are stored or synthesized.

Although the current analysis suggests the involvement of ligninlike compounds in the larvicidal effect of dietary alder leaf litter through a possible induction of an oxidative stress in the mosquito midgut, the origin and the exact constitution of the toxic ligninlike compounds during the decaying process of the leaf material in mosquito breeding sites need further investigation.

Acknowledgments—This investigation was supported by a grant from the Entente Interdépartementale pour la Démoustication (EID) Ain-Isère-Rhône-Savoie (Director: R. Gruffaz). We thank I. Thiery (Institut Pasteur, Paris) for supplying the *A. aegypti* strain, and J. Patouraux and G. Girard for technical assistance.

REFERENCES

ABBOTT, W. S. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265–267.

ALI, M. I., BI, J. L., YOUNG, S. Y., and FELTON, G. W. 1999. Do foliar phenolics provide protection to *Heliothis virescens* from a baculovirus? *J. Chem. Ecol.* 25:2193–2203.

ALIBERT, G., MARIGO, G., and BOUDET, A. 1969. Recherches sur la biosynthèse des composés aromatiques chez les végétaux supérieurs. Identification des acides phénoliques de *Quercus pedunculata* Ehrh. *Physiol. Veg.* 7:57–68.

AMELUNG, W., FLACH, K. W., and ZECH, W. 1999. Lignin in particle-size fractions of native grassland soils as influenced by climate. *Soil Sci. Soc. Am. J.* 63:1222–1228.

APPEL, H. M. 1993. Phenolic compounds in ecological interactions: The importance of oxidation. *J. Chem. Ecol.* 19:1521–1552.

ARNOLD, W. N. 1965. β-Fructo furanosidase from grape berries. *Biochem. Biophys. Acta* 110:134–147.

BARBEHENN, R. V., and MARTIN, M. M. 1994. Tannin sensitivity in larvae of *Malacosoma disstria* (Lepidoptera): Roles of the peritrophic envelope and midgut oxidation. *J. Chem. Ecol.* 20:1985–2001.

BENNETT, R. N., and WALLSGROVE, R. M. 1994. Secondary metabolites in plant defence mechanisms. *New Phytol.* 127:617–633.

BOUDET, A. M., GOFFNER, D. P., and GRIMA-PETTENATI, J. 1996. Lignins and lignification: recent biochemical and biotechnological developments. *C.R. Acad. Sci. Paris, Sci. Vie* 319:1035–1042.

CLEMENTS, A. N. 1992. The Biology of Mosquitoes, Vol. 1: Development, Nutrition and Reproduction. Chapman and Hall, London.

CVIKROVÀ, M., NEDELNIK, J., EDER, J., and BINAROVA, P. 1993. Changes in pattern of phenolic acids induced by culture filtrate of *Fusarium oxysporum* in afalfa plants differing in susceptibility to the pathogen. *J. Plant Physiol.* 142:1–5.

DAVID, J. P., REY, D., PAUTOU, M. P., and MEYRAN, J. C. 2000a. Differential toxicity of leaf litter to dipteran larvae of mosquito developmental sites. *J. Invertebr. Pathol.* 75:9–18.

DAVID, J. P., REY, D. MARIGO, G., and MEYRAN, J. C. 2000b. Larvicidal effect of a cell-wall fraction isolated from alder decaying leaves. *J. Chem. Ecol.* 26:901–913.

FAULDS, C. B., KROON, P. A., BARTOLOMÉ, B., and WILLIAMSON, G. 1999. Hydrolysis of hemicelluloses using combinations of xylanases and feruloyl esterases, pp. 183–195, *in* C. Bucke (ed.). Methods in Biotechnology, Vol. 10: Carbohydrates Biotechnology Protocols. Humana Press, Totowa, New Jersey.

GELLERSTEDT, G. 1992. Chemical degradation methods: permanganate oxidation, pp. 322–333, *in* V. Lin and J. Dence (eds.). Methods of Lignin Chemistry, Springer-Verlag, Berlin.

IIYAMA, K., LAM, T. B. T., and STONE, B. A. 1990. Phenolic acid bridges between polysaccharides and lignins in wheat internodes. *Phytochemistry* 29:733–737.

JURD, L. 1962. Spectral properties of flavonoids compounds, pp. 108–155, *in* T. A. Geissman (ed.). The Chemistry of Flavonoid Compounds. Pergamon Press, Oxford.

LEWIS, N. G., and YAMAMOTO, E. 1990. Lignins: Occurrence, biosynthesis and biodegradation. *Annu. Rev. Plant Physiol. Mol. Biol.* 41:455–496.

MARIGO, G. 1973. Sur une méthode de fractionnement et d'estimation des composés phénoliques chez les végétaux. *Analusis* 2:106–110.

MINDERMAN, G. 1968. Addition, decomposition and accumulation of organic matter in forests. *J. Ecol.* 56:355–362.

MOLLOY, L. F., BRIDGER, B., and CAIRNS, A. 1977. Studies on a climosequence of soils in tussock grasslands. 13. Structural carbohydrates in tussock leaves, roots, and litter soil light fraction. *N.Z. J. Sci.* 20:443–451.

NEWMAN, R. M. 1991. Herbivory and detritivory on freshwater macrophytes by invertebrates: A review. *J. North Am. Benth. Soc.* 10:89–114.

PAUTOU, G., and J. GIREL. 2000. The floodplain forest of the French Upper Rhône river valley, *in* E. Klimo and H. Hager (eds.). Research Report of the European Forest Institute, Joensuu, Finland. Brill Academic Publishers Amsterdam. In Press.

REY, D., MARIGO, G., and PAUTOU, M. P. 1996. Composés phénoliques chez *Alnus glutinosa* et contrôle des populations larvaires de Culicidae. *C.R. Acad. Sci. Paris, Sci. Vie* 319:1035–1042.

REY, D., CUANY, A., MARIGO, G., HOUGARD, J. M., BISSAN, Y., KONE, Y., PAUTOU, M. P., LONG, A., and MEYRAN, J. C. 1998a. Alder–mosquito interactions in alpine hydrosystems: Possible applications in dipteran pest control. *Acta Parasitol. Port.* 5:40.

REY, D., LONG, A., PAUTOU, M. P., and MEYRAN, J. C. 1998b. Comparative histopathology of *Bacillus thuringiensis* var *israelensis* on some dipteran larvae and Crustacea of aquatic hydrosystems. *Entomol. Exp. Appl.* 8:255–263.

REY, D., PAUTOU, M. P., and MEYRAN, J. C. 1999a. Histopathological effects of tannins on the midgut epithelium of aquatic Diptera larvae. *J. Invertebr. Pathol.* 73:173–181.

REY, D., CUANY, A., PAUTOU, M. P., and MEYRAN, J. C. 1999b. Differential sensitivity of mosquito taxa against vegetable tannins. *J. Chem. Ecol.* 25:537–548.

REY, D., DAVID, J. P., MARTINS, D., PAUTOU, M. P., LONG, A., MARIGO, G., and MEYRAN, J. C. 2000a. Role of vegetable tannins in habitat selection among mosquito communities from the Alpine hydrosystems. *C.R. Acad. Sci. Paris, Sci. Vie* 323:391–398.

REY, D., DAVID, J. P., CUANY, A., AMICHOT, M., and MEYRAN, J. C. 2000b. Comparative ability to detoxify alder leaf litter in field larval mosquito strains. *Arch. Insect Biochem. Physiol.* 44:1–8.

SCRIBER, J. M., LINDROTH, R. L., and NITAO, J. 1989. Differential toxicity of a phenolic glycoside from quaking aspen leaves by *Papilio glaucus* subspecies, their hybrids, and backcrosses. *Oecologia* 81:186–191.

STAMOPOULOS, D. C. 1988. Toxic effect of lignin extracted from the tegument of *Phaseolus vulgaris* seeds on the larvae of *Acanthoscelides obtectus* (Say) (Coleoptera, Bruchidae). *J. Appl. Entomol.* 105:317–320.

STEINLY, B. A., and BERENBAUM, M. 1985. Histopathological effects of tannins on the midgut epithelium of *Papilio polyxenes* and *Papilio glaucus. Entomol. Exp. Appl.* 39:3–9.

SUMMER, C. B., and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Molec. Biol.* 24:943–953.

WAINHOUSE, D., CROSS, D. J., and HOWELL, R. S. 1990. The role of lignin as a defence against the spurce bark beetle *Dendroctonus micans:* Effect on larvae and adults. *Oecologia* 85:257–265.

WAINHOUSE, D., ASHBURNER, R., WARD, E., and BOSWELL, R. 1998. The effect of lignin and bark wounding on susceptibility of spruce to *Dendroctonus micans. J. Chem. Ecol.* 24:1551–1561.

WALLACE, G., and FRY, C. 1994. Phenolic components of the plant cell wall. *Int. Rev. Cytol.* 151:229–267.

WALLACE, G., CHESSON, A., LOMAX, J. A., and JARVIS, M. C. 1991. *Anim. Feed Sci. Technol.* 32:193–199.

WHO. 1981. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. WHO/VBC/81, 807. World Health Organization Geneva.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

THIN-LAYER CHROMATOGRAPHY ASSESSING FEEDING STIMULATION BY LABIAL GLAND SECRETION COMPARED TO SYNTHETIC CHEMICALS IN THE SUBTERRANEAN TERMITE *Reticulitermes santonensis*

JUDITH REINHARD[1,*] and MANFRED KAIB[2]

[1]*Bundesanstalt für Materialforschung und -prüfung*
12200 Berlin, Germany
[2]*Universität Bayreuth, Lehrstuhl Tierphysiologie*
95440 Bayreuth, Germany

(Received November 3, 1999; accepted September 8, 2000)

Abstract—The labial gland of the French subterranean termite *Reticulitermes santonensis* De Feytaud contains a polar, heat-resistent, and persistent chemical signal that is released onto the food during food exploitation and stimulates feeding in nestmates. Separation of the labial gland secretion by thin-layer chromatography on cellulose plates revealed that the secretion contains components with reducing and amino groups. In feeding bioassays conducted on the cellulose plate after TLC, termites preferred the area between R_f 0.46 and 0.88 (biologically active zone) for feeding, indicating the location of the feeding-stimulating signal. Thirty-five synthetic chemicals with similar chemical properties as the feeding-stimulating signal were analyzed with TLC. None of them covered the biologically active zone. Therefore, all chemical classes tested, such as sugars, amino acids, and salts, are unlikely as possible sources for the signal structure. In feeding choice tests with synthetic chemicals, termites showed clear feeding preference only for sugarlike components with physiologically excessive concentrations of 10 mmol and 100 mmol. Amino acids induced only light feeding preference. The intensity of feeding stimulation by the natural signal from the labial gland as compared to synthetic phagostimulants is discussed.

Key Words—termites, labial gland, phagostimulants, thin-layer chromatography, sugars, amino acids.

*To whom correspondence should be addressed at current address: CNRS, Laboratoire de Neurobiologie-UPR 9024, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France.

INTRODUCTION

In termite societies communal tasks, such as food exploitation, involve large numbers of nestmates and are in many ways regulated by the secretions of exocrine glands. The paired labial glands play a major role. They are found in all termite species, located in the meso- and metathorax. Their histology and fine structure have been described for several termite species (e.g., Czolij and Slaytor, 1988; Billen et al., 1989; Costa-Leonardo and Cruz-Landim, 1991; Kaib and Ziesmann, 1992; Mednikova, 1996; Costa-Leonardo, 1997). The glandular ducts open into the oral cavity, and from there termites release the watery labial gland secretion onto the substrate.

The labial gland secretion is reported to have various species-specific functions in nest construction, including regulation of nest climate, defense, fungistatic action, and source of digestive enzymes (e.g., Noirot, 1969; Hewitt et al., 1971; Maschwitz and Tho, 1974; Martin and Martin, 1978; Grassé, 1982; Brian, 1983; Hogan et al., 1988; Olagbemiro et al., 1988; Veivers et al., 1982, 1991; Mednikova, 1991). The labial gland secretion also plays an important role during communal food exploitation, as recently documented for *Schedorhinotermes lamanianus* and *Reticulitermes santonensis* (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995; Reinhard et al., 1997). It contains a feeding-stimulating chemical signal released onto the food by termite workers while feeding. It stimulates additional workers to feed at the same site, resulting in a faster and more efficient exploitation of the food source. This feeding stimulating signal is polar, heat-resistant, and nonvolatile (Kaib and Ziesmann, 1992; Reinhard et al., 1997). However, further research is required for its chemical characterization.

Organization of termite feeding is of major economic interest, as it results in the destruction of the material they feed upon. The damage done by termites worldwide reaches several billion U.S. dollars per year (Lewis and Haverty, 1996). There has been extensive research on toxic food baits as a means of controlling termites (e.g., Esenther and Grey, 1968; Jones, 1984; Su, 1994; Peters and Fitzgerald, 1999), but only recently did it become clear that inducing termites to preferentially feed on the toxic bait is of critical importance to the viability of the baits. Therefore, bait additives acting as phagostimulants have been sought among chemicals of nutritional value such as sugars (Waller and Curtis, 1996) and amino acids (Mishra, 1992; Chen and Henderson, 1996). A natural phagostimulant, however, such as the signal found in the labial gland secretion of *S. lamanianus* and *R. santonensis* (Kaib and Ziesmann, 1992; Reinhard et al., 1997), could present a much more effective way to make toxic bait systems attractive to termites.

We investigated chemically the feeding stimulating signal from the labial gland secretion of *R. santonensis* with thin-layer chromatography (TLC) com-

bined with feeding bioassays. Furthermore, the phagostimulating effect of the signal was compared with the effect of an array of synthetic chemicals of similar chemical properties and possible nutritional value.

METHODS AND MATERIALS

Collection and Culture of Termites. A new colony of the subterranean termite *Reticulitermes santonensis* De Feytaud was collected in Ile d'Oleron, France, and kept in the laboratory under constant conditions of 25°C, 75% relative humidity. The colony was housed in a metal container (length × height × width: 150 × 50 × 100 cm) for several months with moist vermiculite as soil substrate and water source and pine wood as food. From the container the termites had access to a foraging arena via a silicone tube (5 mm ID). The foraging arena (10 × 10 cm and 5 mm high) had a sand-blasted glass plate as floor and was covered by a glass plate.

At a preliminary stage of the work, the experiments also were carried out with three older laboratory colonies of *R. santonensis* (reared for up to six years). We could not observe obvious colony-specific differences during the feeding bioassays and chemical analyses. Nevertheless, to avoid possible rearing-related artifacts, only the freshly collected field colony was used for the final experiments.

Preparation of Gland Extract and Synthetic Chemicals. Termite workers were killed by deep-freezing, fixed in a ventral position in paraffin, and covered with distilled water. The paired labial glands were removed through an incision in the thorax. Lots of 100 labial glands were simultaneously extracted in 100 μl distilled water for 12 hr at 4°C and then deep-frozen for 24 hr. Thereafter, the glands were removed from the extract and the extract again deep-frozen until tested. Thirty-five synthetic chemicals (see Table 2 below) were chosen according to physicochemical properties corresponding to the feeding stimulating signal from the labial gland (polar, heat-resistant, nonvolatile: Reinhard et al., 1997). Chemicals were dissolved in water or ethanol at concentrations of 100, 10, and 1 mmol.

Thin-Layer Chromatography. The labial gland extract and the synthetic chemicals were chromatographed on cellulose TLC plates of 10 × 10 cm (TLC aluminum sheets, cellulose F, layer thickness 0.1 mm, Merck). Labial gland extract (10 μl) and 100 mmol solutions of each of the synthetics were applied separately on TLC plates with a microcapillary. The plates were chromatographed at room temperature for 70 min with a mixture of 2-propanol, 25% ammonia solution, water, and saturated boric acid solution (6 : 2 : 1 : 1), pH 9. For detection of spots we used UV light (254 nm) was well as the reagents bromcresol green, aniline phthalate, and ninhydrin/collidine (Merck, 1970).

Bioassays on Feeding Stimulation. Termites are light sensitive, so all tests were made under red light illumination. Feeding behavior in termite workers can be recognized easily by the hypognathous head position and wriggling head movements while gnawing and tearing off food fibers. Feeding spots can also be recognized afterwards by marks or holes in the substrate due to removal of food particles by the termites. Frequencies and spatial distributions of workers that were feeding according to the behavior described above were recorded over time by video techniques (IR-sensitive Kappa camera) and subsequently analyzed. To determine feeding stimulation by labial gland components, 10 μl of the labial gland extract was applied as a 1-cm-long line on cellulose TLC plates (10 cm long $\times$ 3 cm wide) and chromatographed as described above. The TLC plate was then offered in the foraging arena for 1 hr. As control, a TLC plate chromatographed without extract was offered simultaneously. The spatial distribution of all termites feeding between the start and front line on the TLC plates during the 1-hr duration of the experiment was analyzed to determine preferred feeding zones. Termites displaying different behaviors, such as arresting or aggregating without actually feeding, were not considered. The bioassay was repeated 10 times.

For the feeding bioassay with synthetic chemicals, pieces of moist filter paper were used as described in Reinhard et al. (1997). Four indepenent tests were run simultaneously in the foraging arena. Each test consisted of two semicircles (2.5 cm diameter) of filter paper. One of the two semicircles was randomly chosen for application of 25 μl of a synthetic solution and moistened with water. The other semicircle was just moistened (control). For each test the distribution of the first 20 feeding termites on the two semicircles was determined. Termites arresting or aggregating without actually feeding as described above were not considered. Up to three concentrations per chemical were tested for feeding preference. Tests for each chemical and each concentration were replicated 20 times. Enzymes (not heat-resistant) and stearic acid (lower polarity) were not tested for feeding preference.

Data Analysis. The distribution of feeding termites on TLC plates was analyzed via the dispersion index (Elliott, 1979) and the R_f value of the biologically active zone determined. The sign-test was used to analyze the data of the semicircle bioassays.

RESULTS

Analysis of Labial Gland Extract. Thin-layer chromatography of labial gland extract of *R. santonensis* revealed two bands in the lower half of the plate visible under 254 nm UV light (Figure 1A). With bromcresol green (general reagent for organic components), aniline phthalate (reagent for reducing groups

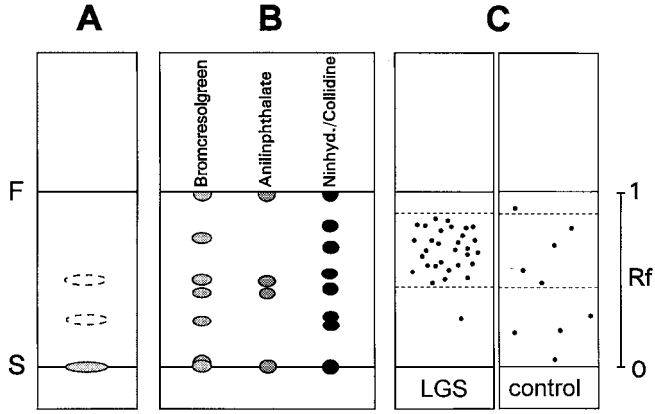


FIG. 1. Thin-layer chromatography of labial gland secretion (LGS) of *Reticulitermes santonensis* on cellulose plates. S: start line, F: front line. (A) Spots visible under UV 254 nm; (B) spots visible after detection with bromcresol green, aniline phthalate, and ninhydrin/collidine; (C) distribution of all termites (dots) feeding on a TLC plate within 1 hr of observation, left on LGS, right on control. Dotted lines: biologically active zone.

as in sugars), and ninhydrin/collidine (reagent for amino groups, amines, amino acids, and peptides) several further spots could be detected (Figure 1B). Consequently the labial gland secretion of *R. santonensis* contains components with reducing and/or amino groups.

When a TLC plate with chromatographed labial gland extract was offered to the termites, at least 95% of all termites showing feeding behavior were found between R_f 0.46 and 0.88 (Figure 1C, Table 1), leaving clear gnawing marks in the cellulose layer. Statistical analysis showed a difference from a random distribution at $P < 0.05$ for all 10 replicates. The feeding stimulating signal of the labial gland secretion must, therefore, be located in this area. In contrast, feeding termites were randomly distributed on the control plates (Figure 1C, Table 1), leaving gnawing marks all over the cellulose layer. Furthermore, more termites fed on the treated TLC plates (28.5 ± 5.6) within the 1-hr duration of the experiment compared to the control (2.7 ± 1.6, mean ± SD, $N = 10$, $P = 0.005$, Wilcoxon matched-pairs test).

Analysis of Synthetic Chemicals. Thirty-five synthetic chemicals with similar physicochemical properties to the feeding stimulating signal from the labial gland have been analyzed with TLC. Spot detection with bromcresol green showed that none of them covered the entire biologically active zone from R_f 0.46 to R_f 0.88 (Figure 2). Mono- and disaccharides were found only within the lower half of the active zone. Two of the sugar alcohols, amino sugars, methyl and phenyl sugars, as well as anhydrosugars/-sugar alcohols were located within or at least at the border

TABLE 1. NUMBER AND DISTRIBUTION OF FEEDING *Reticulitermes santonensis* WORKERS CONTINUOUSLY OBSERVED FOR 1 HOUR ON CELLULOSE TLC PLATES[a]

	Number of feeding termites						
Test	$\sum$ Total	R_f 0–0.25	R_f 0.25–0.5	R_f 0.5–0.75	R_f 0.75–1.0	X^2	P
LGS							
1	36	1	1	14	20	30.4	[b]
2	35	0	2	10	23	37.4	[b]
3	17	1	0	7	9	13.5	[b]
4	29	0	1	8	20	35.0	[b]
5	29	0	0	12	17	30.6	[b]
6	28	1	1	5	21	38.8	[b]
7	26	1	2	10	13	16.1	[b]
8	25	0	0	8	17	31.4	[b]
9	26	0	0	11	15	27.2	[b]
10	34	0	1	11	22	37.2	[b]
Control							
1	6	2	1	1	2	0.7	NS
2	3	1	0	2	0	3.7	NS
3	1	0	1	0	0	3.0	NS
4	2	1	1	0	2	1.9	NS
5	1	0	0	1	0	3.0	NS
6	4	1	1	0	2	1.9	NS
7	2	0	0	2	0	6.0	NS
8	4	1	2	1	0	1.9	NS
9	3	0	0	2	1	3.7	NS
10	1	0	0	1	0	3.0	NS

[a] LGS: 10 replicates after TLC of labial gland secretion (LGS). Control: 10 replicates chromatographed without the secretion as control. Chi-square statistics as in Elliott (1979).
[b] Difference from random distribution at $P < 0.05$, NS: no significant difference.

of the biologically active zone. Amino acids, peptides, enzymes, small carboxylic acids, and salts in general had much lower R_f values, while longer-chain carboxylic acids such as decanoic and stearic acid had higher ones.

The feeding choice tests on filter paper showed that few of the 31 chemicals tested had considerable feeding-stimulating effect, and this was only so at high concentrations (Table 2). A strong feeding-stimulating effect was found for sugars and methyl and phenyl sugars at a concentration of 100 mmol, and for glucose, sucrose, and cellobiose also at 10 mmol. Sugar acids, amino sugars, anhydrosugar, and amino acids showed some activity at 100 mmol. No significant feeding stimulation for any of the chemicals was recorded at 1 mmol (Table 2). Decanoic acid was repellent, and the termites fed more on the control. This repellency, though, is nonspecific and can be expected for various similarly volatile compounds such as ketones and alcohols.

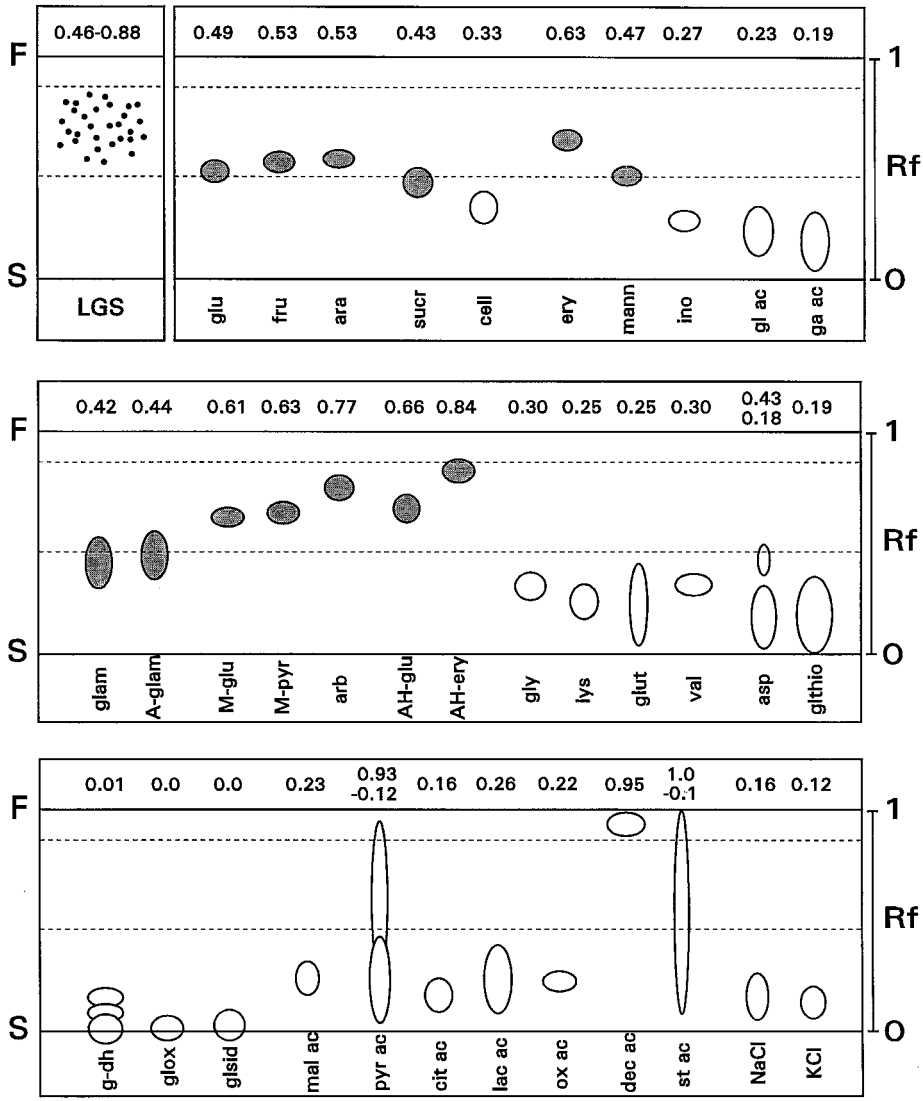


FIG. 2. Thin-layer chromatography of 35 synthetic chemicals on cellulose plates after spot detection with bromcresol green. Above left for comparison TLC of labial gland secretion (LGS) of *Reticulitermes santonensis* with distribution of feeding termites (dots). Dotted lines: biologically active zone. Grey areas: chemicals within the active zone; white areas: chemicals outside the active zone. Numbers above: R_f values of the active zone and the chemicals. S: start line, F: front line. For abbreviations, see Table 2.

TABLE 2. FEEDING STIMULATION IN *Reticulitermes santonensis* BY SYNTHETIC CHEMICALS[a]

Chemical class	Substance	Concentration (mmol)		
		100	10	1
Monosaccharides	glucose (glu)	+++	+++	+/−
	fructose (fru)	+++	−	n.t.
	arabinose (ara)	+++	+/−	n.t.
Disaccharides	sucrose (sucr)	+++	+++	+/−
	cellobiose (cell)	+++	+++	+/−
Sugar alcohols	*m*-erythritol (ery)	−	−	n.t.
	D-mannitol (mann)	−	−	n.t.
	m-inositol (ino)	−	−	n.t.
Sugar acids	glucuronic acid (gl ac)	+	+/−	−
	galacturonic acid (ga ac)	+	+/−	−
Amino sugars	glucosamine HCl (glam)	+/−	+/−	−
	N-acetylglucosamine (A-glam)	+	+/−	−
Methyl sugars	3-*O*-methylglucose (M-glu)	++	+	+/−
	methyl-α,D-glucopyranoside (M-pyr)	++	+	+/−
Phenyl sugars	β-arbutin (arb)	+++	+	+/−
Anhydrosugars/ -sugar alcohols	anhydro-β,D-glucose (AH-glu)	+	+/−	−
	anhydro-m-erythritol (AH-ery)	−	−	n.t.
Amino acids	glycine (gly)	+/−	+/−	+/−
	lysine (lys)	+	+	+/−
	glutamine (glut)	+/−	+/−	+/−
	valine (val)	+/−	+/−	−
Peptides	aspartame (asp)	−	−	n.t.
	glutathione (glthio)	−	−	n.t.
Enzymes	glucose 6P-dehydrogenase (g-dh)	n.t.	n.t.	n.t.
	glucoseoxidase (glox)	n.t.	n.t.	n.t.
	β-glucosidase (glsid)	n.t.	n.t.	n.t.
Carboxylic acids	malic acid (mal ac)	−	−	n.t.
	pyruvic acid (pyr ac)	−	−	n.t.
	citric acid (cit ac)	−	−	n.t.
	lactic acid (lac ac)	−	−	n.t.
	oxalic acid (ox ac)	−	−	n.t.
	decanoic acid (dec ac)	−!	−!	n.t.
	stearic acid (st ac)	n.t.	n.t.	n.t.
Salts	NaCl	−	−	n.t.
	KCl	−	−	n.t.

[a] Abbreviations refer to Figure 2. +++: very strong feeding stimulation ($P < 0.001$), ++: strong feeding stimulation ($P < 0.01$), +: feeding stimulation ($P < 0.05$, +/−: tendency for feeding preference ($P = 0.05$–0.15), −: no feeding stimulation, −!: repellent, n.t.: not tested. P levels refer to original data of feeding choice assays: distribution of the first 20 feeding termites on two pieces of filter paper, treated vs. untreated, analyzed using the sign test, $N = 20$ per chemical and concentration.

DISCUSSION

The process of feeding and communal food exploitation in termites is strongly connected with their labial glands. Labial glands in the African dampwood termite *Schedorhinotermes lamanianus* and the French subterranean termite *Reticulitermes santonensis* contain a polar, heat-resistant, and nonvolatile chemical signal that stimulates feeding (Kaib and Ziesmann, 1992; Reinhard et al., 1997).

Analysis of the labial gland secretion of *R. santonensis* by TLC chromatography revealed the presence of numerous components. This corresponds with literature describing a variety of different compounds within the labial gland secretion of termites, such as proteins (digestive enzymes), lipids, mucopolysaccharides, and salts (Noirot, 1969; Veivers et al., 1982; Hogan et al., 1988; Mednikova, 1991; Tokuda et al., 1997; Watanabe et al., 1997).

The bioassay on TLC plates demonstrated that after chromatography the feeding-stimulating signal from the labial gland secretion is found in a large spot, spreading from R_f 0.46 to R_f 0.88. The fact that the termites respond to the labial gland secretion with feeding even after the separation of its constituents by TLC strongly indicates that the phagostimulant is a single component running as a streak in the TLC rather than a blend. The biologically active zone does not correspond to the spots detected in the labial gland secretion under UV 254 nm and after treatment with reagents or to any of the 35 reference chemicals analyzed. Therefore, it is unlikely that the feeding stimulating signal is found within the common chemical classes with appropriate physicochemical properties such as sugars, salts, amino acids, peptides, and carboxylic acids. Due to their low R_f value the digestive enzymes of the labial gland secretion can be excluded as a feeding-stimulating signal as well, confirming the findings of Reinhard et al. (1997). Detailed fractionation and GC-MS analyses of labial gland secretion are in progress to elucidate the molecular structure of the signal.

In feeding bioassays with cellulose TLC plates as food source, only very small numbers of termites could be induced to feed on the plates if these were untreated or had only synthetic chemicals applied to them. Cellulose TLC plates are therefore much less suitable as a food source for *R. santonensis*, compared to filter paper used in the former feeding bioassays (Reinhard et al., 1997). However, the feeding-stimulating signal of *R. santonensis* is powerful enough to make termites feed even on unsuitable food such as TLC plates. Considerable numbers of termites fed on plates treated with labial gland secretion. This confirms previous results with *R. santonensis*, where the termites could even be made to gnaw on a glass surface when labial gland secretion was applied (Reinhard et al., 1997).

The selected synthetic chemicals were much less effective as phagostimulants. A good feeding response in *R. santonensis* could only be induced on filter

paper and only with physiologically excessive concentrations of sugars and sugar derivatives. Amino acids proved to be only weak phagostimulants even in high concentrations.

The feeding stimulating activity of sugars has been shown earlier in *R. flavipes* and *R. virginicus* (Waller and Curtis, 1996), as well as in *Microtermes traegardhi* (Abushama and Kambal, 1976). Numerous other insect species react with increased feeding to artificial diets offering molar concentrations of diverse mono- and disaccharides (e.g., Ma and Jermy, 1976; Weibull, 1990; Schmidt and Friend, 1991; Allsopp, 1992; van der Meer et al., 1995). Our study shows considerable phagostimulatory activity of methyl and phenyl sugars, which has also been reported in a mosquito (Schmidt and Friend, 1991). The phagostimulatory effect of other sugar derivatives such as sugar acids, amino sugars, and the anhydrosugar are minor and likely to be due to the glucose component in them. Sugar alcohols had no effect on *R. santonensis* or on other insect species (Ma and Jermy, 1976; van der Meer et al., 1995).

The phagostimulant effect of amino acids on termites also has been shown for *Zootermopsis*, *Kalotermes*, and *Reticulitermes* species, as well as *Gnathamitermes tubiformans*, *Neotermes bosei*, and *Coptotermes formosanus* (Hungate, 1941; Spears and Ueckert, 1976; Mishra, 1992; Chen and Henderson, 1996). The feeding stimulation induced by amino acids differs between species, which might be due to the experimental setup used by the authors. Inconsistencies between results of feeding tests have been reported previously (Meehan and Wilde, 1989; Chen and Henderson, 1996). The phagostimulatory activity of amino acids also has been documented in other insect species (e.g., Sogawa, 1972; Viswanathan and Kalode, 1990; Allsopp, 1992). As in sugars, the increased feeding activity is likely to be due to the nutritional value of amino acids, which is especially relevant for termites. Generally, termite foods are low in nitrogen (Higashi et al., 1992) and termites are dependent on intestinal bacteria for nitrogen fixation (Breznak, 1975; Potrikus and Breznak, 1977; Ohkuma et al., 1996; Slaytor and Chappell, 1994). Because nitrogen is limited in naturally occurring termite food (Cowling and Merrill, 1966), nitrogen additives may increase food consumption.

Our results are consistent with the literature in which synthetic phagostimulants in termites are found among chemicals of nutritional value, such as sugars and amino acids. However, these compounds are only active in very high concentrations, as compared to the natural phagostimulant from the labial gland secretion of *R. santonensis*. Obviously this natural signal is much more effective, inducing feeding behavior at naturally low concentrations and even on substrates unsuitable as food sources (Reinhard et al., 1997). This indicates that once its chemical nature is identified, addition of the natural feeding-stimulating signal to a termite bait could increase food consumption and improve bait efficacy much more than the hitherto considered synthetic phagostimulants.

Acknowledgments—We are grateful to Dr. Michael Lacey, CSIRO Entomology, Australia, and Dr. Johannes Steidle, FU Berlin, Germany, for critical comments on an earlier draft of this paper. Dr. Monika Hilker, FU Berlin, Germany, provided laboratory facilities for the chemical analyses. Financial support was provided by a scholarship from the Bundesanstalt für Materialforschung und -prüfung, Berlin, Germany.

REFERENCES

ABUSHAMA, F. T., and KAMBAL, M. A. 1976. The role of sugars in the food selection of the termite *Microtermes traegardhi* (Sjöst.). *Z. Angew. Entomol.* 84:250–255.

ALLSOPP, P. G. 1992. Sugars, amino acids, and ascorbic acid as phagostimulants for larvae of *Antitrogus parvulus* and *Lepidiota negatoria* (Coleoptera: Scarabaeidae). *J. Econ. Entomol.* 85:106–111.

BILLEN, J., JOYE, L., and LEUTHOLD, R. H. 1989. Fine structure of the labial gland in *Macrotermes bellicosus* (Isoptera, Termitidae). *Acta Zool.* 70:37–45.

BREZNAK, J. A. 1975. Symbiotic relationships between termites and their intestinal microbiota. *Soc. Exp. Biol. Symbiosis*, *Cambridge University Press* 559–590.

BRIAN, M. V. 1983. Social Insects: Ecology and Behavioural Biology. Chapman and Hall, London.

CHEN, J., and HENDERSON, G. 1996. Determination of feeding preference of Formosan subterranean termite (*Coptotermes formosanus* Shiraki) for some amino acid additives. *J. Chem. Ecol.* 22:2359–2369.

COSTA-LEONARDO, A. M. 1997. Secretion of salivary glands of the Brazilian termite *Serritermes serrifer* Hagen & Bates (Isoptera: Serritermitidae). *Ann. Soc. Entomol. Fr.* 33:29–37.

COSTA-LEONARDO, A. M., and CRUZ-LANDIM, C. 1991. Morphology of the salivary gland acini in *Grigiotermes bequaerti* (Isoptera: Termitidae: Apicotermitinae). *Entomol. Gen.* 16:13–21.

COWLING, E. B., and MERRILL, W. 1966. Nitrogen in wood and its role in wood deterioration. *Can. J. Bot.* 44:1539–1554.

CZOLIJ, R. T., and SLAYTOR, M. 1988. Morphology of the salivary glands of *Mastotermes darwiniensis* Froggatt (Isoptera: Mastotermitidae). *Int. J. Insect Morphol. Embryol.* 17:207–220.

ELLIOTT, J. M. 1979. Some methods for the statistical analysis of samples of benthic invertebrates. Freshwater Biological Association Scientific Publication 25. Cumbria, UK.

ESENTHER, G. R., and GREY, D. E. 1968. Subterranean termite studies in southern Ontario. *Can. Entomol.* 100:827–834.

GRASSÉ, P.-P. 1982. Termitologia, Vol. 1, Masson, Paris.

HEWITT, P. H., NEL, J. J. C., and SCHOEMAN, I. 1971. Influence of group size on water imbibition by *Hodotermes mossambicus* alate termites. *J. Insect Physiol.* 17:587–600.

HIGASHI, M., ABE, T., and BURNS, T. 1992. Carbon-nitrogen balance and termite ecology. *Proc. R. Soc. London Ser. B* 249:303–308.

HOGAN, M., VEIVERS, P. C., SLAYTOR, M., and CZOLIJ, R. T. 1988. The site of cellulose breakdown in higher termites (*Nasutitermes walkeri* and *Nasutitermes exitiosus*). *J. Insect Physiol.* 34:891–899.

HUNGATE, R. E. 1941. Experiments on the nitrogen economy of termites. *Ann. Entomol. Soc. Am.* 34:467–489.

JONES, S. C. 1984. Evaluation of two insect growth regulators for the bait-block method of subterranean termite (Isoptera: Rhinotermitidae) control. *J. Econ. Entomol.* 77:1086–1091.

KAIB, M., and ZIESMANN, J. 1992. The labial gland in the termite *Schedorhinotermes lamanianus* (Isoptera: Rhinotermitidae): Morphology and function during communal food exploitation. *Insectes Soc.* 39:373–384.

LEWIS, V. R., and HAVERTY, M. I. 1996. Evaluation of six techniques for control of the western drywood termite (Isoptera: Kalotermitidae) in structures. *J. Econ. Entomol.* 89:922–934.

MA, W. C., and JERMY, T. 1976. Mouth parts and receptors involved in feeding behaviour and sugar perception in the African armyworm, *Spodoptera exempta* (Lepidoptera, Noctuidae), pp. 139–151, *in* T. Jermy (ed.). The Host-Plant in Relation to Insect Behavior and Reproduction. Plenum Press, New York.

MARTIN, M. M., and MARTIN, J. S. 1978. Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis:* The role of acquired digestive enzymes. *Science* 119:1453–1455.

MASCHWITZ, U., and THO, Y. P. 1974. Chinone als Wehrsubstanzen bei einigen orientalischen Macrotermitinen. *Insectes Soc.* 31:231–234.

MEDNIKOVA, T. K. 1991. Effect of caste differences on salivary gland functions in the termite *Anacanthotermes ahngerianus. Zh. Evol. Biokhim. Fiziol.* 27:86–91.

MEDNIKOVA, T. K. 1996. The age- and caste-dependent structural features of the salivary glands of the termite, *Anacanthotermes ahngerianus* Jacobson (Isoptera, Hodotermitidae). *Entomol. Rev.* 75:1–15.

MEEHAN, M., and WILDE, G. 1989. Screening for sorghum line and hybrid resistance to chinch bug (Hemiptera: Lygaeidae) in the greenhouse and growth chamber. *J. Econ. Entomol.* 82:616–620.

MERCK, E. 1970. Anfärbereagenzien für Dünnschicht- und Papierchromatographie. Merck Verlag, Darmstadt.

MISHRA, S. C. 1992. Attraction and acceptance responses of termite *Neotermes bosei* Snyder (Kalotermitidae) to organic compounds and solvents. *Indian J. For.* 15:245–249.

NOIROT, C. 1969. Glands and secretions, pp. 89–123, *in* K. Krishna and F. Weesner (eds.). Biology of the Termites, Vol. 1. Academic Press, New York.

OHKUMA, M., NODA, S., USAMI, R., HORIKOSHI, K., and KUDO, T. 1996. Diversity of nitrogen fixation genes in the symbiotic intestinal microflora of the termite *Reticulitermes speratus. Appl. Environ. Microbiol.* 62:2747–2752.

OLAGBEMIRO, T. O., LAJIDE, L., SANI, K. M., and STADDON, B. W. 1988. 2-Hydroxy-5-methyl-1,4-benzoquinone from the salivary gland of the soldier termites *Odontotermes magdalenae. Experientia* 44:1022–1024.

PETERS, B. C., and FITZGERALD, C. J. 1999. Field evaluation of the effectiveness of three timber species as bait stakes and the bait toxicant hexaflumuron in eradicating *Coptotermes acinaciformis* (Froggatt) (Isoptera: Rhinotermitidae). *Sociobiology* 33:227–238.

POTRIKUS, C. J., and BREZNAK, J. A. 1977. Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. *Appl. Environ. Microbiol.* 33:392–399.

REINHARD, J., and KAIB, M. 1995. Interaction of pheromones during food exploitation by the termite *Schedorhinotermes lamanianus. Physiol. Entomol.* 20:266–272.

REINHARD, J., HERTEL, H., and KAIB, M. 1997. Feeding stimulating signal in labial gland secretion of the subterranean termite *Reticulitermes santonensis. J. Chem. Ecol.* 23:2371–2381.

SCHMIDT, J. M., and FRIEND, W. G. 1991. Ingestion and diet destination in the mosquito *Culiseta inornata:* Effects of carbohydrate configuration. *J. Insect Physiol.* 37:817–828.

SLAYTOR, M., and CHAPPELL, D. J. 1994. Nitrogen metabolism in termites. *Comp. Biochem. Physiol.* 107B:1–10.

SOGAWA, K. 1972. Studies on the feeding habits of the brown planthopper. III. Effects of amino acids and other compounds on the sucking response. *Jpn. J. Appl. Entomol. Zool.* 16:1–7.

SPEARS, B. M., and UECKERT, D. N. 1976. Survival and food consumption by the desert termite *Gnathamitermes tubiformans* in relation to dietary nitrogen source and levels. *Environ. Entomol.* 5:1022–1025.

SU, N.-Y. 1994. Field evaluation of hexaflumuron bait for population suppression of subterranean termites (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* 87:389–397.

TOKUDA, G., WATANABE, H., MATSUMOTO, T., and NODA, H. 1997. Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagoensis* (Shiraki): Distribution of cellulases and properties of endo-β-1,4-glucanase. *Zool. Sci.* 14:83–93.

VAN DER MEER, R. K., LOFGREN, C. S., and SEAWRIGHT, J. A. 1995. Specificity of the red imported fire ant (Hymenoptera: Formicidae) phagostimulant response to carbohydrates. *Fla. Entomol.* 78:144–154.

VEIVERS, P. C., MUSCA, A. M., O'BRIEN, R. W., and SLAYTOR, M. 1982. Digestive enzymes of the salivary glands and gut of *Mastotermes darwiniensis. Insect Biochem.* 12:35–40.

VEIVERS, P. C., MÜHLEMANN, R., SLAYTOR, M., LEUTHOLD, R. H., and BIGNELL, D. E. 1991. Digestion, diet and polyethism in two fungus-growing termites: *Macrotermes subhyalinus* Rambur and *M. michaelseni* Sjöstedt. *J. Insect Physiol.* 37:675–682.

VISWANATHAN, K., and KALODE, M. B. 1990. Biochemical aspects of varietal resistance to rice green leafhoppers, *Nephotettix virescens* (Distant) and *Nephotettix nigropictus* (Stal.). *Proc. Indian Acad. Sci., Anim. Sci.* 99:57–66.

WALLER, D. A., and CURTIS, A. D. 1996. Termite (Rhinotermitidae; *Reticulitermes*) preference for sugar-coated foods. Presentation at the Annual Meeting of the Entomological Society of America, December 8–12, 1996, Louisville, Kentucky.

WATANABE, H., NAKAMURA, M., TOKUDA, G., YAMAOKA, I., SCRIVENER, A. M., and NODA, H. 1997. Site of secretion and properties of endogenous endo-β-1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. *Insect Biochem. Mol. Biol.* 27:305–313.

WEIBULL, J. 1990. Host plant discrimination in the polyphagous aphid *Rhopalosiphum padi:* The role of leaf anatomy and storage carbohydrate. *Oikos* 57:167–174.

Journal of Chemical Ecology, Vol. 27, No. 1, 2001

FOOD EXPLOITATION IN TERMITES: INDICATION FOR A GENERAL FEEDING-STIMULATING SIGNAL IN LABIAL GLAND SECRETION OF ISOPTERA

JUDITH REINHARD[1,*] and MANFRED KAIB[2]

[1]*Bundesanstalt für Materialforschung und -prüfung*
12200 Berlin, Germany
[2]*Universität Bayreuth, Lehrstuhl Tierphysiologie*
95440 Bayreuth, Germany

(Received November 3, 1999; accepted September 8, 2000)

Abstract—The paired labial glands are located in all termite species in the thorax. During food exploitation workers of the French termite *Reticulitermes santonensis* and the African termite *Schedorhinotermes lamanianus* release the secretion of their labial glands directly onto the food. The secretion carries a water-soluble, heat-resistant, nonvolatile signal that stimulates gnawing and feeding and leads to aggregations of feeding workers. In a feeding bioassay, extracts of the labial glands of 11 termite species from five families all proved to have this feeding-stimulating effect both on *R. santonensis* and *S. lamanianus*. The heat resistance of the feeding-stimulating signal also could be shown for selected species from all termite families tested. A combined thin layer chromatography–feeding bioassay on cellulose TLC plates showed that after chromatography of labial gland secretion, the feeding-stimulating signal is located in all 11 species in the same area from R_f 0.46 to 0.88. An extract of labial glands of cockroaches stimulated feeding in *R. santonensis* and *S. lamanianus* as well, but was not active after heat treatment and after TLC. This points towards a general feeding-stimulating signal having evolved only in the labial gland secretion of termites.

Key Words—Termites, *Reticulitermes*, *Schedorhinotermes*, labial gland secretion, chemical signal, phagostimulant.

*To whom correspondence should be addressed at current address: CNRS, Laboratoire de Neurobiologie-UPR 9024, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France.

INTRODUCTION

The performance of labor in termite societies includes large numbers of nestmates, and its organization depends predominantly on chemical signals produced by various exocrine glands, such as the paired labial glands. These are located in the meso- and metathorax of all termite species, and the glandular ducts open into the oral cavity (Pasteels, 1965; Noirot, 1969; Billen et al., 1989; Costa-Leonardo and Cruz-Landim, 1991; Kaib and Ziesmann, 1992; Mednikova, 1996; Costa-Leonardo, 1997).

During communal food exploitation of termites, the labial gland secretion is released onto the food by feeding workers, as was recently demonstrated in the African termite *Schedorhinotermes lamanianus* and the French species *Reticulitermes santonensis* (Kaib and Ziesmann, 1992; Reinhard and Kaib, 1995; Reinhard et al., 1997a). It contains a chemical signal that stimulates additional workers to feed at the same site, thereby initiating clusters of feeding termites, aiding in efficient food exploitation. The feeding-stimulating signal was shown to be highly polar, heat-resistant, and nonvolatile in both *S. lamanianus* and *R. santonensis* (Kaib and Ziesmann, 1992; Reinhard et al., 1997a).

Both termite species belong to the same family, Rhinotermitidae, but they differ in many aspects of their biology, such as habitat, nesting habits, and foraging and recruitment strategies. While *S. lamanianus* is an arboreal termite that lives in humid forests of tropical Africa, *R. santonensis* occurs in a temperate zone of France in subterranean nests (Harris, 1968; Becker, 1970; Kaib and Brandl, 1992; Brandl et al., 1996). In *S. lamanianus* foraging is initiated by soldiers (Schedel and Kaib, 1987); in *R. santonensis*, in contrast, foraging is initiated by workers (Reinhard et al., 1997b). Nevertheless, both species form distinctive feeding clusters on the food source induced by release of the feeding-stimulating signal. This may indicate that feeding stimulation by the chemical signal from the labial glands is a general strategy for organization of communal food exploitation in termites.

Our work was undertaken to examine the existence of a non-species-specific feeding-stimulating signal in the labial gland secretion of termites. Using feeding bioassays with *R. santonensis* and *S. lamanianus* as test species, we investigated the labial gland secretions of 11 different termite species from five families, collected all over the world, as well as the labial gland secretion of one cockroach species for comparison.

METHODS AND MATERIALS

Collection and Culture of Termites. One colony of the subterranean termite *Reticulitermes santonensis* De Feytaud (Rhinotermitidae) was collected in

Ile d'Oléron, France, and kept in the laboratory under constant conditions of 25°C, 75% relative humidity. The colony was housed in a metal container with moist vermiculite as soil substrate and water source and pine wood as food. One carton nest of the termite *Schedorhinotermes lamanianus* Sjöstedt (Rhinotermitidae) was collected near a campsite at Shimba Hills National Reserve, Kwale District, Kenya, and kept in the laboratory under constant conditions of 28°C, 70–80% relative humidity, LD 12L : 12D h). The colony was housed in a plastic container, provided with water from a connected tank, and fed ab libitum with filter paper. From the containers the termites had access to foraging arenas via a silicone tube (5 mm ID). The foraging arenas (10 × 10 cm; height: *R. santonensis:* 0.5 cm; *S. lamanianus:* 2.5 cm) had a sand-blasted glass plate as floor and were covered by a glass plate.

Labial glands were obtained from workers of the above as well as the following termite species [reared at the Bundesanstalt für Materialforschung und -prüfung (BAM), Berlin, Germany]: *Kalotermes flavicollis* (Kalotermitidae, Mediterranean), *Mastotermes darwiniensis* (Mastotermitidae, Australia), *Hodotermes mossambicus* (Hodotermitidae, West Africa), *Zootermopsis angusticollis* (Hodotermitidae, North America), *Heterotermes indicola* (Rhinotermitidae, India), *Coptotermes formosanus* (Rhinotermitidae, Southeast Asia), and *Nasutitermes nigriceps* (Termitidae, Central America). *Macrotermes subhyalinus* (Termitidae, West Africa) was provided from a colony held at the University of Bern, Switzerland, and *Trinervitermes trinervoides* (Termitidae) was collected in the field near Grahamstown, South Africa. All termite species used are wood-eating, except *H. mossambicus*, *M. subhyalinus*, and *T. trinervoides*, which are grass-feeding. Cockroaches (*Periplaneta americana*) were provided by the Institut für Wasser-Boden-Luft-Hygiene (WABOLU), Berlin, Germany.

Earlier experiments (Reinhard and Kaib, 1995, 2001; Reinhard et al. 1997a) with other colonies of the same species did not show any intraspecific variation in individual feeding behavior or analyses of the labial gland secretion, as compared to the present work. Experiments were, therefore, carried out with only one colony of each species.

Preparation of Labial Gland Extracts. Labial gland extracts of termite workers and cockroaches were prepared as described in Reinhard and Kaib (2001). Ten labial glands per 100 μl water were extracted in the case of *R. santonensis*. As gland sizes differ species-specifically, lots of labial glands corresponding in volume to 10 labial glands of *R. santonensis* were pooled for gland extracts of the other species.

Bioassays on Feeding Stimulation. All tests were conducted in the foraging arenas and made under red light illumination. Frequencies and spatial distribution of workers that were displaying feeding behavior as defined in Reinhard and Kaib (2001) were recorded over time by video techniques (IR-sensitive CCD camera CF 15/2, Kappa) and subsequently analyzed.

Choice feeding tests were carried out both with *R. santonensis* and *S. lamanianus* on pieces of moist filter paper. In each test two semicircles (2.5 cm diameter) of filter paper were offered to the termites. On one of the two semicircles 25 μl of a labial gland extract was applied, the other semicircle was only moistened (control). For each test the distribution of the first 20 termites feeding on the two semicircles during the test duration of ca. 1 hr was determined. Tests were replicated 20 times. For the experiments on heat resistance, the labial gland extracts of *K. flavicollis*, *M. darwiniensis*, *H. mossambicus*, *R. santonensis*, *S. lamanianus*, and *M. subhyalinus* as selected representatives of the termite families as well as *P. americana* were chosen. The extracts were exposed for 15 min to 95°C in a waterbath before applying them to the semicircles of filter paper.

Feeding tests on thin-layer chromatography plates were carried out with *R. santonensis* only. Twenty-five microliters of labial gland extract each from the cockroach and the 11 termite species were applied separately as 1-cm-long lines on cellulose TLC plates and chromatographed as described in Reinhard and Kaib (2001). The TLC plates were then offered as food, and the spatial distribution of all termites feeding between start and front line on the TLC plates during the 1-hr duration of the experiment was analyzed. The bioassay was repeated five times for each labial gland extract.

Data Analysis. The sign test was used to analyze the data of the choice feeding tests on filter paper. The distribution of termites feeding on TLC plates was analyzed via the dispersion index (Elliott, 1979) and the R_f value of the preferred feeding zones determined.

RESULTS

Labial gland extracts of all 11 termite species and the cockroach had a strong feeding-stimulating effect both on *Reticulitermes santonensis* and *Schedorhinotermes lamanianus* (Figure 1). Neither *R. santonensis* nor *S. lamanianus* responded more strongly to their own labial gland secretion than to the secretions of the other species. Labial gland extracts of the three grass-feeding termite species had the same effect as extracts of the wood-feeding termite species.

After heat treatment of labial gland extracts of selected representatives of each family, only the termite secretions still had the same feeding-stimulating effect on *R. santonensis* and *S. lamanianus* (Table 1). Heat-treated labial gland extract of the cockroach species *Periplaneta americana* did not show any phagostimulatory effect.

When TLC plates with chromatographed labial gland extracts of the 11 termite species were offered to *R. santonensis*, the feeding termites showed a clumped distribution between R_f 0.46 and 0.88 for all extracts in all five replicates (Figure 2, Table 2). In contrast, feeding termites were randomly distributed

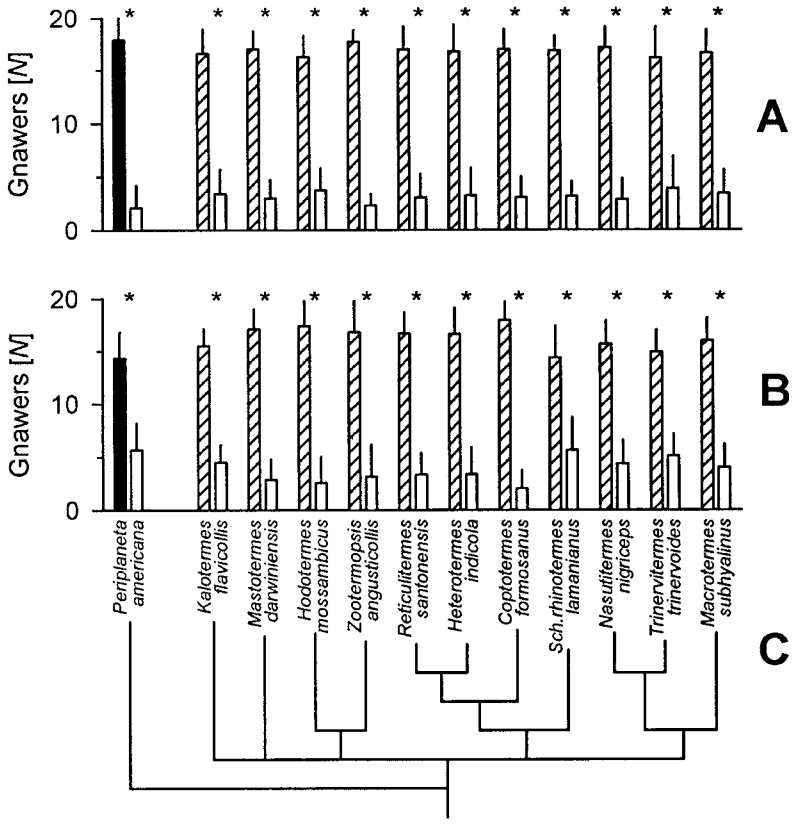


FIG. 1. Feeding stimulating effect of labial gland extracts of one cockroach and 11 termite species on *Reticulitermes santonensis* (A) and *Schedorhinotermes lamanianus* (B). Distribution of the first 20 termites (gnawers) feeding on two semicircles of filter paper within 1 hr of observation (mean number per semicircle ± SD, $N = 20$). Choice test represented by bars; black/hatched bars: 25 μl LGS, empty bars: water. *Significant difference at $P < 0.001$, sign test. (C) Phylogenetic tree of tested species (Abe, 1987), no genetic distances presented.

on the TLC plates when cockroach labial gland secretion had been applied, leaving gnawing marks all over the cellulose layer.

DISCUSSION

The labial gland secretion of *Schedorhinotermes lamanianus* and *Reticulitermes santonensis* contains a feeding-stimulating chemical signal, inducing

TABLE 1. PHAGOSTIMULANT EFFECT AFTER HEAT TREATMENT OF COCKROACH AND TERMITE LABIAL GLAND SECRETION (LGS) ON *Reticulitermes santonensis* (*R.s.*) AND *Schedorhinotermes lamanianus* (*S.l.*)[a]

LGS of:	Bioassay with	Termites (*N*) on Semicircle with LGS	Termites (*N*) on Semicircle without LGS	*P*
P. americana	*R.s.*	12.3 ± 4.2	7.7 ± 4.2	0.058
	S.l.	9.5 ± 3.0	10.5 ± 3.0	0.868
K. flavicollis	*R.s.*	14.5 ± 3.4	5.5 ± 3.4	<0.001
	S.l.	15.1 ± 2.9	4.9 ± 2.9	<0.001
M. darwiniensis	*R.s.*	15.3 ± 1.9	4.7 ± 1.9	<0.001
	S.l.	14.8 ± 2.4	5.2 ± 2.4	<0.001
H. mossambicus	*R.s.*	18.0 ± 1.2	2.0 ± 1.2	<0.001
	S.l.	17.4 ± 1.7	2.6 ± 1.7	<0.001
R. santonensis	*R.s.*	16.6 ± 2.4	3.4 ± 2.4	<0.001
	S.l.	17.6 ± 1.9	2.4 ± 1.9	<0.001
S. lamanianus	*R.s.*	15.8 ± 2.7	4.2 ± 2.7	<0.001
	S.l.	16.4 ± 3.1	3.6 ± 3.1	<0.001
M. subhyalinus	*R.s.*	16.2 ± 2.0	3.8 ± 2.0	<0.001
	S.l.	15.5 ± 2.9	4.5 ± 2.9	<0.001

[a] Distribution of the first 20 termites feeding on two semicircles of filter paper with and without 25μl LGS within 1 hr of observation (mean number per semicircle ± SD, $N = 20$, P as determined with sign test). For full names of species, see text and Figure 1.

clusters of feeding nestmates to form where applied (Kaib and Ziesmann, 1992; Reinhard et al., 1997a). The experiments presented here showed that labial gland secretion of various other termite species also induced the same feeding response in *R. santonensis* and *S. lamanianus*. The feeding-stimulating signal was heat-resistant in all termite labial gland secretions and was located within the same biologically active zone of R_f 0.46–0.88 after TLC separation. This corresponds to the zone determined as the location of the feeding-stimulating signal in *R. santonensis* after TLC (Reinhard and Kaib, 2001). These results strongly suggest that the feeding-stimulating signal from the labial glands in all termite species is of the same or very similar chemical composition, independent of phylogeny, geographical origin, or nesting or feeding habits.

This is supported by preliminary GC-MS analyses of termite labial gland secretions, which identified several metabolic compounds, common for *R. santonensis*, *S. lamanianus* (both Rhinotermitidae), *M. subhyalinus* (Termitidae), and *M. darwiniensis* (Mastotermitidae) (Reinhard, 1998; Ibarra, unpublished results). A variety of compounds are reported to be produced in the various cell types of termite labial glands (Noirot, 1969; Veivers et al., 1982; Hogan et al., 1988; Mednikova, 1991; Tokuda et al., 1997; Watanabe et al., 1997). It is quite possible

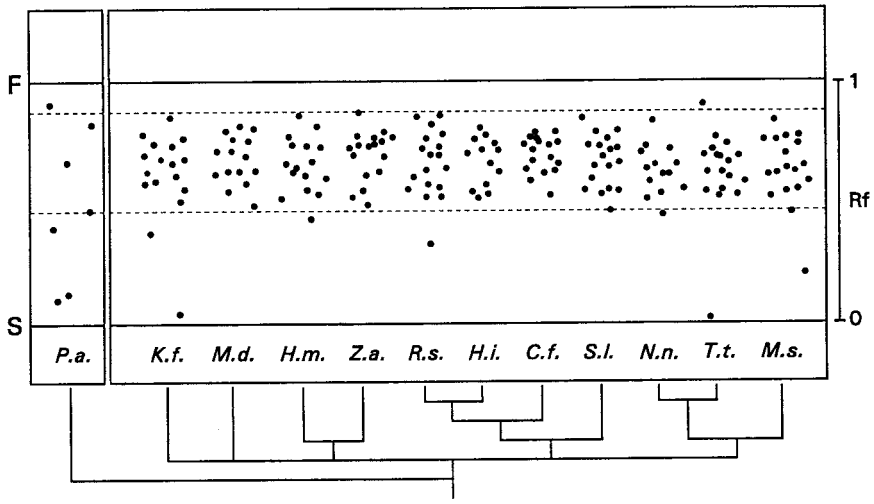


FIG. 2. Exemplary distribution of *Reticulitermes santonensis* termites (dots) feeding on a cellulose TLC plate within 1 hr, after thin-layer chromatography of labial gland secretion of one cockroach and 11 termite species. S: start line, F: front line. Dotted lines indicate biologically active zone. *P.a.: Periplaneta americana*, *K.f.: Kalotermes lavicollis*, *M.d.: Mastotermes darwiniensis*, *H.m.: Hodotermes mossambicus*, *Z.a.: Zootermopsis angusticollis*, *R.s.: Reticulitermes santonensis*, *H.i.: Heterotermes indicola*, *C.f.: Coptotermes formosanus*, *S.l.: Schedorhinotermes lamanianus*, *N.n.: Nasutitermes nigriceps*, *T.t.: Trinervitermes trinervoides*, and *M.s.: Macrotermes subhyalinus*.

that the chemicals extracted from the glands for the analyses are only partially the same as that deposited on the food by feeding workers. However, the fact that the termites respond to the labial gland extracts with feeding in the choice tests and even after the separation of its constituents by TLC supports the inference that the extracts do contain the relevant component(s). Further work on elucidation of the chemical structure of this general feeding-stimulating signal in termites is in progress.

The labial gland secretion of the cockroach, however, could not induce feeding after heat treatment or TLC separation. The observed feeding stimulation by untreated cockroach secretion can therefore not be caused by the persistent phagostimulant signal as found in termites. It must be due to other factors, for example, possibly digestive enzymes of the secretion (Slaytor, 1992) that metabolize the cellulose in the filter paper during the bioassay. Thus, the feeding-stimulating signal is not present in cockroaches, but was found in all 11 termite species, representing all termite families (except the rare Serritermitidae), coming from all continents, and including both xylophagous and harvester ter-

TABLE 2. TOTAL NUMBER AND DISTRIBUTION OF *Reticulitermes santonensis* TERMITES FEEDING ON CELLULOSE TLC PLATES WITHIN 1 HOUR[a]

Test no.	Number of feeding termites						
	Total	R_f 0–0.25	R_f 0.25–0.5	R_f 0.5–0.75	R_f 0.75–1.0	X^2	P
P.a.							
1	6	2	1	1	2	0.7	[b]
2	3	0	1	0	2	3.7	[b]
3	2	0	2	0	0	6.0	[b]
4	7	2	2	1	2	0.4	[b]
5	4	1	1	0	2	1.9	[b]
K.f.							
1	23	0	1	7	15	24.8	[b]
2	17	0	0	4	13	26.5	[b]
3	23	1	2	8	12	14.0	[b]
4	30	2	0	11	17	24.9	[b]
5	22	1	1	7	13	17.9	[b]
M.d.							
1	22	0	0	13	9	23.5	[b]
2	22	0	1	4	17	33.6	[b]
3	24	0	1	13	10	20.9	[b]
4	25	1	2	7	15	19.6	[b]
5	20	0	1	7	12	18.8	[b]
H.m.							
1	13	0	0	2	11	25.4	[b]
2	22	0	0	14	8	25.3	[b]
3	23	1	2	3	17	29.7	[b]
4	27	0	0	12	15	27.7	[b]
5	18	0	0	8	10	18.4	[b]
Z.a.							
1	19	0	0	7	12	21.6	[b]
2	25	0	1	14	10	22.5	[b]
3	26	1	2	8	15	19.2	[b]
4	26	1	0	11	14	22.9	[b]
5	28	1	0	8	19	32.9	[b]
R.s.							
1	22	0	1	12	9	19.1	[b]
2	29	1	0	8	20	35.1	[b]
3	24	0	2	10	13	18.7	[b]
4	23	0	0	8	15	27.3	[b]
5	27	0	0	10	17	30.6	[b]
H.i.							
1	27	1	1	12	13	19.7	[b]
2	24	0	1	8	15	24.3	[b]
3	31	1	2	11	17	22.5	[b]

TABLE 2. CONTINUED

Test no.	Number of feeding termites: Total	R_f 0–0.25	R_f 0.25–0.5	R_f 0.5–0.75	R_f 0.75–1.0	X^2	P
4	21	0	0	9	13	23.5	[b]
5	29	0	1	10	18	29.6	[b]
C.f.							
1	24	0	1	7	16	26.9	[b]
2	17	0	0	5	12	22.8	[b]
3	26	0	0	9	17	30.9	[b]
4	25	1	0	10	14	22.5	[b]
5	27	0	0	10	17	30.6	[b]
S.l.							
1	28	0	0	8	20	38.3	[b]
2	24	0	0	6	17	33.5	[b]
3	28	1	2	11	14	17.9	[b]
4	28	0	0	10	18	32.8	[b]
5	26	0	1	13	12	22.3	[b]
N.n.							
1	21	1	0	4	16	31.0	[b]
2	15	0	0	5	10	18.3	[b]
3	24	1	0	12	11	20.3	[b]
4	24	1	1	14	8	19.7	[b]
5	31	1	1	9	20	31.3	[b]
T.t.							
1	15	1	0	4	10	16.2	[b]
2	20	0	0	7	13	23.6	[b]
3	22	0	1	10	11	18.3	[b]
4	22	0	0	8	14	25.2	[b]
5	28	1	0	11	16	25.9	[b]
M.s.							
1	22	1	0	10	11	18.3	[b]
2	21	1	0	7	13	20.7	[b]
3	27	0	0	9	18	32.9	[b]
4	26	0	0	11	15	27.2	[b]
5	26	0	0	16	10	28.8	[b]

[a] Five tests each after thin layer chromatography of labial gland secretion of one cockroach and 11 termite species. Chi-square as in Elliott (1979). *P.a.: Periplaneta americana, K.f.: Kalotermes flavicollis, M.d.: Mastotermes darwiniensis, H.m.: Hodotermes mossambicus, Z.a.: Zootermopsis angusticollis, R.s.: Reticulitermes santonensis, H.i.: Heterotermes indicola, C.f.: Coptotermes formosanus, S.l.: Schedorhinotermes lamanianus, N.n.: Nasutitermes nigriceps, T.t.: Trinervitermes trinervoides,* and *M.s.: Macrotermes subhyalinus*.

[b] Difference from random distribution at $P < 0.05$.

mites. Cockroaches are considered ancestral to termites (Krishna and Weesner, 1969/70; Terra, 1988; Kambhampati, 1995). Hence the feeding stimulant from the labial gland secretion must be an apomorphic character for the Isoptera that evolved during the rise of the whole order and is linked to the evolution of eusociality and the need to organize efficient communal exploitation of food sources. It is notable that this trait has been retained unchanged across the order.

The biological meaning of the signal lies in the formation of feeding clusters, rendering food exploitation more efficient. If several nestmates feed at the same spot, repeated application of saliva humidifies the food and repeated biting enhances the mechanical break-up of its surface. By concentrating feeding efforts, a termite colony can exploit a food source faster and more efficiently. The system of feeding clusters also guarantees that the released signal as well as other important compounds of the labial gland secretion, such as digestive enzymes (Martin and Martin, 1978; Veivers et al., 1982, 1991), are not lost, but again taken up by nestmates joining a group of feeding workers.

Feeding clusters can be observed easily in xylophagous termites such as *S. lamanianus*, which feed on clumped food sources (Kaib and Ziesmann, 1992). Other species, such as the harvester termites *H. mossambicus*, *T. trinervoides*, and *M. subhyalinus*, feed on dispersed food sources. They collect grass as a direct food source or even culture fungus on partly digested grass for food, respectively (Abe, 1987; Badertscher et al., 1983; Hewitt et al., 1990). Feeding clusters seem to be of little importance for them. However, preliminary observations have shown that even in harvester termites, several workers group to bite off pieces of longer grass stems (Reinhard, 1998). Furthermore, *H. mossambicus* workers preferred the spots on a grass stem where labial gland secretion had been applied before by another worker or artificially in a bioassay. Thus, even harvester termites employ the feeding-stimulating chemical signal for organization of communal food exploitation.

Although this is not required by definition (Nordlund and Lewis, 1976), it is generally acknowledged that many insect pheromones are rather species-specific. The existence of a non-species-specific phagostimulant in termites is remarkable. However, although the same feeding-stimulating signal may be used by sympatric species, a multitude of behaviors has evolved, preventing constant interspecific conflicts on common food sources. There are highly species-specific foraging and recruitment signals, specialized behavioral patterns and strategies, the use of soldier defense lines for delineating a given feeding area, and constructing physical barriers, such as galleries and cover sheets (e.g., Jander and Daumer, 1974; Howard et al., 1976; Kaib et al., 1982; Badertscher et al. 1983; Heidecker and Leuthold, 1984; Hall and Traniello, 1985; Traniello and Busher, 1985; Lys and Leuthold, 1987, 1991; Schedel and Kaib, 1987; Miura and Matsumoto, 1995; Reinhard, 1998). They ensure that several termite species may share common food sources without permanent conflicts. Furthermore, a com-

mon signal can even be of mutual advantage for rival colonies or species. Delaplane and La Fage (1989) reported that termites prefer timber that had previously been attacked by conspecifics, or even a different species, over sound wood. Termites recognize and accept food sources faster when previously used and thus marked with the common phagostimulatory signal.

Acknowledgments—We are grateful to Dr. Michael Lacey, CSIRO Entomology, Australia, and Dr. Johannes Steidle, FU Berlin, Germany, for critical comments on an earlier draft of this paper. Unpublished data by Fernando Ibarra, University of Hamburg, Germany, were part of a Master's thesis. Financial support was provided by a scholarship from the Bundesanstalt für Materialforschung und -prüfung, Berlin, Germany.

REFERENCES

ABE, T. 1987. Evolution of life types in termites, pp. 128–148, *in* S. Kawano, J. H. Connell, and T. Hidaka (eds.). Evolution, Coadaptation and Biotic Communities. University of Tokyo Press, Tokyo, Japan.

BADERTSCHER, S., GERBER, C., and LEUTHOLD, R. H. 1983. Polyethism in food supply and processing in termite colonies of *Macrotermes subhyalinus* (Isoptera). *Behav. Ecol. Sociobiol.* 12:115–119.

BECKER, G. 1970. *Reticulitermes* (Ins., Isopt.) in Mittel- und Westeuropa. *Z. Angew. Entomol.* 65:268–278.

BILLEN, J., JOYE, L., and LEUTHOLD, R. H. 1989. Fine structure of the labial gland in *Macrotermes bellicosus* (Isoptera, Termitidae). *Acta Zool.* 70:37–45.

BRANDL, R., BAGINE, R. N. K., and KAI, M. 1996. The distribution of *Schedorhinotermes lamanianus* (Isoptera: Rhinotermitidae) and its termitophile *Paraclystis* (Lepidoptera: Tineidae) in Kenya: its importance for understanding east African biogeography. *Glob. Ecol. Biogeo. Lett.* 5:143–148.

COSTA-LEONARDO, A. M. 1997. Secretion of salivary glands of the Brazilian termite *Serritermes serrifer* Hagen & Bates (Isoptera: Serritermitidae). *Ann. Soc. Entomol. Fr.* 33:29–37.

COSTA-LEONARDO, A. M., and CRUZ-LANDIM, C. 1991. Morphology of the salivary gland acini in *Grigiotermes bequaerti* (Isoptera: Termitidae: Apicotermitinae). *Entomol. Gen.* 16:13–21.

DELAPLANE, K. S., and LA FAGE, J. P. 1989. Preference of the Formosan subterranean termite (Isoptera: Rhinotermitidae) for wood damaged by conspecifics. *J. Econ. Entomol.* 82(5):1363–1366.

ELLIOTT, J. M. 1979. Some methods for the statistical analysis of samples of benthic invertebrates. Freshwater Biological Association Scientific Publication 25. Cumbria, UK.

HALL, P., and TRANIELLO, J. F. A. 1985. Behavioral bioassays of termite trail pheromones: Recruitment and orientation effects of cembrene-A in *Nasutitermes costalis* (Isoptera: Termitidae) and discussion of factors affecting termite response in experimental contexts. *J. Chem. Ecol.* 11:1503–1513.

HARRIS, W. V. 1968. African termites of the genus *Schedorhinotermes* (Isoptera: Rhinotermitidae) and associated termitophiles (Lepidoptera: Tineidae). *Proc. R. Entomol. Soc. London* 37:103–113.

HEIDECKER, J. L., and LEUTHOLD, R. H. 1984. The organisation of collective foraging in the harvester termite *Hodotermes mossambicus* (Isoptera). *Behav. Ecol. Sociobiol.* 14:195–202.

HEWITT, P. H., NEL, J. J. C., and SCHOEMAN, I. 1971. Influence of group size on water imbibition by *Hodotermes mossambicus* alate termites. *J. Insect Physiol.* 17:587–600.

HEWITT, P. H., VAN DER WESTHUIZEN, M. C., VAN DER LINDE, T. C. DE K., and MITCHELL, J. 1990.

The dry matter, energy and nitrogen budget of the harvester termite *Hodotermes mossambicus* (Hagen). *S. Afr. J. Sci.* 86:30–34.

HOGAN, M., VEIVERS, P. C., SLAYTOR, M., and CZOLIJ, R. T. 1988. The site of cellulose breakdown in higher termites (*Nasutitermes walkeri* and *Nasutitermes exitiosus*). *J. Insect Physiol.* 34:891–899.

HOWARD, R., MATSUMURA, F., and COPPEL, H. C. 1976. Trail-following pheromones of the Rhinoteremitidae: Approaches to their authentication and specificity. *J. Chem. Ecol.* 2:147–166.

JANDER, R., and DAUMER, K. 1974. Guide-line and gravity orientation of blind termites foraging in the open (Termitidae: *Macrotermes*, *Hospitalitermes*). *Insectes Soc.* 21(1):45–69.

KAIB, M., and BRANDL, R. 1992. Distribution, geographic variation and between-colony compatibility of *Schedorhinotermes lamanianus* in Kenya (Isoptera: Rhinotermitidae), pp. 121–131, *in* J. Billen (ed.). Biology and Evolution of Social Insects. Leuven University Press, Leuven, Belgium.

KAIB, M., and ZIESMANN, J. 1992. The labial gland in the termite *Schedorhinotermes lamanianus* (Isoptera: Rhinotermitidae): Morphology and function during communal food exploitation. *Insectes soc.* 39:373–384.

KAIB, M., BRUINSMA, O., and LEUTHOLD, R. H. 1982. Trail-following in termites: Evidence for a multicomponent system. *J. Chem. Ecol.* 8:1193–1205.

KAMBHAMPATI, S. 1995. A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes. *Proc. Natl. Acad. Sci. U.S.A.* 92:2017–2020.

KRISHNA, K., and WEESNER, F. M. 1969/70. Biology of termites. Academic Press, New York.

LYS, J. A., and LEUTHOLD, R. H. 1987. Caste polyethism during the process of food acquisition in the termite *Macrotermes bellicosus* (Smeathman), pp. 116–117, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, Munich.

LYS, J. A., and LEUTHOLD, R. H. 1991. Task-specific distribution of the two worker castes in extranidal activities in *Macrotermes bellicosus* (Smeathman): Observation of behaviour during food acquisition. *Insectes Soc.* 38:161–170.

MARTIN, M. M., and MARTIN, J. S. 1978. Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis:* The role of acquired digestive enzymes. *Science* 119:1453–1455.

MEDNIKOVA, T. K. 1991. Effect of caste differences on salivary gland functions in the termite *Anacanthotermes ahngerianus. Zh. Evol. Biokhim. Fiziol.* 27:86–91.

MEDNIKOVA, T. K. 1996. The age- and caste-dependent structural features of the salivary glands of the termite, *Anacanthotermes ahngerianus* Jacobson (Isoptera, Hodotermitidae). *Entomol. Rev.* 75:1–15.

MIURA, T., and MATSUMOTO, T. 1995. Worker polymorphism and division of labor in the foraging behavior of the black marching termite *Hospitalitermes medioflavus* on Borneo Island. *Naturwissenschaften* 82:564–567.

NOIROT, C. 1969. Glands and secretions, pp. 89–123, *in* K. Krishna and F. Weesner (eds.). Biology of the Termites, Vol. 1. Academic Press, New York.

NORDLUND, D. A., and LEWIS, W. J. 1976. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *J. Chem. Ecol.* 2:211–220.

PASTEELS, J. M. 1965. Polyéethisme chez les ouvriers de *Nasutitermes lujae* (Termitidae: Isoptères). *Biol. Gabon.* 1:191–205.

REINHARD, J. 1998. Nahrungssuche und Nahrungsausbeute der Erdtermite *Reticulitermes santonensis:* Rolle chemischer Signale, PhD thesis. Bayreuther Forum Ökologie Vol. 54.

REINHARD, J., and KAIB, M. 1995. Interaction of pheromones during food exploitation by the termite *Schedorhinotermes lamanianus. Physiol. Entomol.* 20:266–272.

REINHARD, J., and KAIB, M. 2001. Thin-layer chromatography assessing feeding stimulation by labial gland secretion compared to synthetic chemicals in the subterranean termite *Reticulitermes santonensis. J. Chem. Ecol.* 27:175–187.

REINHARD, J., HERTEL, H., and KAI, M. 1997a. Feeding stimulating signal in labial gland secretion of the subterranean termite *Reticulitermes santonensis*. *J. Chem. Ecol.* 23:2371–2381.

REINHARD, J., HERTEL, H., and KAI, M. 1997b. Systematic search for food in the subterranean termite *Reticulitermes santonensis* De Feytaud (Isoptera, Rhinotermitidae). *Insectes Soc.* 44:147–158.

SCHEDEL, A., and KAIB, M. 1987. Polyethism during foraging in *Schedorhinotermes lamanianus* in unprotected areas: the role of exocrine glands, p. 416, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, Munich.

SLAYTOR, M. 1992. Cellulose digestion in termites and cockroaches: What role do symbionts play? *Comp. Biochem. Physiol.* 103B:775–784.

TERRA, W. R. 1988. Physiology and biochemistry of insect digestion: an evolutionary perspective. *Braz. J. Med. Biol. Res.* 21:675–734.

TOKUDA, G., WATANABE, H., MATSUMOTO, T., and NODA, H. 1997. Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagoensis* (Shiraki): Distribution of cellulases and properties of endo-β-1,4-glucanase. *Zool. Sci.* 14:83–93.

TRANIELLO, J. F. A., and BUSHER, C. 1985. Chemical regulation of polyethism during foraging in the neotropical termite *Nasutitermes costalis*. *J. Chem. Ecol.* 11:319–332.

VEIVERS, P. C., MUSCA, A. M., O'BRIEN, R. W., and SLAYTOR, M. 1982. Digestive enzymes of the salivary glands and gut of *Mastotermes darwiniensis*. *Insect Biochem.* 12:35–40.

VEIVERS, P. C., MÜHLEMANN, R., SLAYTOR, M., LEUTHOLD, R. H., and BIGNELL, D. E. 1991. Digestion, diet and polyethism in two fungus-growing termites: *Macrotermes subhyalinus* Rambur and *M. michaelseni* Sjöstedt. *J. Insect Physiol.* 37:675–682.

WATANABE, H., NAKAMURA, M., TOKUDA, G., YAMAOKA, I., SCRIVENER, A. M., and NODA, H. 1997. Site of secretion and properties of endogenous endo-β-1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. *Insect. Biochem. Mol. Biol.* 27:305–313.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

DEFENSIVE CHEMISTRY OF AN APOSEMATIC BUG, *Pachycoris stallii* UHLER AND VOLATILE COMPOUNDS OF ITS HOST PLANT *Croton californicus* MUELL.-ARG.

LIVY WILLIAMS III,[1,*] PHILIP E. EVANS,[2] and WILLIAM S. BOWERS[2]

[1]*USDA-ARS, Southern Insect Management Research Unit*
P.O. Box 346, Stoneville, Mississippi 38776-0346

[2]*Department of Entomology*
The University of Arizona
Forbes 401, Tucson, Arizona 85721

(Received March 15, 2000; accepted October 9, 2000)

Abstract—Volatile components of *Pachycoris stallii* scent gland secretions and the bug's host plant, *Croton californicus*, were identified by gas chromatography and mass spectroscopy. The predominant compounds isolated from *C. californicus* fruit and leaves were β-myrcene and β-caryophyllene. Metathoracic gland secretions of *P. stallii* contained mostly (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, (*E*)-2-hexenyl acetate, and *n*-tridecane. In males, *n*-tridecane was present throughout the metathoracic gland, but in females this compound was found only in the median reservoir/accessory gland. (*E*)-2-Hexenal was present throughout the gland of female bugs, but in males was primarily present in the median reservoir/accessory gland. (*E*)-4-Oxo-2-hexenal and *n*-dodecane were isolated from the median reservoir/accessory gland of male and female bugs. Metathoracic glands were sexually monomorphic. Data from chemical analyses and anatomical observations suggest that dorsal abdominal glands of adults were apparently obsolescent. In nymphs, dorsal abdominal glands produced (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, *n*-dodecane, *n*-tridecane, and tetradecanal. The proportion of the predominant constituent, (*E*)-4-oxo-2-hexenal, decreased from 72% in the first instar to 47% in the fourth instar. Proportions of tetradecanal and *n*-tridecane were greater in the fourth instar than in the first instar. Observations of dissected glands indicated that median and posterior dorsal abdominal glands of all nymphal instars were more developed than anterior dorsal abdominal glands. Scanning electron micrography revealed the presence of polygonal microsculpturing on the integument surrounding the ostioles of metathoracic and dorsal abdominal glands. Chemical, anatomical,

*To whom correspondence should be addressed.

0098-0331/01/0200-0203$19.50/0

and behavioral data indicated that *P. stallii* has a chemical defense system based on short-chain carbonyl compounds and that this system is directed against arthropods. The abundance of arthropod natural enemies apparently has forced *P. stallii* to maintain this defense system despite feeding on a toxic host plant.

Key Words—*Pachycoris stallii*, Pentatomoidea, Scutelleridae, allomone, *Croton californicus*, Euphorbiaceae, terpenoids, exocrine glands, aposematic, carbonyl defense.

INTRODUCTION

Heteroptera are well-known for their chemical defense systems (Aldrich, 1988). Heteropteran allomones are typically produced in and expelled from: (1) adult metathoracic glands located ventrally in the metathorax and with lateral ostia between the meso- and metathoracic legs, and (2) adult and nymphal dorsal abdominal glands arranged in a metameric series of up to four dorsally located glands on abdominal tergites III to VI (Staddon, 1979; Aldrich, 1988, 1995). The defensive secretions are usually mixtures of unbranched aliphatic compounds, most with carbon chains of C_6, C_8, and C_4, as well as some aromatic compounds (Staddon, 1979; Aldrich, 1995). These chemicals function as nonspecific toxins, irritants, and repellents, and are generally more effective against arthropods than birds or mammals (Eisner, 1970; Blum, 1981).

Aposematic heteropterans, like other apparent insects, have a high likelihood of discovery by natural enemies and are thus expected to be chemically well-defended (Pasteels et al., 1983). Aposematic bugs sometimes sequester compounds that are toxic to predators from host plants (Duffey and Scudder, 1972; Aldrich, 1988; Aldrich et al., 1996), or produce the typical allomones de novo or from simple precursors (Blum, 1981; Staddon et al., 1987; Krall et al., 1999). In many cases, sequestration of host toxins relaxes the selection pressure for production of typical exocrine gland secretions (Staddon et al., 1987; Aldrich, 1995; Aldrich et al., 1996).

Pachycoris stallii Uhler is an aposematic scutellerid bug of neotropical distribution. Adults are ca. 12 mm in length and are brown–black with 22 orange spots on the dorsum that sometimes coalesce. Nymphs are red with metallic blue–green markings on the abdomen, pronotum, head, and legs. *Pachycoris stallii* appears to be highly host specific to the euphorb *Croton californicus* Muell.-Arg. (Williams, unpublished observations), a perennial shrub that occurs in the southwestern United States and northwestern Mexico (Shreve and Wiggins, 1964). *Pachycoris stallii* is also a subsocial insect. Females oviposit on *C. californicus* leaves and guard the egg masses and first-instar nymphs from natural enemies (Williams, unpublished observations). With the exception of

first-instar nymphs that do not feed, all nymphal instars as well as adults feed on *C. californicus* seeds developing within capsular fruit. Early instar nymphs occur in aggregations on *C. californicus*. Field observations suggest that arthropods are the predominant natural enemies of *P. stallii* (Williams, unpublished observations). Like many Euphorbiaceae, *Croton* species produce an abundance of chemicals toxic and irritating to vertebrates (Upadhyay and Hecker, 1976; Chavez et al., 1982; Alexander et al., 1991). Phytochemicals in *C. californicus* apparently render it unpalatable, because herbivores, vertebrate and invertebrate alike, generally avoid it.

Pachycoris stallii is an aposematic insect that is relatively large and long-lived, apparently monophagous on a toxic host plant, and lives in aggregations in open habitat where it is attacked by other arthropods. Ecological theory predicts that such an insect should possess a fortified chemical defense system (Pasteels et al., 1983). The objectives of this study were to: (1) test the hypothesis that despite feeding on a toxic host plant that may confer protection against vertebrate predators, *P. stallii* maintains a potent exocrine gland defensive system directed at arthropods, and (2) describe the volatiles produced by *C. californicus*.

METHODS AND MATERIALS

Preparation of Chemical Extracts. Croton californicus and *P. stallii* were field-collected in Baja California, Mexico. Immediately after collection, three to seven *Croton* fruit or leaves were placed in a glass vial containing 1 ml ethyl acetate for 2 min, after which all plant tissue was removed and the vial was sealed.

Pachycoris stallii were held in plastic vials with several fresh *Croton* leaves until collection of secretions (<4 hr after collection). Metathoracic gland secretions were obtained from living bugs by two methods: dissection of whole glands and collection of rinsates from whole bugs. Whole glands were dissected in a paraffin-coated Petri dish with tap water and were separated into two groups: (1) median reservoirs with accessory glands (MR/AG) and (2) lateral reservoirs with secretory tubules (LR/ST). Excess water was drawn from the glands with tissue paper, after which the glands were placed in a glass vial with 0.5 ml ethyl acetate and macerated with a capillary tube. Whole-bug rinsates were collected by placing one to four adults of the same gender (females guarding egg masses, females not guarding egg masses, and males) into a glass vial with 1 ml ethyl acetate for 3 min, after which adults were removed. (Preliminary analyses indicated that dorsal abdominal glands of adult *P. stallii* produce only trace amounts of volatiles; thus, we attribute results from whole-bug rinses solely to metathoracic glands). Samples from one to six bugs were pooled for analysis.

Dorsal abdominal gland secretions were obtained from adults by dissection similar to that described for metathoracic glands. Anterior (tergites III–IV),

median (tergites IV–V), and posterior (tergites V–VI) dorsal abdominal glands were dissected separately. Samples from three to six bugs were pooled and stored in 0.5 ml ethyl acetate for analysis. Nymphal dorsal abdominal gland secretions were collected by placing 5–40 nymphs of the same instar in a glass vial with 1 ml ethyl acetate for 3 min, after which nymphs were removed. All extracts were held at −20°C until chemical analysis.

Chemical Analysis. Extracts were analyzed on a Hewlett-Packard 5890 gas chromatograph with a 15-m × 0.32-mm-ID column coated with 0.25 μm DB-5 film (J & W Scientific, Folsom, California) and equipped with a flame ionization detector. The injector temperature was 200°C and the detector temperature was 240°C. The temperature was held at 60°C for 1 min then increased at 10°C/min to 220°C and held for 4 min. Nitrogen (20 cm/sec) was used as the carrier gas.

Gas chromatographic–mass spectrometric (GC-MS) analyses were conducted on a Hewlett-Packard 5890 gas chromatograph and a 5970 mass spectrometer. Data were obtained by using a 25-m × 0.32-mm-ID column coated with 0.25 μm film of DB-5 MS (J & W Scientific). The injector temperature was 250°C and the detector temperature was 280°C. The column temperature was held at 60°C for 2 min, then programmed at 10°C/min to 230°C and held for 2 min. The helium flow rate was 28.7 cm/sec. Electron impact (EI) mass spectra were measured at 70 eV.

GC-MS data were analyzed on a Hewlett-Packard ChemStation by using a NBS mass spectral library. Compounds were identified by comparison of the obtained mass spectra to library spectra and spectra of known standards, and by matching the obtained chromatographic retention times to those of known standard compounds.

Gland Anatomy. Pachycoris stallii adults and all five nymphal instars were killed and preserved in 70% ethanol. These bugs were dissected and observed with a dissecting scope to obtain gross morphological descriptions of metathoracic and dorsal abdominal glands. Scanning electron microscopy was used to study the efferent systems of metathoracic and dorsal abdominal glands. Specimens killed in 70% ethanol were sonicated for 1 min to remove debris, then passed through a graded ethanol series for dehydration, after which they were critical-point dried with carbon dioxide. Specimens were then mounted on SEM stubs using conductive silver paint, and were ion sputter-coated with gold before viewing with a Philips 501 scanning electron microscope (3.6–7.2 kV).

Behavior of P. stallii. Field observations of *P. stallii* behavior aided interpretation of the chemical and anatomical data.

RESULTS

Plant Chemistry. GC-MS analysis showed that two compounds, β-myrcene and β-caryophyllene, were the major constituents in extracts of *C. californicus*

TABLE 1. COMPOUNDS PRESENT IN *Croton californicus*

Retention time (min)	Compound	Composition (%)[a]	
		Fruit	Leaves
6.19	3-thujene	2	3
7.21	β-pinene	2	0
7.32	β-myrcene	58	46
8.62	δ-terpinene	3	4
9.96	Unidentified	0	3
11.47	*n*-tridecane	2	5
14.63	β-caryophyllene	23	30
15.14	α-caryophyllene	3	0
15.47	Unidentified sesquiterpene	7	5

[a] Percentages based on total ion area.

(Table 1). Together these terpenoids comprised at least 75% of the volatile compounds identified in fruit and leaves. Small quantities of other terpenoids and an unbranched aliphatic compound were also identified.

Metathoracic Gland Chemistry. The predominant secretory components identified in rinsates of adult *P. stallii* were (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, (*E*)-2-hexenyl acetate, and *n*-tridecane (Table 2). Small quantities of unbranched aliphatic compounds were also present in rinsates of both genders. Aside from males having a greater proportion of (*E*)-2-hexenyl acetate than females, major differences in the relative proportions of chemical constituents were not observed between the genders (Table 2). In females, the most abundant constituents, in order of greatest proportion, were *n*-tridecane, (*E*)-2-hexenal, and (*E*)-4-oxo-2-hexenal. Together these compounds made up approximately 90% of the total volatiles collected from female metathoracic glands. In males, the most abundant constituents, in order of greatest proportion, were *n*-tridecane, (*E*)-2-hexenyl acetate, (*E*)-2-hexenal, and (*E*)-4-oxo-2-hexenal. These constituents comprised 96% of the total volatiles in male metathoracic glands. A possible explanation for the greater relative abundance of (*E*)-2-hexenyl acetate in male glands than in female glands may be that at the time the rinse was made some male bugs in the sample had not converted as much of the ester to the aldehyde as females had.

Results of chemical analyses of dissected metathoracic glands are presented in Table 3. Major differences were not observed in the chemical composition of metathoracic glands between females guarding egg masses and females not guarding egg masses. However, metathoracic gland secretions of males appeared to have a greater proportion of *n*-tridecane than was observed in females. In males, *n*-tridecane was present in MR/AG and LR/ST, but in females this compound was found only in MR/AG. (*E*)-2-Hexenal was present in both MR/AG

TABLE 2. COMPOUNDS PRESENT IN WHOLE-BUG RINSATES OF ADULT *Pachycoris stallii*

Retention time (min)	Compound	Composition (%)[a]		
		Female guarding egg mass	Female not guarding egg mass	Male
3.95	(*E*)-2-hexenal	32	36	20
5.85	(*E*)-4-oxo-2-hexenal	19	15	11
6.84	(*E*)-2-hexenyl acetate	4	trace	21
8.28	*n*-undecane	1	0	0
9.97	*n*-dodecane	4	3	2
11.42	1-tridecene	1	1	1
11.61	*n*-tridecane	38	44	44
13.00	*n*-tetradecane	trace	trace	0
14.38	*n*-pentadecane	1	trace	1

[a] Percentages based on GC peak areas.

and LR/ST of female bugs, but in males was primarily present in the MR/AG. (*E*)-4-Oxo-2-hexenal and *n*-dodecane were isolated from the MR/AG of male and female bugs.

Dorsal Abdominal Gland Chemistry. No volatiles were isolated from dorsal abdominal glands of adult female *P. stallii*. Adult males produced trace amounts of *n*-tridecane in the anterior and median dorsal abdominal glands, but no volatiles were isolated from posterior dorsal abdominal glands. Table 4 presents the volatile components identified from dorsal abdominal glands of first through fourth nymphal instar *P. stallii*. Rinsates of nymphs revealed the presence of (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, *n*-dodecane, *n*-tridecane, and tetradecanal. Of these compounds, (*E*)-4-oxo-2-hexenal comprised the greatest proportion of total constituents in the four nymphal instars tested (Table 4). The proportion of (*E*)-4-oxo-2-hexenal decreased from 72% in the first instar to 47% in the fourth instar. Tetradecanal and *n*-tridecane were present in similar proportions in all instars except the second, and relative proportions of both compounds were greater in the fourth instar than in the first instar.

Gland Anatomy. Metathoracic glands of *P. stallii* were sexually monomorphic. Median reservoirs were bright orange, while lateral reservoirs were translucent and without pigment. Microsculpturing of the integument was present surrounding the metathoracic gland ostia on the mesepisternum and metepisternum (Figure 1). Ostioles of adult anterior dorsal abdominal glands were of the type 1 (undivided ostiole) described by Remold (1962), while median and posterior ostioles were of a gradient between types 1 and 2 (divided ostiole). Adult dorsal abdominal glands were small and apparently obsolescent. These glands were mostly translucent, but with a bit of orange pigment. Anterior dorsal abdominal glands were divided half-glands and were spherical, while median and posterior

TABLE 3. COMPOUNDS OBTAINED FROM DISSECTION OF *Pachycoris stallii* METATHORACIC GLANDS

Retention time (min)	Compound	Composition (%)[a]					
		Female guarding egg mass		Female not guarding egg mass		Male	
		MR/AG[b]	LR/ST[c]	MR/AG[b]	LR/ST[c]	MR/AG[b]	LR/ST[c]
2.76	(*E*)-2-hexenal	31	100	43	100	18	trace
3.92	(*E*)-4-oxo-2-hexenal	4	0	5	0	3	trace
4.75	(*E*)-2-hexenyl acetate	1	0	0	0	1	0
7.52	*n*-dodecane	1	0	0	0	1	0
8.98	*n*-tridecane	63	0	52	0	77	99

[a] Percentages based on GC peak areas.
[b] Median reservoir and accessory gland.
[c] Lateral reservoir and secretory tubules.

TABLE 4. COMPOUNDS PRESENT IN FIRST THROUGH FOURTH NYMPHAL INSTARS OF *Pachycoris stallii* DORSAL ABDOMINAL GLANDS

Retention time (min)	Compound	Composition (%)[a]			
		First instar	Second instar	Third instar	Fourth instar
2.71	(*E*)-2-hexenal	5	1	3	5
3.94	(*E*)-4-oxo-2-hexenal	72	61	52	47
7.51	*n*-dodecane	0	1	0	1
8.99	*n*-tridecane	12	23	22	22
13.15	tetradecanal	11	14	23	25

[a] Percentages based on GC peak areas.

glands were undivided and elongate. All five nymphal instars possessed ostioles of a similar arrangement as adults (anterior, type 1; median and posterior, type 2) (Figure 2). Anterior dorsal abdominal glands of all nymphal instars resembled those of adults in relative size, shape, and color. Compared to the anterior glands, median and posterior dorsal abdominal glands of all nymphal instars were relatively well developed. These glands were elongate translucent swollen sacs, with some orange pigmentation. Scanning electron micrography showed the presence of a polygonal pattern of cuticular microsculpturing associated with the ostioles of dorsal abdominal glands (Figure 3).

DISCUSSION

The most prevalent compounds found in *C. californicus*, β-myrcene and β-caryophyllene, are terpenoids commonly found in higher plants. These compounds are known from several species of *Croton* (Neto et al., 1994; Menut et al., 1995). The function of β-myrcene and β-caryophyllene in *C. californicus* is not known, although they are present in the defensive secretions of some Heteroptera (Gough et al., 1985; Krall et al., 1997). *Pachycoris stallii* may use the relative ratios of these compounds to differentiate between structures on which to feed (fruit) and those for oviposition (leaves). The ratio of β-myrcene to β-caryophyllene in *C. californicus* fruit is 2.5 : 1, while in leaves it is 1.5 : 1. It is also possible that *P. stallii* uses other plant characteristics, such as minor chemical constituents or physical factors (e.g., shape and texture of plant structures), to recognize different plant structures. The presence of *n*-tridecane in fruit and leaves is probably the result of degradation of hydrocarbon components of cuticular waxes (Goodwin and Mercer, 1986).

Results of chemical analyses demonstrated that *P. stallii* employs a carbonyl-based scent gland chemistry [C_6 alkenal (*E*)-2-hexenal, the C_6 4-oxo-

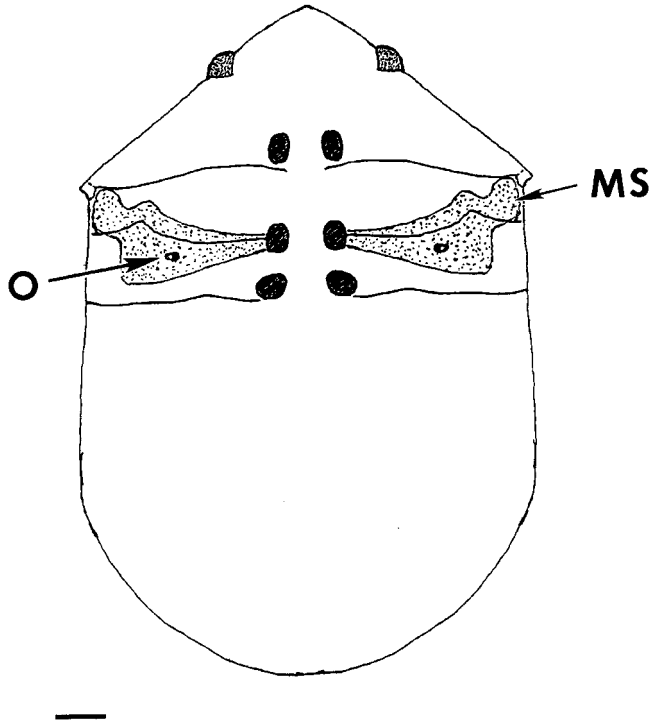


FIG. 1. Ventral view of *Pachycoris stallii* female showing the position of ostia (O) and microsculpturing (MS) on mesepisternum and metepisternum; scale bar = 1 mm.

2-alkenal (*E*)-4-oxo-2-hexenal, and the C_{13} alkane *n*-tridecane], which conforms to the general pattern for other Pentotomoidea (Staddon, 1979; Aldrich, 1988, 1995). As expected, the corresponding ester of (*E*)-2-hexenal, (*E*)-2-hexenyl acetate, was also present (Aldrich, 1988). Dorsal abdominal glands in adults were apparently obsolescent, while those of nymphs produced (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, *n*-dodecane, *n*-tridecane, and the C_{14} alkanal tetradecanal. With the exception of tetradecanal, these compounds are typical of those found in dorsal abdominal glands of Pentotomoidea. Anatomical data indicate that defensive secretions are produced in well-developed metathoracic or dorsal abdominal glands, and field observations indicated that the allomones are delivered via an efferent system that allows the concoction to be squirted (Williams, unpublished observations). Light and electron microscopy revealed the presence of microsculptured cuticle surrounding the ostia of these glands, presumably to aid in the accumulation and evaporation of the secretion (Staddon, 1979).

Current evidence on the cytological sources of the compounds produced

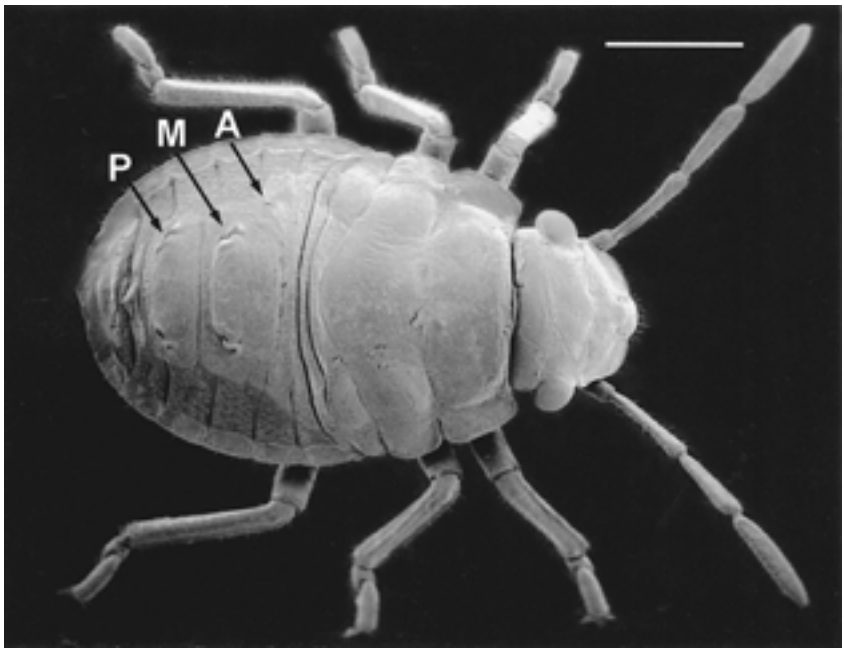


FIG. 2. Scanning electron micrograph of second-instar *Pachycoris stallii* showing arrangement of anterior (A), median (M), and posterior (P) dorsal abdominal gland ostia; scale bar = 1 mm, magnification 18×.

in the metathoracic glands indicates that esters are synthesized in the secretory tubules, and alkanals and related scent carbonyls are generally restricted to the median reservoir (Gilby and Waterhouse, 1967; Aldrich et al., 1978; Everton et al., 1979). Results of the present study conflicted somewhat with this trend. Analyses from dissected metathoracic glands suggest that (*E*)-2-hexenyl acetate is secreted in MR/AG, after which it is converted to (*E*)-2-hexenal, and possibly to (*E*)-4-oxo-2-hexenal, as in other Heteroptera (Staddon, 1979). Small quantities of esters produced in secretory tubules are usually found in the median reservoir (Staddon, 1979), but this does not explain the absence of the ester in the LR/ST extracts in *P. stallii*. It is possible that the bugs from which extracts were prepared had, upon being agitated prior to dissection, used all available (*E*)-2-hexenyl acetate to produce (*E*)-2-hexenal. As expected, (*E*)-2-hexenal was found in extracts of female MR/AG, but was also detected in extracts of LR/ST. This suggests that in *P. stallii* (*E*)-2-hexenal is formed in the lateral reservoir as well as the median reservoir. In male bugs, *n*-tridecane was present in extracts of MR/AG, as expected, but also in extracts of LR/ST, suggesting that *P. stallii* is

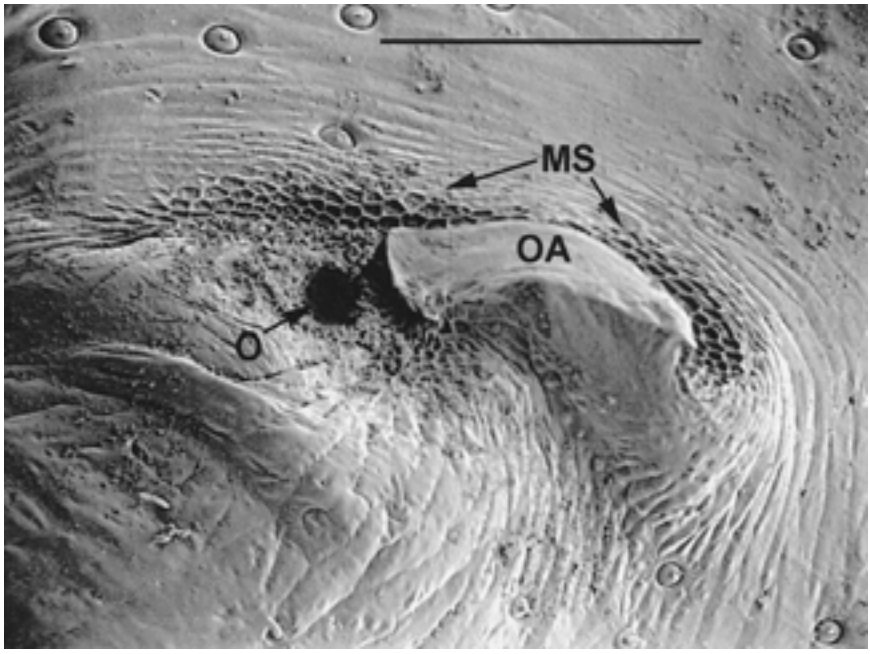


FIG. 3. Scanning electron micrograph of median dorsal abdominal gland of second instar *Pachycoris stallii* showing microsculpturing (MS), ostiole (O), and occlusion arm (OA); scale bar = 0.1 mm, magnification 416×.

capable of producing *n*-tridecane in the secretory tubules and lateral and median reservoirs. Gilby and Waterhouse (1967) detected *n*-tridecane in the secretory tubules of *Nezara viridula* (L.).

Attributing biological significance to the differences between the chemical composition of allomones produced by *P. stallii* adults and nymphs is difficult in the absence of behavioral data. It is possible that the differences are not of great importance, as long as the chemical and physical characteristics of the mixtures result in an effective defense (Tschinkel, 1975). If this is true for *P. stallii*, then the differences may reflect different metabolic costs of synthesis between adults and nymphs. The differences observed may also reflect differences in natural enemies between nymphs and adults (Pasteels et al., 1982). Pasteels et al. (1983) suggested that such differences in composition might be a strategy to avoid counteradaptation by natural enemies. The above explanations may also apply to the observed changes in chemical composition of dorsal abdominal gland secretions between nymphal instars. Behavioral studies under field conditions will help elucidate the roles of scent gland secretions in different life stages of *P. stallii*.

Field observations supported the theoretical prediction that insects will utilize defensive secretions in a frugal manner and may use other defensive ploys before discharging allomones (Wallace and Blum, 1969; Whitman et al., 1990). The first line of defense for *P. stallii* is to run down stems away from the aggressor or drop to the leaf litter under the plant canopy (Williams, unpublished observations). Brooding females remain with their young and use behavioral defenses, such as kicking, towards an aggressor. These females were rarely induced to eject defensive secretions, possibly to avoid poisoning their offspring. Usually, bugs were induced to discharge allomones only after considerable physical contact, such as being pinched with forceps or after being handled. When bugs did discharge gland contents, they demonstrated the ability to squirt them toward an aggressor from a single ostiole, further suggesting that *P. stallii* uses allomones in a conservative manner.

The chemical nature of the scent glands of *P. stallii* suggests that this defensive system is directed at arthropods. The aliphatic aldehydes [e.g., (*E*)-2-hexenal], ketoaldehyde [(*E*)-4-oxo-2-hexenal], and *n*-alkanes (e.g., *n*-tridecane) produced by *P. stallii* are nonspecific irritants, toxins, or olfactory repellents of arthropods (Blum, 1981; Pasteels et al., 1983). Moreover, *n*-tridecane is believed to act as a surfactant and evaporatory retardant, as well as aid in the penetration of toxic aldehydes through an aggressor's cuticle (Staddon, 1979; Gunawardena and Herath, 1991). The nonspecific volatiles produced by *P. stallii* are known to deter ants (Blum, 1961; Wallace and Blum, 1969), and may also mimic alarm pheromones of ants, allowing bugs to escape (Blum, 1980). In the present study, field observations suggested that ants were the primary arthropod predators of *P. stallii*. *Dorymyrmex bicolor* Wheeler was commonly observed patrolling stems and leaves of *C. californicus*, as well as the soil surface, where they attacked nymphs and adults that fell to the soil and were immobilized by high surface temperatures. The ants were sometimes successful in driving brooding female bugs away from egg masses, after which eggs were consumed. Our results suggest that *P. stallii* is an aposematic heteropteran that, despite feeding on a host plant toxic to vertebrates, has maintained a potent exocrine defense system directed at arthropod predators.

Acknowledgments—Thanks are extended to the following individuals for taxonomic determinations: J. E. Eger, Jr. (*P. stallii*) and R. R. Snelling (*D. bicolor*). Technical assistance was provided by F. Bailey, R. N. Paul, and T. M. Roane, and J. R. Aldrich offered technical advice. We are also grateful to V. Manrique and anonymous reviewers for comments on the manuscript.

REFERENCES

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.
ALDRICH, J. R. 1995. Chemical communication in the true bugs and parasitoid exploitation, pp.

318–363, *in* R. T. Cardé and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman & Hall, New York.

ALDRICH, J. R., BLUM, M. S., LLOYD, H. A., and FALES, H. M. 1978. Pentatomid natural products: Chemistry and morphology of the III–IV dorsal abdominal glands of adults. *J. Chem. Ecol.* 4:161–172.

ALDRICH, J. R., AVERY, J. W., LEE, C.-J., GRAF, J. C., HARRISON, D. J., and BIN, F. 1996. Semiochemistry of cabbage bugs (Heteroptera: Pentatomidae: *Eurydema* and *Murgantia*). *J. Entomol. Sci.* 31:172–182.

ALEXANDER, I. C., PASCOE, K. O., MANCHARD, P., and WILLIAMS, L. A. D. 1991. An insecticidal diterpene from *Croton linearis. Phytochemistry* 30:1801–1803.

BLUM, M. S. 1961. The presence of 2-hexenal in the scent gland of the pentatomid *Brochymena quadripustulata. Ann. Entomol. Soc. Am.* 54:410–412.

BLUM, M. S. 1980. Arthropods and ecomones: better fitness through ecological chemistry, pp. 207–222, *in* R. Gilles (ed.). Animals and Environmental Fitness. Academic Press, New York.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

CHAVEZ, P. I., JOLAD, S. D., HOFFMANN, J. J., and COLE, J. R. 1982. Four new 12-deoxyphorbol diesters from *Croton californicus. J. Nat. Prod.* 45:745–748.

DUFFEY, S. S., and SCUDDER, G. C. E. 1972. Cardiac glycosides in North American Asclepiadaceae, a basis for unpalatability in brightly coloured Hemiptera and Coleoptera. *J. Insect Physiol.* 18:63–78.

EISNER, T. 1970. Chemical defense against predation in arthropods, pp. 157–217, *in* E. Sondheimer and J. B. Simeone (eds.). Chemical Ecology. Academic Press, New York.

EVERTON, I. J., KNIGHT, D. W., and STADDON, B. W. 1979. Linalool from the metathoracic scent gland of the cotton stainer *Dysdercus intermedius* Distant (Heteroptera: Pyrrhocoridae). *Comp. Biochem. Physiol. B* 63:157–161.

GILBY, A. R., and WATERHOUSE, D. F. 1967. Secretions from the lateral scent glands of the green vegetable bug, *Nezara viridula. Nature* 216:90–91.

GOODWIN, T. W., and MERCER, E. I. 1986. Introduction to Plant Biochemistry. Pergamon Press, New York.

GOUGH, A. J. E., HAMILTON, J. G. C., GAMES, D. E., and STADDON, B. W. 1985. Multichemical defense of plant bug *Hotea gambiae* (Westwood) (Heteroptera: Scutelleridae): Sesquiterpenoids from abdominal gland in larvae. *J. Chem. Ecol.* 11:342–352.

GUNAWARDENA, N. E., and HERATH, H. M. W. K. B. 1991. Significance of medium chain *n*-alkanes as accompanying compounds in hemipteran defensive secretions: An investigation based on the defensive secretion of *Coridius janus. J. Chem. Ecol.* 17:2449–2458.

KRALL, B. S., ZILKOWSKI, B. W., KNIGHT, S. C., BARTELT, R. J., and WHITMAN, D. W. 1997. Chemistry and defensive efficacy of secretion of burrowing bug (*Sehirus cinctus cinctus*). *J. Chem. Ecol.* 23:1951–1962.

KRALL, B. S., BARTELT, R. J., LEWIS, C. J., and WHITMAN, D. W. 1999. Chemical defense in the stink bug *Cosmopepla bimaculata. J. Chem. Ecol.* 25:2477–2494.

MENUT, C., LAMATY, G., BESSEIRE, J. M., SEULEIMAN, A. M., FENDERO, P., MAIDOU, E., and DENAMGANAI, J. 1995. Aromatic plants of tropical Central Africa. XXII. Volatile constituents of *Croton aubrevillei* J. Leonard and *C. zambesicus* Muell. Arg. *J. Essent. Oil Res.* 7:419–422.

NETO, M. A., DE ALENCAR, J. W., CUNHA, A. N., and SILVEIRA, E. R. 1994. Volatile constituents of *Croton lundianus* (F. Diedr.) Muell. and *C. glandulosus* (L.) Muell. *J. Essent. Oil Res.* 6:191–193.

PASTEELS, J. M., BRAEKMAN, J. C., DALOZE, D., and OTTINGER, R. 1982. Chemical defence in chrysomelid larvae and adults. *Tetrahedron* 38:1891–1897.

PASTEELS, J. M., GRÉGOIRE, J.-C., and ROWELL-RAHIER, M. 1983. The chemical ecology of defense in arthropods. *Annu. Rev. Entomol.* 28:263–289.

REMOLD, H. 1962. Über die biologische Bedeutung der Duftdrüsen bei den Landwanzen (Geocorisae). *Z. Vergl. Physiol.* 45:636–694.

SHREVE, F., and WIGGINS, I. L. 1964. Vegetation and Flora of the Sonoran Desert (2 vols.). Stanford University Press, Stanford, California.

STADDON, B. W. 1979. The scent glands of Heteroptera. *Adv. Insect Physiol.* 14:351–418.

STADDON, B. W., THORNE, M. J., and KNIGHT, D. W. 1987. The scent glands and their chemicals in the aposematic cotton harlequin bug, *Tectocoris diophthalmus* (Heteroptera: Scutelleridae). *Aust. J. Zool.* 35:227–234.

TSCHINKEL, W. R. 1975. A comparative study of the chemical defensive system of tenebrionid beetles: Chemistry of the secretions. *J. Insect Physiol.* 21:753–783.

UPADHYAY, R. R., and HECKER, E. 1976. A new cryptic irritant and cocarcinogen from seeds of *Croton sparciflorus. Phytochemistry* 15:1070–1072.

WALLACE, J. B., and BLUM, M. S. 1969. Refined defensive mechanisms in *Chrysomela scripta. Ann. Entomol. Soc. Am.* 62:503–506.

WHITMAN, D. W., BLUM, M. S., and ALSOP, D. W. 1990. Allomones: Chemicals for defense, pp. 289–351, *in* D. L. Evans and J. O. Schmidt (eds.). Insect Defenses: Adaptive Mechanisms and Strategies of Prey and Predators. State University of New York Press, Albany, New York.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

ANGIOSPERM BARK VOLATILES DISRUPT RESPONSE OF DOUGLAS-FIR BEETLE, *Dendroctonus pseudotsugae*, TO ATTRACTANT-BAITED TRAPS

DEZENE P. W. HUBER* and JOHN H. BORDEN

Centre for Environmental Biology
Department of Biological Sciences
Simon Fraser University
8888 University Drive
Burnaby, British Columbia, Canada, V5A 1S6

(Received March 13, 2000; accepted September 29, 2000)

Abstract—Antennally active, bark-derived, angiosperm volatiles were tested singly and in groups for their ability to disrupt the response of the Douglas-fir beetle (DFB), *Dendroctonus pseudotsugae*, to attractant-baited multiple-funnel traps. One compound, conophthorin, was active alone in reducing the response of beetles to the baited traps. Further experiments showed disruptive activity in two aliphatic green-leaf alcohols [1-hexanol and (*Z*)-3-hexen-1-ol], as well as guaiacol and benzyl alcohol, and three aliphatic aldehydes [nonanal, hexanal, and (*E*)-2-hexenal] but not in two aromatic aldehydes (benzaldehyde and salicylaldehyde). Every binary combination that included conophthorin or any two of the other groups, except aromatic aldehydes, significantly reduced the response of beetles to baited traps. Various ternary mixtures and the complete mixture of all the groups were generally the most effective treatments. These results provide evidence that DFBs recognize and avoid nonhosts while flying rather than landing on candidate hosts and testing them while in contact with the tree. Nonhost angiosperm bark volatiles may have practical utility on their own or in combination with the antiaggregation pheromone 3-methylcyclohex-3-en-1-one (MCH) to protect single trees, logs, or stands from attack by the DFB.

Key Words—Douglas-fir beetle, *Dendroctonus pseudotsugae*, Coleoptera, Scolytidae, nonhost volatiles, green-leaf volatiles, disruptant, 3-methylcyclohex-3-en-1-one, MCH.

*To whom correspondence should be addressed.

0098-0331/01/0200-0217$19.50/0

INTRODUCTION

The Douglas-fir beetle (DFB), *Dendroctonus pseudotsugae* (Coleoptera: Scolytidae), is a destructive pest of Douglas fir, *Pseudotsuga menziesii*, forests throughout western North America. Its ability to breed in recently felled trees and slash allows populations to build up in areas of active logging or blown-down timber (Humphreys, 1995). In the first warm days of spring, brood beetles emerge and may mass attack and kill surrounding standing Douglas-fir trees (McMullen and Atkins, 1962). Prevention of DFB outbreaks in managed forests demands the prompt removal of slash following logging and sanitation logging to remove infested standing trees (Humphreys, 1995). However, these practices are not always convenient, feasible, or ecologically sensible. One alternative in the United States is to use the newly registered antiaggregation pheromone, 3-methylcyclohex-3-en-1-one (MCH) that has been shown to be efficacious in deterring beetle attack on susceptible timber (Ross et al., 1996). We hypothesized that supplements or alternatives to MCH might be found in the application of repellent nonhost angiosperm bark volatiles.

DFBs are highly host specific and do not attack angiosperm trees. A number of studies have shown that portions of nonhosts (Schroeder, 1992), as well as individual chemicals or blends of chemicals known to occur in angiosperm tissues (Dickens et al., 1992; Wilson et al., 1996; Borden et al., 1997, 1998; Byers et al., 1998; Deglow and Borden, 1998a,b; Poland et al., 1998; Poland and Haack, 1999; Huber et al., 1999), are active in reducing attraction of coniferophagous bark and ambrosia beetles to their aggregation pheromones or host kairomones. Coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses have revealed a variety of nonhost angiosperm bark volatiles that elicit antennal responses in female DFBs (Huber et al., 2000). We report here the results of experiments testing these EAD-active compounds for their ability to deter the response of DFBs to traps baited with the attractant semiochemicals ethanol (Ross and Daterman, 1995), frontalin (Ross and Daterman, 1995) and 1-methylcyclohex-2-en-1-ol (MCOL) (Lindgren et al., 1992).

METHODS AND MATERIALS

Field trapping experiments were conducted approximately 18 km north of Lytton, British Columbia, Canada. The stand at the site was a mixture of mostly decadent Douglas fir, *Pseudotsuga menziesii*, and ponderosa pine, *Pinus ponderosa*, and much of the area had been recently logged. All experiments utilized 12-unit multiple funnel traps (Lindgren, 1983) (Phero Tech Inc., Delta, British Columbia) placed 15–20 m apart in a line along a logging road in randomized complete blocks. Release devices and rates for components used in attractive

baits (DFB lure), as well as synthetic nonhost volatiles that were tested for repellency, are given in Table 1. A small block of Vapona No-Pest Strip (Green Cross, Fisons Horticulture Inc., Mississauga, Ontario, Canada) was placed in each collecting cup so that captured predaceous beetles did not consume captured bark beetles.

Experiment 1 tested 20 EAD-active compounds individually for activity in reducing attraction of DFBs to attractant-baited traps. One other compound, conophthorin, was already known to be repellent to DFBs (Huber et al., 1999). The treatments were unbaited traps, traps baited with the three-component DFB lure (Table 1), or traps baited with the DFB lure plus either benzyl alcohol, benzaldehyde, 3-carene, decanal, guaiacol, heptanal, hexanal, 1-hexanol, 2-hexanone, 3-hexanone, (*E*)-2-hexenal, limonene, methyl salicylate, nonanal, octanoic acid, phenylacetaldehyde, α-pinene, β-pinene, salicylaldehyde, or toluene or a mixture of all of the above compounds in equal proportions (equal-ratio blend; Table 1). The experiment comprised 12 replicates carried out two or four replicates at a time between May 7–9, 7–12, 9–12, and 12–15, 1997, and with bait positions rerandomized between replicates.

Experiment 2 tested the effect of (*E*)-ocimene with three treatments: unbaited traps and traps baited with the DFB lure alone or with ocimene (72 *E*-isomer). Fifteen replicates were run simultaneously from April 24 to 29, 1998.

Experiments 3 and 4 tested three groups of synthetic nonhost compounds: (1) four green-leaf volatiles (GLVs) [hexanal, (*E*)-2-hexenal, 1-hexanol, and (*Z*)-3-hexen-1-ol]; (2) six volatiles (salicylaldehyde, conophthorin, guaiacol, nonanal, benzaldehyde, and benzyl alcohol) that each elicited antennal responses from ≥3 of the five scolytid species tested by Huber et al. (2000)[1]; and (3) seven volatiles [4-allylanisole, heptanal, (*E*)-2-heptenal, methyl salicylate, decanal, thymol methylether, and (*E*)-nerolidol] that elicited antennal responses from fewer than three of the five scolytid species. The six treatments in experiment 3 were: unbaited traps, traps baited with the DFB lure, and traps baited with the DFB lure plus the GLVs in group 1, the volatiles in group 2, the volatiles in group 3, or all the volatiles in groups 1–3. In experiment 4 the three groups of nonhost volatiles were tested in all three binary combinations and in the ternary combination with the following treatments: unbaited traps, traps baited with the DFB lure, traps baited with the DFB lure plus volatiles in groups 1 and 2, 1 and 3, 2 and 3, or 1–3. Fifteen replicates in each experiment were run from May 5 to 7, 1998 (experiment 3), and May 7–11, 1998 (experiment 4). Most of the volatiles in experiments 3 and 4 were tested as mixtures in common release devices (Table 1). No monoterpenes were included in experiments 3 and 4, as many such compounds are known to attractant various coniferophagous Scolytidae.

[1]Beetles tested in addition to the DFB were: *Dendroctonus ponderosae*, *D. rufipennis*, *Dryocoetes confusus*, and *Ips pini*.

TABLE 1. DESCRIPTION OF CHEMICALS AND RELEASE DEVICES UTILIZED IN TRAPPING EXPERIMENTS

Volatile category and compound[a]	Source	Purity (%)	Release device and experiment number	Release rate (mg/24 hr) and temperature
DFB lure				
Frontalin (racemic)	Phero Tech[b]	>99	0.4 ml PE[c] vial, closed (all experiments)	2.6 at 23°C
Ethanol	Phero Tech	95	40 cm Phero Tech pouch (all experiments)	40 at 20°C
MCOL	Phero Tech	98	Phero Tech bubblecap (all experiments)	2.0 at 20°C
Equal-ratio blend	Various		1.5 ml PP[c] vial, open (Exp. 1)	39 at 28°C
4-Allylanisole	Sigma[d]	98	15 ml PE bottle also containing heptanal, (*E*)-2-heptenal, methyl salicylate, decanal, and (*E*)-nerolidol (Exp. 3, 4)	3.8 at 25°C
Benzyl alcohol	Fisher[e]	98	1.5 ml PP vial, open (Exp. 1)	6 at 28°C
			15 ml Phero Tech bubblecap (Exp. 3–8)	1.3 at 20°C
Benzaldehyde	Fisher	>99	0.6 PP vial, open (Exp. 1)	15 at 28°C
			15 ml PE bottle also containing guaiacol, nonanal, and salicylaldehyde (Exp. 3, 4)	1.7 at 25°C
			Phero Tech flexlure (Exp. 5–10)	3.5 at 20°C
3-Carene	Sigma	90	0.6 ml PP vial, open (Exp. 1)	31 at 28°C
Conophthorin (racemic)	S.F.U.[f]	87	0.4 ml PE vial, closed (Exp. 3–10)	3.0 at 28°C
Decanal	Sigma	95	1.5 ml PP vial, open (Exp. 1)	11 at 28°C
			15 ml PE bottle also containing heptanal, (*E*)-2-heptenal, methyl salicylate, 4-allylanisole, and (*E*)-nerolidol (Exp. 3, 4)	2.5 at 25°C
Guaiacol	Sigma	>98	1.5 ml PP vial, open (Exp. 1)	19 at 28°C
			15 ml PE bottle also	1.9 at 25°C

TABLE 1. CONTINUED

Volatile category and compound[a]	Source	Purity (%)	Release device and experiment number	Release rate (mg/24 hr) and temperature
			containing benzaldehyde, salicylaldehyde, and nonanal (Exp. 3, 4)	
			Phero Tech bubblecap (Exp. 5–10)	5.0 at 20°C
Heptanal	Sigma	95	1.5 ml PP vial, open (Exp. 1)	27 at 28°C
			15 ml PE bottle also containing decanal, (*E*)-2-heptenal, methyl salicylate, 4-allylanisole, and (*E*)-nerolidol (Exp. 3, 4)	4.0 at 25°C
(*E*)-2-Heptenal	Bedoukian[g]	95	15 ml PE bottle also containing decanal, heptenal, methyl salicylate, 4-allylanisole, and (*E*)-nerolidol (Exp. 3, 4)	3.7 at 25°C
Hexanal	Sigma	98	0.4 ml PE vial, open (Exp. 1)	18 at 28°C
			15 ml PE bottle also containing 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexenal (Exp. 3, 4)	8.2 at 25°C
			Phero Tech flexlure (Exp. 5–10)	3.0 at 20°C
1-Hexanol	Sigma	98	0.6 ml PP vial, open (Exp. 1)	23 at 28°C
			15 ml PE bottle also containing hexanal, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexenal (Exp. 3, 4)	2.8 at 25°C
			Phero Tech bubblecap (Exp. 5–10)	2.5 at 20°C
2-Hexanone	Sigma	98	0.4 ml PE vial, open (Exp. 1)	28 at 28°C
3-Hexanone	Sigma	98	0.4 ml PE vial, open (Exp. 1)	32 at 28°C

TABLE 1. CONTINUED

Volatile category and compound[a]	Source	Purity (%)	Release device and experiment number	Release rate (mg/24 hr) and temperature
(*E*)-2-Hexenal	Bedoukian	98	0.6 ml PP vial, open (Exp. 1)	10 at 28°C
			15 ml PE bottle also containing hexanal, (*Z*)-3-hexen-1-ol, and 1-hexanol (Exp. 3, 4)	34 at 25°C
			Phero Tech flexlure (Exp. 5–10)	3.5 at 20°C
(*Z*)-3-Hexen-1-ol	Bedoukian	98	15 ml PE bottle also containing hexanal, hexanol, and (*E*)-2-hexenal (Exp. 3, 4)	3.7 at 25°C
			Phero Tech bubblecap (Exp. 5–10)	3.0 at 20°C
Limonene (racemic)	Sigma	96	0.6 ml PP vial, open (Exp. 1)	31 at 28°C
Methyl salicylate	Sigma	98	1.5 ml PP vial, open (Exp. 1)	17 at 28°C
			15 ml PE bottle also containing decanal, (*E*)-2-heptenal, heptanal, 4-allylanisole, and (*E*)-nerolidol (Exp. 3, 4)	3.6 at 25°C
(*E*)-Nerolidol (racemic)	Bedoukian	>97	15 ml PE bottle also containing decanal, (*E*)-2-heptenal, heptanal, 4-allylanisole, and methyl salicylate (Exp. 3, 4)	0.1 at 25°C
Nonanal	Sigma	95	1.5 ml PP vial, closed (Exp. 1)	31 at 28°C
			15 ml PE bottle also containing benzaldehyde, salicylaldehyde and guaiacol (Exp. 3, 4)	0.8 at 25°C
			Phero Tech flexlure (Exp. 5–10)	3.5 at 20°C
Ocimene (72% *E* isomer)	IFF[h]	>95	0.4 ml PE vial, closed (Exp. 2)	32 at 28°C
Octanoic acid	Sigma	99.5	1.5 ml PP vial, open (Exp. 1)	1 at 28°C
Phenylacet-aldehyde	Sigma	>90	1.5 ml PE vial, open (Exp. 1)	26 at 28°C

TABLE 1. CONTINUED

Volatile category and compound[a]	Source	Purity (%)	Release device and experiment number	Release rate (mg/24 hr) and temperature
α-Pinene (racemic)	Sigma	98	0.6 ml PP vial, open (Exp. 1)	65 at 28°C
β-Pinene [99% (−)-enantiomer]	Sigma	99	0.6 ml PP vial, open (Exp. 1)	38 at 28°C
Salicylaldehyde	Sigma	99	1.5 ml PP vial, open (Exp. 1)	45 at 28°C
			15 ml PE bottle also containing benzaldehyde, nonanal, and guaiacol (Exp. 3, 4)	1.5 at 25°C
			Phero Tech bubblecap (Exp. 5–10)	5.0 at 20°C
Thymol methylether	S.F.U.	98	0.4 ml PE vial, closed (Exp. 3, 4)	2.7 at 28°C
Toluene	Sigma	>99	0.4 ml PE vial, closed (Exp. 1)	120 at 28°C

[a] I.U.P.A.C. names if different than trivial name in table: 4-allylanisole is 1-methoxy-4-(2-propenyl) benzene; conophthorin is (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane; frontalin is 1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane; guaiacol is 2-methoxyphenol; limonene is *p*-mentha-1,8-diene; MCOL is 1-methylcyclohex-2-en-1-ol; methylsalicylate is methyl 2-hydroxybenzoate; (*E*)-nerolidol is (*E*)-3,7,11-trimethyl-1,6,10-dodecatrien-3-ol; ocimene is 3,7-dimethyl-1,3,6-octatriene; α-pinene is 2-pinene; β-pinene is 2(10)-pinene; salicylaldehyde is 2-hydroxybenzaldehyde; thymol methylether is 1-isopropyl-2-methoxy-4-methylbenzene.
[b] Phero Tech, 7572 Progress Way, Delta, British Columbia, V4G 1E9.
[c] PE = polyethylene, PP = polypropylene.
[d] Sigma-Aldrich Canada Ltd., 2149 Winston Park Drive, Oakville, Ontario, L6H 6J8.
[e] Fisher Scientific Ltd., 112 Colonnade Road, Nepean, Ontario, K2E 7L6.
[f] Synthesized by H. D. Pierce, Jr., and E. Czyzewska, Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, V5A 1S6.
[g] Bedoukian Research, Inc., 21 Finance Drive, Danbury, Connecticut 06810.
[h] International Flavors and Fragrances Inc. 521 West 57th St., New York, New York 10019.

All volatiles in experiments 5–10 were tested in separate release devices for each compound (Table 1). Experiments 5 and 6 tested the active synthetic compounds from experiments 3 and 4 in groups based on their chemical functionalities: (1) the bicyclic acetal, conophthorin, alone; (2) aldehydes [salicylaldehyde, benzaldehyde, hexanal, (*E*)-2-hexenal, and nonanal], and (3) alcohols and phenols [benzyl alcohol, (*Z*)-3-hexen-1-ol, 1-hexanol, and the phenol, guaiacol]. The treatments in both experiments included unbaited traps and traps baited with the DFB lure. In experiment 5 the other treatments were traps baited with the DFB lure plus the volatiles in groups 1, 2, 3, or 1–3. In experiment 6 the other treat-

ments were the DFB lure plus the volatiles in groups 1 and 2, 1 and 3, 2 and 3, or 1–3. Experiment 5 was run from April 24 to 26, 1999, and experiment 6 was run from April 26 to 30, 1999. Both had 15 replicates.

Experiment 7 tested the nonhost alcohols from experiments 5 and 6 in five treatments: unbaited traps, traps baited with the DFB lure and traps baited with the DFB lure plus aliphatic alcohols [1-hexanol and (*Z*)-3-hexen-1-ol], plus benzyl alcohol and guaiacol, or plus the aliphatic alcohols, benyl alcohol, and guaiacol all together. The experiment ran from May 14 to 22, 1999, with 17 replicates.

Experiment 8 tested the two groups of compounds from experiment 7 in binary combinations with each other and with conophthorin. The six treatments were: unbaited traps, traps with the DFB lure, and traps with the DFB lure plus aliphatic alcohols and benzyl alcohol plus guaiacol, aliphatic alcohols and conophthorin, benzyl alcohol plus guaiacol and conophthorin, or aliphatic alcohols, benzyl alcohol plus guaiacol and conophthorin all together. The experiment ran from May 22 to 24, 1999, with 15 replicates.

Experiment 9 tested the nonhost aldehydes from experiments 5 and 6 in five treatments: unbaited traps, traps baited with the DFB lure, and traps baited with the DFB lure plus either aromatic aldehydes [salicylaldehyde and benzaldehyde], aliphatic aldehydes [hexanal, (*E*)-2-hexenal, and nonanal], or both aldehyde groups together. The experiment ran from April 30–May 14, 1999, with 17 replicates.

Experiment 10 tested the aldehyde groups from experiment 9 in binary combinations with each other and with conophthorin. The six treatments were: unbaited traps, traps with the DFB lure, and traps with the DFB lure plus aromatic aldehydes and conophthorin, aliphatic aldehydes and conophthorin, aromatic and aliphatic aldehydes, or aromatic aldehydes, aliphatic aldehydes and conophthorin. The experiment ran from May 24 to 26, 1999, with 15 replicates.

Captured beetles were frozen until sexed and counted. Data were transformed by $\log_{10}(x + 1)$ (Zar, 1984) to meet the assumptions of homoscedasticity and normality and were analyzed by ANOVA followed by comparison of means by the Ryan-Einot-Gabriel-Welsch Multiple Q-test (REGWQ procedure) (SAS Institute Inc., 1988; Day and Quinn, 1989) by using SAS software (GLM procedure) (SAS Institute, Inc., 1988). In all cases $\alpha = 0.05$. Due to the large number of treatments, experiment 1 was best represented with descriptive statistics; no further statistical analysis was completed.

RESULTS

Of the 20 individual compounds tested in experiment 1, only 1-hexanol and benzyl alcohol appeared to weakly reduce attraction of DFBs to the attractant bait, a result similar to that obtained with the mixture of all compounds together (Figure 1). Some compounds appeared to enhance the attractiveness of the bait,

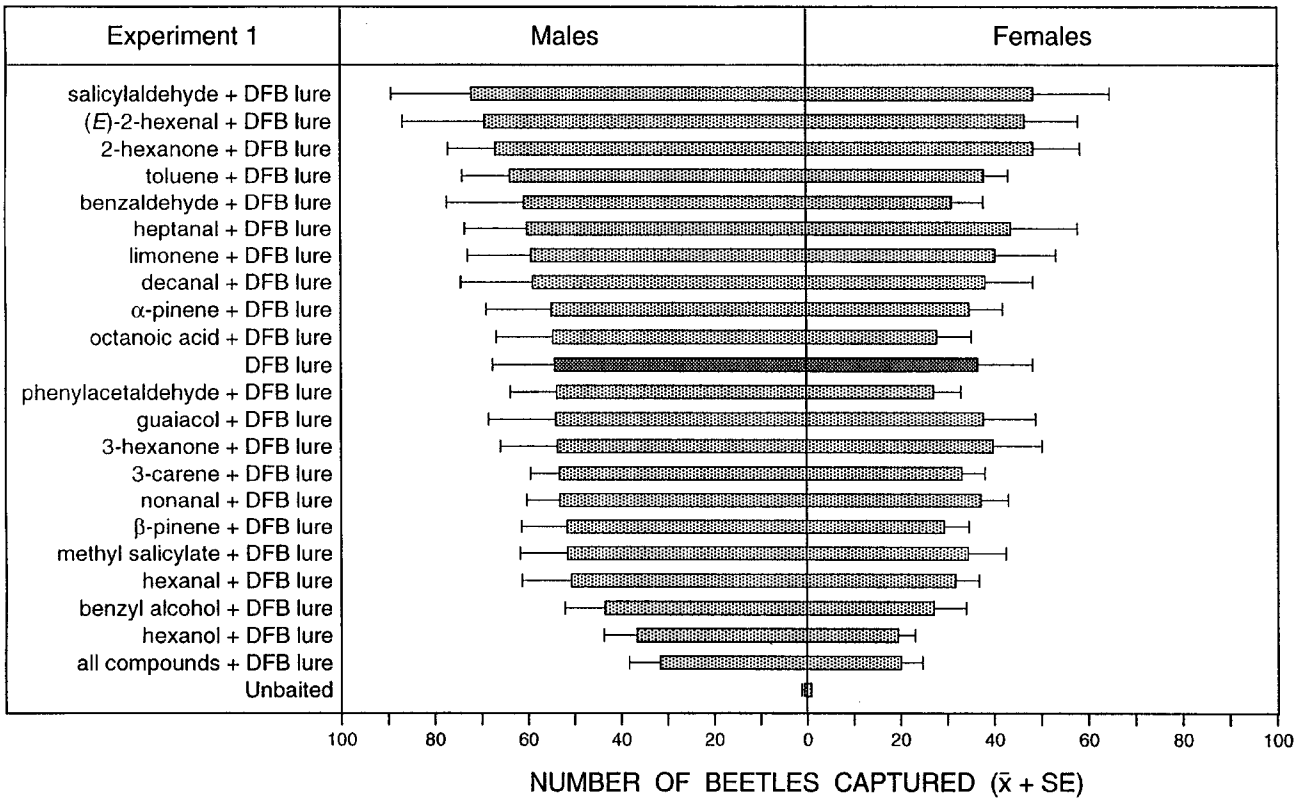


FIG. 1. Catches of *Dendroctonus pseudotsugae* in multiple-funnel traps baited with DFB lure alone or with single or combined nonhost bark volatiles in experiment 1, $N = 12$, May 7–15, 1997, near Lytton, British Columbia.

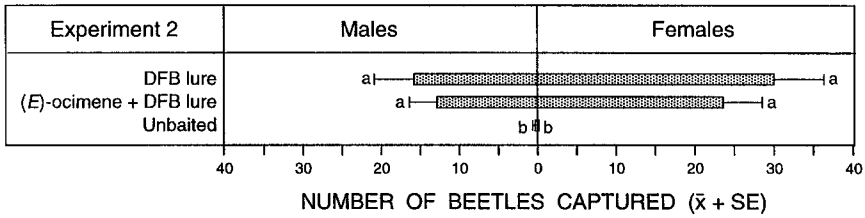


FIG. 2. Catches of *Dendroctonus pseudotsugae* in multiple-funnel traps baited with DFB lure alone or with ocimene [72% (*E*)-isomer] in experiment 2, $N = 15$, April 24–29, 1998, near Lytton, British Columbia. Bars for each sex with the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$).

while most appeared to be benign. (*E*)-Ocimene did not significantly reduce the attraction of DFB to attractant-baited traps in experiment 2 (Figure 2).

In experiment 3, the GLVs reduced attraction of female DFBs, and the group of volatiles that elicited antennal responses from ≥3 scolytid species reduced attraction of both sexes (Figure 3). The combination of all three groups of compounds had the greatest disruptant effect, reducing catch to 13.5% and 11.2% of that in the attractant-baited control traps for males and females, respectively. Each of the binary combinations of the above groups tested in experiment 4 significantly reduced the attraction of both sexes of beetles to attractant-baited traps, as did the ternary blend, which reduced the total trap catch to 9.0% and 11.2% of the attractant-baited control traps in males and females, respectively (Figure 3).

Each treatment in experiment 5 reduced attraction of both sexes to attractant-baited traps, including conophthorin, which was active alone (Figure 4). The ternary combination of aldehydes, alcohols, and conophthorin was the most disruptive, reducing catch to 6.1% and 7.5% of that in attractant-baited control traps for males and females, respectively. Each of the binary combinations in experiment 6 reduced attraction of both sexes to attractant-baited traps (Figure 4). The ternary blend again resulted in the lowest trap catches, reducing trap catch to 7.4% and 4.1% of that in attractant-baited traps for males and females, respectively. Trap catch of female DFB was reduced to a level not significantly different from that in the unbaited control traps.

In experiment 7, benzyl alcohol plus guaiacol reduced the attraction of males to attractant-baited traps, and the aliphatic alcohols, 1-hexanol and (*Z*)-3-hexen-1-ol, reduced attraction of both sexes to attractant-baited traps (Figure 5). The treatment consisting of both groups reduced the catch to 23.4% and 24.9% of the attractant-baited control traps for males and females, respectively. Each of the two groups tested with conophthorin, and the two groups together reduced

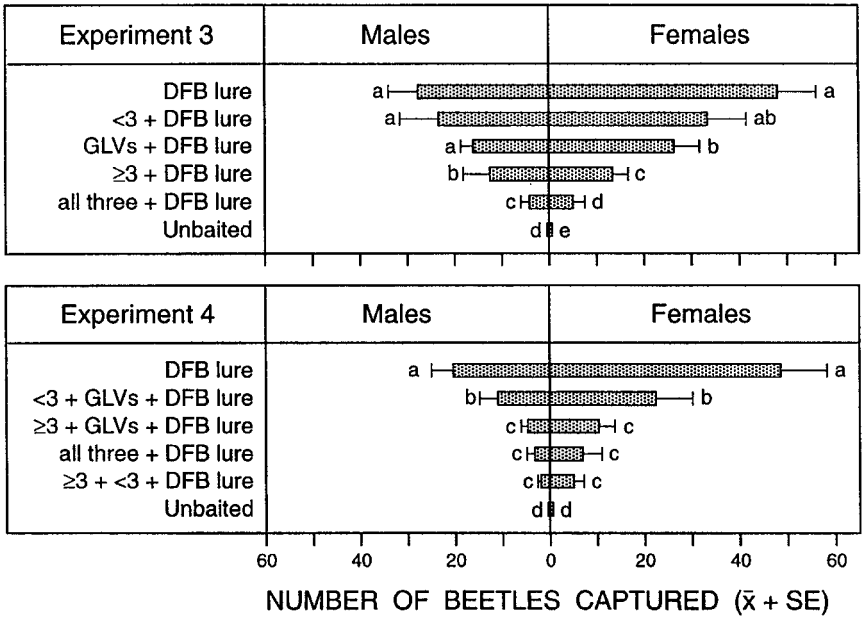


FIG. 3. Catches of *Dendroctonus pseudotsugae* in multiple-funnel traps baited with DFB lure alone or with three blends of nonhost compounds, tested singly in experiment 3 (top) and in binary and ternary combination in experiment 4 (bottom). The blends were green-leaf volatiles (GLVs), compounds eliciting antennal responses from at least three of five species of scolytids previously tested in electroantennogram studies (≥3), and compounds eliciting antennal responses from fewer than three of the five species tested (<3). Experiment 3, $N = 15$, May 5–7, 1998; experiment 4, $N = 15$, May 7–11, 1998, both near Lytton, British Columbia. Bars within each experiment and sex with the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$).

attraction of both sexes to attractant-baited traps in experiment 8 and the blend of all compounds reduced trap catch to 29.4% and 21.8% of the attractant-baited control traps for males and females, respectively (Figure 5).

Only the aliphatic aldehydes, hexanal, (*E*)-2-hexenal, and nonanal, reduced attraction of both sexes of DFBs in experiment 9, reducing trap catch to 36.4% and 37.9% of that in attractant-baited control traps for males and females, respectively (Figure 6). The aromatic aldehyde treatment, salicylaldehyde and benzaldehyde, was inactive alone, but appeared to modify the disruptant effect of the aliphatic aldehydes when both groups were combined. Combining the two groups of aldehydes with each other or with conophthorin resulted in treatments that significantly reduced attraction of both sexes to attractant-baited traps in experiment 10 (Figure 6). The aliphatic aldehydes plus conophthorin reduced

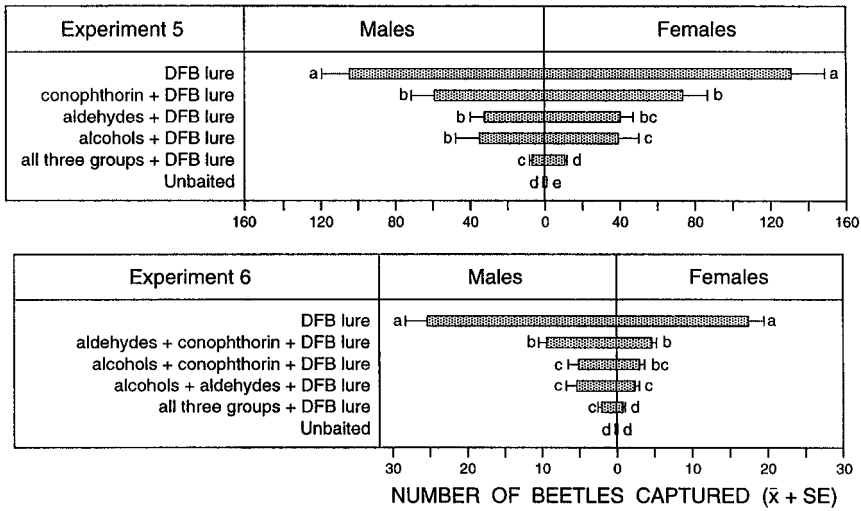


FIG. 4. Catches of *Dendroctonus pseudotsugae* in multiple-funnel traps baited with DFB lure alone or with the nonhost volatile, conophthorin, an aldehyde blend, and an alcohol blend tested singly in experiment 5 (top) and in binary and ternary combination in experiment 6 (bottom). Experiment 5, $N = 15$, April 24–26, 1999; experiment 6, $N = 15$, April 26–30, 1999, both near Lytton, British Columbia. Bars within each experiment and sex with the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$).

the catch to 38.6% and 29.1% of that in the attractant-baited control traps for males and females, respectively.

DISCUSSION

We have shown that a variety of compounds known to be present in the bark of nonhost angiosperm trees strongly reduce attraction of DFBs to attractant semiochemicals. This is the first report of blends of angiosperm-derived compounds having such an effect on the DFB, and builds on the evidence of disruption caused by the known antiaggregation pheromone, MCH (Rudinsky et al., 1972; Ross et al., 1996) and the apparently ubiquitous spiroketal, conophthorin (Huber et al., 1999). Conophthorin is a known pheromone for other insects (Francke et al., 1978), including scolytids (Kohnle et al., 1992; Dallara et al., 1994; Pierce et al., 1995; Birgersson et al., 1995), but it is also found in *Acer*, *Betula*, *Populus*, and *Salix* spp. trees (Byers et al., 1998; Huber et al., 1999; R. Gries and C. Broberg, personal communication). It generally has a repellent or disruptive effect.

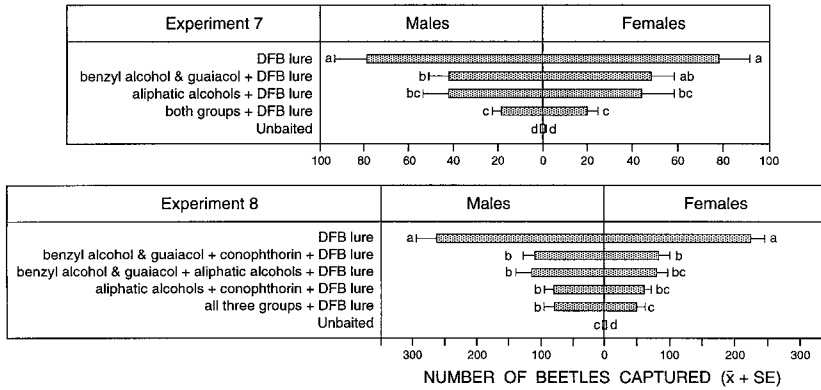


FIG. 5. Catches of *Dendroctonus pseudotsugae* in multiple-funnel traps baited with DFB lure alone or with blends of aliphatic alcohols or benzyl alcohol plus guaiacol tested singly in experiment 7 (top) and in binary and ternary combinations with each other and with conophthorin in experiment 8 (bottom). Experiment 7, $N = 17$, May 14–22, 1999; experiment 8, $N = 15$, May 22–24, 1999, both near Lytton, British Columbia. Bars within each experiment and sex with the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$).

The monoterpene, (*E*)-ocimene, was tested alone in experiment 2 because it had not been included in the initial screening of compounds in experiment 1. It was not tested further, nor were any of the other monoterpenes, many of which are known to be common host conifer volatiles (Norin, 1996; Persson et al., 1996; Sjödin et al., 1996; Wang et al., 1997; Wibe et al., 1998). Although there is some possibility that (*E*)-ocimene or another monoterpene might augment the bioactivity of the most active nonhost volatiles in experiments 3–10, the strong disruptant effects that we obtained in this study are good evidence that the nonhost message can be obtained in the absence of these compounds. Additionally, several compounds tested in experiment 1 were not tested in further experiments, because additional GC-EAD and mass spectrometric data cast some doubt on their authenticity.

The only group of two or more of the EAD-active compounds tested in experiments 3–10 (Table 1) that did not reduce attraction of DFB to baited traps was the aromatic aldehyde group, salicylaldehyde and benzaldehyde (Figure 6). Different blends of compounds had similar disruptive effects, and the highest degree of disruption occurred when the largest number of compounds were grouped together, for example in experiments 5 and 6. This redundant and additive effect is similar to, but more complex than, that found by Borden et al. (1998) for the mountain pine beetle among 1-hexanol, benzyl alcohol, benzaldehyde, and nonanal.

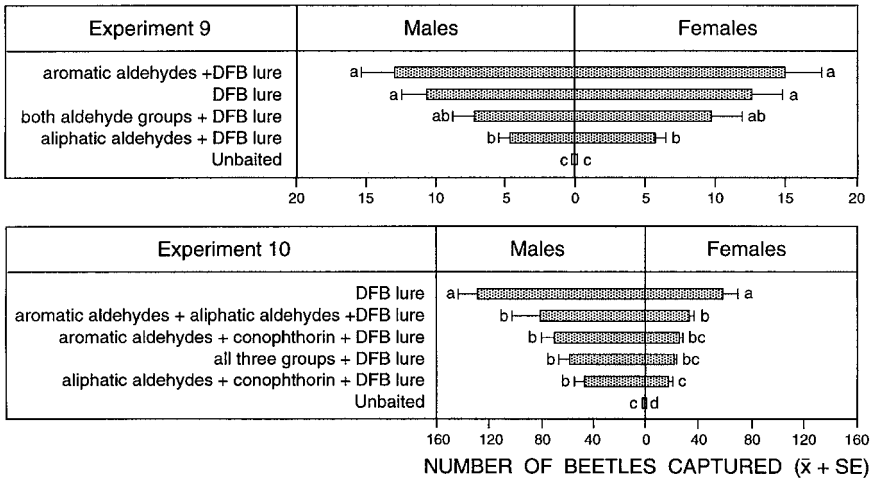


FIG. 6. Catches of *Dendroctonus pseudotsugae* in multiple-funnel traps baited with DFB lure alone or with blends of aliphatic or aromatic nonhost aldehydes tested singly in experiment 9 (top) and in binary and ternary combinations with each other and with conophthorin in experiment 10 (bottom). Experiment 9, $N = 17$, April 30–May 14, 1999; experiment 10, $N = 15$, May 24–26, 1999, both near Lytton, British Columbia. Bars within each experiment and sex with the same letter are not significantly different (Ryan-Einot-Gabriel-Welsch Multiple Q-test, $P < 0.05$).

These effects are not always evident in studies of responses by conifer-ophagous bark beetles to angiosperm volatiles (e.g., Dickens et al., 1991; Byers et al., 1998). However, it is probable that testing of varied combinations of EAD-active nonhost volatiles would reveal such effects to be common in the conifer-ophagous Scolytidae. This is likely for two reasons. A beetle that was too sensitive to any one nonhost volatile would presumably be repelled from such a wide area that it could miss suitable hosts that grew in close proximity to nonhost angiosperm trees. The perception and avoidance of a number of compounds in an additive manner would require a foraging beetle to come fairly close to a nonhost tree before it was repelled. It has also been hypothesized (Borden et al., 1998) that the redundancy effect for nonhost angiosperm bark volatiles would allow a foraging coniferophagous bark beetle to perceive and avoid different blends of compounds that are indicative of a variety of nonhost angiosperm taxa.

Our results provide good evidence that the DFB is able to detect and avoid nonhost angiosperms while in flight rather than randomly landing on and testing individual trees, because most DFBs that attempt to land on a multiple-funnel trap are subsequently caught (D.P.W.H., personal observation). A reduction in

numbers of beetles captured when nonhost volatiles are added to attractive baits is then an indication of repellency, a result borne out by numerous other studies on coniferophagous Scolytidae (Dickens et al., 1992; Wilson et al., 1996; Borden et al., 1997, 1998; Byers et al., 1998; Deglow and Borden, 1998a,b; Poland et al., 1998; Poland and Haack, 1999; Huber et al., 1999). It is possible that the hypothesis of random landing by dispersing beetles on host and nonhost trees, followed by close-range acceptance or rejection while in contact with the tree (Moeck et al., 1981), applies only to some other species of coniferophagous Scolytidae, applies only for differentiation between host and nonhost gymnosperms, or is just a by-product of beetles landing for different reasons such as to wait out inclement weather or to rest.

Although the compounds tested in our experiments were based on antennal responses by female beetles, responses by both sexes were generally similar. This result is in opposition to the hypothesis that because females are required to make the initial decision regarding the suitability of a given tree, they would respond more strongly than males to nonhost volatiles. On the other hand, few beetles probably initiate an attack and female and male beetles entering a pheromone plume should be able to distinguish between host and nonhost trees in order to accurately locate a tree undergoing attack.

The strong disruptant effect of nonhost angiosperm bark volatiles suggests that, as for the mountain pine beetle (Wilson et al., 1996; Borden et al., 1998), they could be used alone or in conjunction with the antiaggregation pheromone, MCH, to protect fallen trees and susceptible standing timber from attack by the DFB.

Acknowledgments—We thank M. Stastny, J. Allison, J. Burleigh, K. Chapman, R. McIntosh, D. Pureswaran and C. Wong, for assistance in the field; E. W. Senger of the British Columbia Ministry of Forests and C. N. O'Connor of Lytton Lumber Ltd. for access to field sites; and G. Gries and L. Safranyik for critical review of the manuscript. This research was supported by the Natural Sciences and Engineering Research Council of Canada, the Canadian Forest Service, Forest Renewal B.C., the Science Council of B.C., Ainsworth Lumber Co. Ltd., B.C. Hydro and Power Authority, Bugbusters Pest Management Inc., Canadian Forest Products Ltd., Crestbrook Forest Industries Ltd., Donohue Forest Industries Inc., Gorman Bros. Ltd., International Forest Products Ltd., Lignum Ltd., Manning Diversified Forest Products Ltd., Northwood Pulp and Timber Ltd., Phero Tech Inc., Riverside Forest Products Ltd., TimberWest Ltd., Tolko Industries Ltd., Weldwood of Canada Ltd., West Fraser Mills Ltd., and Weyerhaeuser Canada Ltd.

REFERENCES

BIRGERSSON, G., DEBARR, G. L., DE GROOT, P., DALUSKY, M. J., PIERCE, H. D., JR., BORDEN, J. H., MEYER, H., FRANCKE, W., ESPELIE, K. E., and BERISFORD, C. W. 1995. Pheromones in the white pine cone beetle, *Conophthorous coniperda* (Schwarz) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 21:143–167.

BORDEN, J. H., CHONG, L. J., SAVIOE, A., and WILSON, I. M. 1997. Responses to green leaf volatiles

in two biogeoclimatic zones by striped ambrosia beetle, *Trypodendron lineatum. J. Chem. Ecol.* 27:2479–2491.

BORDEN, J. H., WILSON, I. M., GRIES, R., CHONG, L. J., PIERCE, H. D., JR., and GRIES, G. 1998. Volatiles from the bark of trembling aspen, *Populus tremuloides* Michx. (Salicaceae) disrupt secondary attraction by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *Chemoecology* 8:69–75.

BYERS, J. A., ZHANG, Q.-H., and SCHLYTER, G. 1998. Volatiles from nonhost birch trees inhibit pheromone response in spruce bark beetles. *Naturwissenschaften* 85:557–561.

DALLARA, P. L., SEYBOLD, S. J., FRANCKE, W., and WOOD, D. L. 1994. The chemical ecology of *Pityophthorus Eichhoff* (Coleoptera: Scolytidae) in central coastal California, Appendix, pp. Ixviii–Ixix, *in* D. H. Adams, J. E. Rios, and A. J. Storer (eds.). Proceedings, 43rd Anual Meeting of the California Forest Pest Council, Rancho Cordova, California, .

DAY, R. W., and QUINN, G. P. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecol. Monogr.* 59:433–463.

DEGLOW, E. K., and BORDEN, J. H. 1998a. Green leaf volatiles disrupt and enhance response by the ambrosia beetle, *Gnathotrichus retusus* (Coleoptera: Scolytidae) to pheromone-baited traps. *J. Entomol. Soc. BC.* 95:9–15.

DEGLOW, E. K., and BORDEN, J. H. 1998b. Green leaf volatiles disrupt and enhance response to aggregation pheromones by the ambrosia beetle, *Gnathotrichus sulcatus* (Coleoptera: Scolytidae). *Can. J. For. Res.* 28:1697–1705.

DICKENS, J. C., BILLINGS, R. F., and PAYNE, T. L. 1992. Green leaf volatiles interrupt aggregation pheromone response in bark beetles infesting southern pines. *Experientia* 48:523–524.

FRANCKE, W., HINDORF, G., and REITH, W. 1978. Methyl-1,6-dioxaspiro[4,5]decanes as odors of *Paravespula vulgaris* (L.). *Angew. Chem. Int. Ed. Engl.* 17:862.

HUBER, D. P. W., GRIES, R., BORDEN, J. H., and PIERCE, H. D., JR. 1999. Two pheromones of coniferophagous bark beetles (Coleoptera: Scolytidae) found in the bark of nonhost angiosperms. *J. Chem. Ecol.* 25:805–816.

HUBER, D. P. W., GRIES, R., BORDEN, J. H., and PIERCE, H. D., JR. 2000. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. *Chemoecology*. In press.

HUMPHREYS, N. 1995. Douglas-fir beetle in British Columbia. Forest Pest Leaflets. Natural Resources Canada, Canadian Forest Service. Cat. No. Fo 29-6/14-1995E.

KOHNLE, U., DENSBORN, S., KÖLSCH, P., MEYER, H., and FRANCKE, W. 1992. (*E*)-7-Methyl-1,6-dioxaspiro[4.5]decane in the chemical communication of European Scolytidae and Nitidulidiae (Coleoptera). *J. Appl. Entomol.* 114:187–192.

LINDGREN, B. S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

LINDGREN, B. S., GRIES, G., PIERCE, H. D., JR., and MORI, K. 1992. *Dendroctonus pseudotsugae* Hopkins (Coleoptera: Scolytidae): Production of and response to enantiomers of 1-methylcyclohex-2-en-1-ol.

MCMULLEN, L. S., and ATKINS, M. D. 1962. On the flight and host selection of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopk. (Coleoptera: Scolytidae). *Can. Entomol.* 94:1309–1325.

MOECK, H. A., WOOD, D. L., and LINDAHL, K. Q. 1981. Host selection behavior of bark beetles (Coleoptera: Scolytidae) attacking *Pinus ponderosae*, with special emphasis on the western pine beetle, *Dendroctonus brevicomis. J. Chem. Ecol.* 7:49–83.

NORIN, T. 1996. Chiral chemodiversity and its role for biological activity. Some observations from studies on insect/insect and insect/plant relationships. *Pure Appl. Chem.* 68:2043–2049.

PERSSON, M., SJÖDIN, K. BORG-KARLSON, A-K., NORIN, T., and EKBERG, I. 1996. Relative amounts and enantiomeric compositions of monoterpene hydrocarbons in xylem and needles of *Picea abies.* Phytochemistry 42:1289–1297.

PIERCE, H. D., JR., DE GROOT, P., BORDEN, J. H., RAMASWAMY, S., and OEHLSCHLAGER, A. C. 1995. Pheromones in red pine cone beetle, *Conophthorus resinosae* Hopkins, and its synonym, *C. banksianae* McPherson (Coleoptera: Scolytidae). *J. Chem. Ecol.* 21:169–185.

POLAND, T. M., and HAACK, R. A. 1999. Pine shoot beetle, *Tomicus piniperda* (Coleoptera: Scolytidae), responses to common green leaf volatiles. *J. Appl. Entomol.* In press.

POLAND, T. M., BORDEN, J. H., STOCK, A. J., and CHONG, L. J. 1998. Green leaf volatiles disrupt responses by the spruce beetle, *Dendroctonus rufipennis*, and the western pine beetle, *Dendroctonus brevicomis* (Coleoptera: Scolytidae) to attractant-baited traps. *J. Entomol. Soc. BC.* 95:17–24.

ROSS, D. W., and DATERMAN, G. E. 1995. Response of *Dendroctonus pseudotstugae* (Coleoptera: Scolytidae) and *Thanasimus undatulus* (Coleoptera: Cleridae) to traps with different semiochemicals. *J. Econ. Entomol.* 88:106–111.

ROSS, D. W., GIBSON, K. E., THIER, R. W., and MUNSON, A. S. 1996. Optimal dose of an antiaggregation pheromone (3-methylcyclohex-2-en-1-one) for protecting live Douglas-fir from attack by *Dendroctonus pseudotsugae* (Coleoptera: Scolytidae). *J. Econ. Entomol.* 89:1204–1207.

RUDINSKY, J. A., FURNISS, M. M., KLINE, L. N., and SCHMITZ, R. F. 1972. Attraction and repression of *Dendroctonus pseudotsugae* (Coleoptera: Scolytidae) by three synthetic pheromones in traps in Oregon and Idaho. *Can. Entomol.* 104:815–822.

SAS INSTITUTE, INC. 1988. SAS/STAT Users Guide, Release 6.03 Edition. Cary, North Carolina.

SCHROEDER, L. M. 1992. Olfactory recognition of nonhosts aspen and birch by conifer bark beetles *Tomicus piniperda* and *Hylurgops palliatus. J. Chem. Ecol.* 18:1583–1593.

SJÖDIN, K., PERSSON, M., BORG-KARLSON, A-K., and TORBJÖRN, H. 1996. Enantiomeric compositions of monoterpene hydrocarbons in different tissues of four individuals of *Pinus sylvestris*. Phytochemistry 41:439–445.

WANG, X., LIU, Y-S., NAIR, U. B., ARMSTRONG, D. W., ELLIS, B., and WILLIAMS, K. M. 1997. Enantiomeric composition of monoterpenes in conifer resins. *Tetrahedron Asymmetry* 8:3977–3984.

WIBE, A., BORG-KARLSON, A-K., PERSSON, M., NORIN, T., and MUSTAPARTA, H. 1998. Enantiomeric composition of monoterpene hydrocarbons in some conifers and receptor neuron discrimination of α-pinene and limonene enantiomers in the pine weevil, *Hylobius abiietis. J. Chem. Ecol.* 24:273–287.

WILSON, I. M., BORDEN, J. H., GRIES, R., and GRIES, G. 1996. Green leaf volatiles as antiaggregants for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *J. Chem. Ecol.* 22:1861–1875.

ZAR, J. H. 1984. Data Transformations. Biostatistical Analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey, pp. 236–242.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

FIELD ATTRACTION OF MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (WIEDEMANN) TO SYNTHETIC STEREOSELECTIVE ENANTIOMERS OF THE CERALURE B1 ISOMER

ERIC B. JANG,[1] ANDRE S. RAW,[2] and LORI A. CARVALHO[1]

[1]*US Pacific Basin Agricultural Research Center*
USDA-ARS
Hilo Hawaii

[2]*USDA-ARS-PSI, Insect Chemical Ecology Laboratory*
Beltsville, Maryland 20705

(Received July 13, 2000; accepted October 1, 2000)

Abstract—Stereoselectively synthesized enantiomers of ethyl *cis*-5-iodo-*trans*-2-methylcyclohexane-1-carboxylate (ceralure B1), a potent lure for male Mediterranean fruit flies, were tested in the laboratory and the field against laboratory reared sterile flies. The (−)-ceralure B1 enantiomer was significantly more attractive than the (+)-ceralure B1 antipode. Dose–response studies of the above compounds demonstrated that (−)-ceralure B1 and to a lesser extent, racemic ceralure B1, captured consistently more male flies than trimedlure, the current male attractant used in detection programs. Longevity tests demonstrated that, over a three-week period, both (−)-ceralure B1 and racemic ceralure B1 caught significantly more flies than trimedlure. The synthesis of specific enantiomers of ceralure B1 shows great promise as a male attractant that could replace trimedlure for detection and delimitation in action programs aimed at this exotic pest.

Key Words—Mediterranean fruit fly, attractants, detection, male annihilation.

INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) is a serious pest of fruits and vegetables throughout many parts of the world. Except for the state of Hawaii, medfly is not established in the United States. If established, this pest would have a serious impact on US agriculture and the environment through increased costs for control and additional pesticide sprays. In addi-

0098-0331/01/0200-0235$19.50/0

tion, the regulatory quarantines by importing countries could severely restrict worldwide trade of US agricultural products (CDFA, 1994; Siebert and Cooper, 1995).

The establishment of this pest within the contiguous United States has been prevented through a vigorous detection, quarantine inspection, and eradication program. Monitoring and detection for the medfly using synthetic attractants is a key step in signaling the need for eradication. Each year states such as California, Florida, and Texas employ more than 100,000 traps in and around agricultural and urban areas to survey and detect possible introductions of medfly. For almost 40 years, trimedlure (TML), developed from an intensive US Department of Agriculture screening and testing program has been widely used as the "standard" synthetic male medfly attractant (Beroza et al., 1961). For commercial use, 2 g of trimedlure is formulated in a polymeric plug-type dispenser that provides controlled release of the attractant for up to eight weeks when deployed in Jackson traps (Leonhardt et al., 1987, 1989; Gilbert and Bingham, 1999).

Commercial trimedlure (TML) is an isomeric mixture of *tert*-butyl esters of 4- and 5-chloro-2-methylcyclohexane-1-carboxylate. Hence, trimedlure is a mixture of 16 possible regio- and stereoisomers. Of these, a single isomer (1*S*,2*S*,4*R*)-*tert*-butyl 4-chloro-2-methylcyclohexane-1-carboxylate (trimedlure-C enantiomer, henceforth referred to as (+)-trimedlure-C based on its optical rotation) was found to be the most attractive (McGovern et al., 1987; Doolittle et al., 1991; Heath et al., 1990). Furthermore, the optical antipode, (−)-trimedlure-C ((1*R*,2*R*,4*S*)-*tert*-butyl 4-chloro-2-methylcyclohexane-1-carboxylate) was shown to have very little biological activity.

McGovern co-workers investigated various halogen and ester analogs of trimedlure and found that ethyl 4- (and 5-) iodo-trans-2-methylcyclohexane-1-carboxylate (ceralure) was more potent and persistent than trimedlure (McGovern and Cunningham, 1988; DeMilo et al., 1994; Warthen et al., 1994). Like trimedlure, commercial ceralure is also a mixture of 16 regio- and stereoisomers. A field study designed to measure the relative attractiveness of the racemates, which were tediously separated by HPLC, demonstrated that racemic ethyl *cis*-5-iodo-*trans*-2-methylcyclohexane-1-carboxylate (ceralure B1) is most active (Warthen et al., 1994). Recently, a highly effective stereoselective synthesis that yields the two enantiomers of ceralure B1 on a multigram scale was developed (Raw and Jang, 2000).

For the current study, laboratory and field tests with released sterile flies on the island of Hawaii were carried out to compare the attractancy, dose–response, and initial persistence of the pure enantiomers as well as the racemic mixture. The purpose of this study was to evaluate the relative attractancy of the two nearly synthesized ceralure B1 enantiomers and to compare these to responses of commercially available trimedlure and ceralure.

METHODS AND MATERIALS

Test Compounds. Liquid trimedlure (UOP Chemicals, East Rutherford, New Jersey) (98% pure; density 1.02 g/ml) and ceralure (Agrisense/Biosys, Palo Alto, California) (98% pure; density 1.43 g/ml) were purchased from commercial sources. Ceralure was stored over a copper coil to prevent discoloration. The enantiomers of ceralure B1, ethyl (1*R*,2*R*,3*R*)-5-iodo-2-methylcyclohexane-1-carboxylate and ethyl (1*S*,2*S*,3*S*)-5-iodo-2-methylcyclohexane-1-carboxylate, henceforth referred to as (−)-ceralure B1 and (+)-ceralure B1, respectively, based on their optical rotations, were synthesized by using a unique nine-step process (Raw and Jang, 2000). This yielded each enantiomer on a multigram scale with high purity (97% ee) and an overall yield of 15%. Racemic ceralure B1 was synthesized via the same synthetic scheme utilized to synthesize the pure enantiomers of ceralure B1, with the exception of using racemic sigluric acid as starting material.

Insects. Sterilized medfly pupae were obtained from the USDA-ARS Pacific Basin Agricultural Research Center (PBARC) rearing facility in Honolulu, Hawaii. The flies were reared on an artificial diet (Tanaka et al., 1969) under standard mass rearing conditions and sterilized as pupae at a dose of 150 Gy two days before emergence. Flies were shipped by air to the PBARC laboratories in Hilo, HI, where 60–150 ml of pupae (both sexes) were placed either in small aluminum cubical screen cages (0.3 × 0.3 × 0.3 m) or in larger holding cages made from 57-liter (15-gallon) Rubbermaid "action packers" (Rubbermaid Inc., Khartoum, Sudan) modified by screened panels on the bottoms and sides of the units. The emerged flies were given water, sugar and hydrolyzed yeast protein. Flies were 2–9 days old when tested.

Rotating Olfactometer Test. Initial tests were conducted in a four-arm outdoor rotating olfactometer (3 × 3 × 2.5 m) as described in Jang et al. (1997). Neat compounds were diluted in reagent-grade acetone to give the following doses: 10, 1, 0.1, and 0.01 μg (in 100 μl acetone). The solutions were pipetted onto a 1.2-cm cotton wick inside of a Jackson trap containing a cardboard insert coated with stickem glue. One cubical aluminum cage of medflies (approximately 3600 flies of mixed sex) was released into the olfactometer. Temperature ranged from 20 to 24°C and relative humidity was 65–88%. Traps were allowed to slowly rotate within the olfactometer cage for 4 hr. At the end of the 4-hr test period, traps were removed and trapped flies were counted and recorded.

Open Field Test. All field tests were conducted by using Jackson traps with a 1.2-cm-long × 0.7-cm-diameter cotton wick that contained the test chemical (Harris et al., 1971). All tests were conducted with a randomized complete block design having five traps of each treatment in the block. The tests were replicated at least five times.

Dose–Response Tests. Traps were baited with (+)-ceralure B1, (−)-ceralure

B1, racemic ceralure B1, commercial trimedlure, commercial ceralure, or a blank (acetone) control. For each compound, the following doses were tested: 10, 1, 0.1, and 0.01 mg. Traps were placed in a macadamia nut orchard on the Island of Hawaii near the town of Hilo. Traps were separated by every other tree in every row (approximately 10 m between traps). Flies (approximately 6000/row) were then released by slowly walking between rows while allowing the flies to escape from the opened action packer. After 48 hr, traps were collected and brought back to the lab for analysis. Both males and females medflies were counted on the sticky inserts.

Longevity Tests. Preliminary tests to determine the effectiveness of the different chemical compounds over time were also carried out using sterile flies released in a macadamia nut orchard. In these tests, all compounds were applied to the cotton wick at a 10 mg dose. Traps were serviced weekly for three weeks. Inserts were changed each week and new flies were released, but the same cotton wicks were left in the field to age throughout the 3-week test period.

Data Analysis. Although male and female medflies were counted on the sticky inserts, the trap captures were overwhelmingly male biased. Thus, only data from male captures are included in the analysis. Data were subjected to analysis of variance (Proc GLM) and Tukey's honestly significant difference test for separation of mean values. Significant differences in response were determined at the $P < 0.05$ level. Data were not transformed prior to analysis. Statistical analysis was performed on SAS ver 6.12 (SAS Institute, Cary, North Carolina) with a PC. Values presented in the tables are means ±SEM.

RESULTS

In outdoor olfactometer cage tests, traps baited with (−)-ceralure B1 captured numerically more male medflies than did other traps at all concentrations. In comparison with commercial ceralure and the control, (−)-ceralure B1 produced significantly greater capture at the 10- and 1-μg mg dosages. At the 1-μg dosage, (−)-ceralure B1 was also different from racemic ceralure B1. There were no significant differences in trap captures among compounds at the 0.01-μg dosage (Table 1).

The field results comparing the relative attractancy of optically active ceralure B1 enantiomers, racemic ceralure B1, commercial trimedlure, and commercial ceralure at various doses are shown in Table 2. Traps baited with (−)-ceralure B1 captured significantly more male medflies than those baited with its (+)-ceralure B1 antipode. In addition, (−)-ceralure B1 and racemic ceralure B1 produced significantly higher capture than commercial trimedlure or ceralure at all dosages. In the course of these studies, it was noted that, at both the 10-mg and 1.0-mg doses of (−)-ceralure B1, trap saturation (overcrowding) was a

TABLE 1. RESPONSE OF RELEASED MEDITERRANEAN FRUIT FLIES TO DIFFERENT DOSAGES OF TML AND CERALURE COMPOUNDS IN ROTATING OLFACTOMETER[a]

Treatment	*N*	Male fly capture (mean ± SE)			
		10 μg[b]	1 μg	0.1 μg	0.01 μg
TML	4	211.5 ± 11.5 abc	176.2 ± 35.5 ab	91 ± 23.4 ab	4.5 ± 0.8 a
Com. Ceralure	4	87.5 ± 0.5 bc	42.5 ± 10 c	92 ± 25.3 ab	3.5 ± 1.5 a
(+)-Ceralure B1	4	241.5 ± 28.5 ab	234.2 ± 27.7 ab	131 ± 9.7 ab	8 ± 2.2 a
(±) Ceralure B1	4	188 ± .5 abc	131 ± 30.6 bc	121.5 ± 9.8 ab	7.2 ± .8 a
(−)-Ceralure B1	4	275 ± 16 a	296.7 ± 31.8 a	161.5 ± 38.5 a	11.5 ± 3.4 a
Control	4	50 ± 30 c	48 ± 15.2 c	38.5 ± 4.3 b	7.2 ± 1.6 a

[a] Data analyzed by Proc GLM; means followed by different letters in a column are significantly different ($P < 0.05$) by Tukey's test.
[b] Two replicates.

concern. Thus, the data at those concentrations may not be indicative of the relative attractiveness of (−)-ceralure B1 to the other attractants (e.g., trimedlure or commercial ceralure). At the 0.1-mg and 0.01-mg doses, trap saturation was not an issue. At these dosages, medfly capture by (−)-ceralure B1 outperformed, on average, commercial ceralure by 16-fold, and trimedlure by ninefold.

In longevity studies, all compounds were tested at the 10-mg dose. During the first week, both (−)-ceralure B1 and racemic ceralure B1 captured significantly more males than (+)-ceralure B1, commercial trimedlure, commercial ceralure, and control (Table 3). During the second week, captures at traps baited with (−)-ceralure B1 and racemic ceralure B1 were 20% and 30%, respectively, of the initial response before aging, whereas capture at traps baited with (+)-

TABLE 2. FIELD RESPONSE OF RELEASED MEDITERRANEAN FRUIT FLIES TO DIFFERENT DOSAGES OF TML AND CERALURE COMPOUNDS[a]

Treatment	*N*	Male fly capture (mean ± SE)			
		10 mg	1 mg	0.1 mg	0.01 mg[b]
TML	15	133.3 ± 30.7 c	112.5 ± 20.9 bc	31.1 ± 12.4 c	14 ± 3.4 bc
Com. Ceralure	15	170.5 ± 33.1 c	65.1 ± 9.5 bc	11.3 ± 4.1 c	23.4 ± 6.3 bc
(+)-Ceralure B1	15	151.8 ± 25.3 c	173 ± 42.2 b	57.2 ± 8.3 c	38.3 ± 6.5 b
(±)-Ceralure B1	15	365.5 ± 33.7 b	376.8 ± 36.7 a	179.7 ± 33.5 b	111 ± 12.2 a
(−)-Ceralure B1	15	487.3 ± 33 a	473.1 ± 56.6 a	303.1 ± 34.5 a	117 ± 14.1 a
Control	15	11.5 ± 3 d	8.4 ± 2.7 d	2.1 ± .7 c	2.3 ± 1c

[a] Data analyzed by Proc GLM; means followed by different letters in a column are significantly different ($P < 0.05$) by Tukey's test.
[b] Twenty-five replicates.

TABLE 3. RESPONSE OF RELEASED MEDITERRANEAN FRUIT FLIES TO 10 mg OF TML AND CERALURE COMPOUNDS OVER A THREE WEEK TEST PERIOD.

Treatment	*N*	Male fly capture (mean ± SE)		
		Week 1	Week 2	Week 3
TML	15	111 ± 19.4 bc	0.1 ± .1 b	0 ± 0 a
Com. Ceralure	15	135 ± 16.8 b	3.1 ± 2.2 b	0.5 ± .2 a
(+) Ceralure B1	15	135.7 ± 33.4 b	5.5 ± 4.5 b	0 ± 0 a
(±) Ceralure B1	15	274.5 ± 21.8 a	79.6 ± 33.3 a	4.7 ± 3.7 a
(−) Ceralure B1	15	338.9 ± 40.1 a	69.3 ± 24.9 ab	0.3 ± .3 a
Control	15	11.4 ± 3.9 c	2.3 ± 2.1 b	0.07 ± .1

[a] Data analyzed by Proc GLM; means followed by different letters in a column are significantly different ($P < 0.05$) by Tukey's test.

ceralure B1, commercial trimedlure, and commercial ceralure, were not significantly greater than control. After two weeks, racemic ceralure B1 captured more males than any other compound, although the response was only 2% that of the initial response. By the third week of testing, all compounds were capturing few males flies.

DISCUSSION

In this study, we have evaluated the initial biological activity of a new stereoselectively synthesized enantiomer of the ceralure B1 isomer. The enantiopure (−)-ceralure B1 was more attractive to male medflies than either commercial ceralure (a mixture of 16 isomers), or commercial trimedlure (also a mixture of 16 isomers). This increase in relative attraction was not initially apparent from dose–response studies in the cage olfactometer (Table 1) but was clearly demonstrated in field tests at doses of 0.1–10 mg (Table 2). In addition, longevity tests with 10 mg showed greater capture in the field after two weeks compared to trimedlure or ceralure (Table 3).

From a practical perspective, these results clearly demonstrate that (−)-ceralure B1 and to a lesser extent, racemic ceralure B1, are significantly more potent medfly attractants than commercial trimedlure and ceralure. Capture by (−)-ceralure B1 outperformed the standard trimedlure used in survey and detection of medfly by as much as ninefold. Furthermore, the increased longevity of this compound, and hence the decreased volatility of this attractant compared to trimedlure, suggest that on a molecule-to-molecule basis, (−)-ceralure B1 should have even greater activity compared to trimedlure. Nevertheless, this conservative estimate demonstrates that the activity of (−)-ceralure B1 is greater than that reported for (+)-α-copaene (2–5 times more active than trimedlure) or even the

C isomer of trimedlure. To our knowledge (−)-ceralure B1 is the most potent medfly attractant thus far reported. Studies to demonstrate its effectiveness with wild fly populations have shown (−)-ceralure B1 to outperform trimedlure in the field (unpublished data). Further studies are currently in progress.

The increased effectiveness of (−)-ceralure B1 may justify the use of this attractant as a replacement of trimedlure, which is used annually in over 100,000 baited detection traps for monitoring infestations of the Mediterranean fruit fly in the United States. The effectiveness of (−)-ceralure B1 may have further implication for its potential use for male annihilation trapping of male medfly for control and eradication.

REFERENCES

BEROZA, M., GREEN, N., GERTLER, S. I., STEINER, L. F., and MIYASHITA, D. 1961. New attractants for the Mediterranean fruit fly. *J. Agric. Food Chem.* 9:361–365.

CDFA. 1994. Final programmatic environmental impact report. The exotic fruit fly eradicaton program using aerial application of Malathion and bait. California Department of Food and Agriculture. Clearinghouse number 91043018.

DEMILO, A. B., CUNNINGHAM, R. T., and MCGOVERN, T. P. 1994a. Trimedlure: Effects of structural modifications on its attractiveness to Mediterranean fruit fly males (Diptera: Tephritidae). *J. Econ. Entomol.* 87:1494–1501.

DOOLITTLE, R. E., CUNNINGHAM, R. T., MCGOVERN, T. P., and SONNET, P. E. 1991. Trimedlure enantiomers: Differences in attraction for Mediterranean fruit fly, *Ceratitis capitata* (Weid.) (Diptera: Tephritidae). *J. Chem. Ecol.* 17:475–484.

GILBERT, J., and BINGHAM, R. R. (eds.). 1999. Insect Trapping Guide. California Department of Food and Agriculture. MF 1–9.

HARRIS, E. J., NAKAGAWA, S., and URAGO, T. 1971. Sticky traps for detection and survey of three tephritids. *J. Econ. Entomol.* 64:62–65.

HEATH, R. R., CHAMBERS, D. L., TUMLINSON, J. H., and LANDOLT, P. J. 1990. Controlled release of trimedlure isomer C from a compressed disk and the evaluation of its attractiveness to the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 83(3):819–822.

JANG, E. B., CARVALHO, L., and STARK, J. 1997. Attraction of female Oriental fruit fly, *Bactrocera dorsalis*, to volatile semiochemicals from leaves and extracts of a nonhost plant, Panax (*Polyscias guilfoylei*) in laboratory and olfactometer assays. *J. Chem. Ecol.* 23:1389–1401.

LEONHARDT, B. A., CUNNINGHAM, R. T., RICE, R. E., HARTE, E. M., and MCGOVERN, T. P. 1987. Performance of controlled-release formulations of trimedlure to attract the Mediterranean fruit fly, *Ceratitis capitata. Entomol. Exp. Appl.* 44:45–51.

LEONHARDT, B. A., CUNNINGHAM, R. T., RICE, R. E., HARTE, E. M., and HENDRICHS, J. 1989. Design, effectiveness, and performance criteria of dispenser formulations of trimedlure, and attractant of the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 82:860–867.

MCGOVERN, T. P., and CUNNINGHAM, R. T. 1988. U.S. Patent No. 4,764,366.

MCGOVERN, T. P., CUNNINGHAM, R. T., and LEONHARDT, B. A. 1987. Attractiveness of *trans*-trimedlure and its four isomers in field tests with the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 80:617–620.

RAW, A. S., and JANG, E. B. 2000. Enantioselective synthesis of ceralure B1, ethyl *cis*-5-iodo-*trans*-2-methylcyclohexane-1-carboxylate. *Tetrahedron* 56:3285–3290.

SIEBERT, J. B., and COOPER, T. 1995. Embargo on California produce would cause revenue, job loss. *Calif. Agric.* 49(4):7–12.

TANKA, N., STEINER, L. F., OHINATA, K., and OKAMOTO, R. 1969. Low cost larval rearing medium for mass production of Oriental and Mediterranean fruit flies. *J. Econ. Entomol.* 62:967–968.

WARTHEN, JR., J. D., CUNNINGHAM, R. T., DEMILO, A. B., and SPENCER, S. 1994. *Trans*-ceralure isomers: Differences in attraction for Mediterranean fruit fly, *Ceratitis capitata* (Weid.) (Diptera: Tephritidae). *J. Chem. Ecol.* 20:569–578.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

EFFECTS OF ALLELOCHEMICALS FROM FIRST (BRASSICACEAE) AND SECOND (*Myzus persicae and Brevicoryne brassicae*) TROPHIC LEVELS ON *Adalia bipunctata*

FRÉDÉRIC FRANCIS,[1,*] GEORGES LOGNAY,[2] JEAN-PAUL WATHELET,[2] and ERIC HAUBRUGE[1]

[1]*Unit of General and Applied Zoology*
[2]*Unit of General and Organic Chemistry*
Gembloux Agricultural University
Passage des Déportés 2, B-5030 Gembloux, Belgium

(Received April 9, 2000; accepted October 6, 2000)

Abstract—Three Brassicaceae species, *Brassica napus* (low glucosinolate content), *Brassica nigra* (including sinigrin), and *Sinapis alba* (including sinalbin) were used as host plants for two aphid species: the generalist *Myzus persicae* and the specialist *Brevicoryne brassicae*. Each combination of aphid species and prey host plant was used to feed the polyphagous ladybird beetle, *Adalia bipunctata*. Experiments with Brassicaceae species including different amounts and kinds of glucosinolates (GLS) showed increased ladybird larval mortality at higher GLS concentrations. When reared on plants with higher GLS concentrations, the specialist aphid, *B. brassicae*, was found to be more toxic than *M. persicae*. Identification of GLS and related degradation products, mainly isothiocyanates (ITC), was investigated in the first two trophic levels, plant and aphid species, by high-performance liquid chromatography and gas chromatography–mass spectrometry, respectively. While only GLS were detected in *M. persicae* on each Brassicaceae species, high amounts of ITC were identified in *B. brassicae* samples (allyl-ITC and benzyl-ITC from *B. nigra* and *S. alba*, respectively) from all host plants. Biological effects of allelochemicals from plants on predators through aphid prey are discussed in relation to aphid species to emphasize the role of the crop plant in integrated pest management in terms of biological control efficacy.

Key Words—Allelochemical, glucosinolates, isothiocyanates, ladybird, aphid, toxicity, tritrophic interactions.

* To whom correspondence should be addressed. e-mail: francis.f@fsagx.ac.be

0098-0331/01/0200-0243$19.50/0

INTRODUCTION

Many Brassicaceae species are cultivated as oilseed, vegetable, or fallow plants. In 1997, cabbages (*Brassica oleracea* L.) represented 10.7% of the world vegetable production. More than 11% of plant oil was produced from rape, *Brassica napus* L. (FAO, 1995, 1997). Brassicaceae crops are known to biosynthesize glucosinolates (GLS) as secondary metabolites. Myrosinase, a thioglucosidase enzyme (EC 3.2.3.1.), was found in plants containing GLS but was also detected in insects, such as the cabbage aphid, *Brevicoryne brassicae* L. (MacGibbon and Allison, 1968; Francis, 1999a). In the plant, myrosinase is located in the cytoplasm whereas GLS are stored in vacuoles (Grob and Matile, 1979). Glucosinolate degradation is initiated when cell integrity is destroyed (Birch et al., 1990), leading to the production of nitriles, thiocyanates, oxazolidinethiones, and isothiocyanates (ITC). The latter compounds may act as infochemicals in plant–animal interactions (Lamb, 1989) and can also display toxic effects and constitute part of the plant's defense against fungal and insect infestation (Heaney and Fenwick, 1995). ITC may be repellent or attractant, depending on the insect. While Brassicaceae specialists are stimulated to feed and oviposit by these substances, generalist herbivores are generally deterred.

Chemical cues from plants or herbivores are essential in the effective localization of the host/prey habitat and the searching behavior of entomophagous insects (Sauls et al., 1979). *Adalia bipunctata* L. is known for its polyphagy towards many aphid species (Hodek, 1959) even if some of them can present negative effects on the ladybird development (Hodek and Honek, 1996). The herbivore aphids we studied were *M. persicae* and *B. brassicae*. The latter is a Brassicaceae specialist whereas the first one is a generalist (more than 400 host plants; Quaglia et al., 1993). Due to its high polyphagy, *M. persicae* is found on Brassicaceae plants and tolerates the GLS and their degradation products (Nault and Stayer, 1972).

Host plant suitability for aphids does not necessarily meet the aphidophagous predator needs (Hodek and Honek, 1996). Determination of tritrophic interactions includes both practical and theorical interests. Plant–aphid–predator relations have been little studied in integrated pest management programs and raise some questions: how do the third trophic level interactions evolve in the presence of allelochemicals constituting plant defenses? Is the impact of specific plant secondary metabolites similar on the biology of herbivores and entomophagous insects? What are the active allelochemicals in aphids—native GLS or their degradation products? Indeed, GLS break down by enzymatic degradation to release volatile by-products, including mainly isothiocyanates. Brassicaceae species used in these studies were chosen for their glucosinolate profiles: sinigrin and sinalbin are mainly present (around sixfold more than total GLS in rape) in *Brassica nigra* L. and *Sinapis alba* L., respectively, while low GLS

content rape variety includes several GLS in low concentrations. This work was carried out to determine the toxicity of the Brassicaceae secondary substances on an aphid predator and to identify accurately the chemical compounds involved.

METHODS AND MATERIALS

Plant and Insect Rearing. Broad beans (*Vicia faba* L.), black mustard (*Brassica nigra* L.), white mustard (*Sinapis alba* L.), and oilseed rape (*Brassica napus* L.) were grown in 10-cm-diameter plastic pots in three separate controlled environmental rooms at 20 ± 2°C temperature and 16L : 8D photoperiod. While beans were cultivated in pots containing a 1 : 1 mixture of perlite and vermiculite, Brassicaceae species were sown in 20-cm × 30-cm plastic trays containing ordinary compost and transplanted into plastic pots with the same compost when the plants had two true leaves.

Ladybirds, *Adalia bipunctata* L., *Acyrthosiphon pisum* (Harris), and *Myzus persicae* Sultzer had been reared in the laboratory for several years, whereas *Brevicoryne brassicae* L. was collected from a white mustard field in September 1998. While *A. pisum* was only reared on bean, the two other aphid species were mass reared on rape and white and black mustards. The Brassicaceae species were inoculated when they had five to six true leaves. Each plant species was cultivated in separate climate chambers.

For each combination of aphid species and host plant, 30 newly hatched larvae of *A. bipunctata* were isolated individually in 5-cm-diameter Petri dishes to avoid cannibalism and were kept at 20 ± 2° and 16L : 8D photoperiod. The larvae were collected from different egg clusters of the ladybird stock culture. Adults of the latter were reared in aerated plastic boxes (25 individuals per container) and fed every other day ad libitum with *A. pisum* reared on bean (optimal diet). Each experiment was repeated twice (a total of 60 individuals per beetle diet). Aphids stayed at least two weeks on their host plants before being removed from them and offered to the larvae ad libitum.

Biological Observations. Seven plant–prey combinations were tested in this work as ladybird diet: *M. persicae* reared either on *V. faba* (without GLS, considered as control), *B. napus*, *B. nigra*, or *S. alba* and *B. brassicae* reared either on *B. napus*, *B. nigra*, or *S. alba*. The Brassicaceae specialist aphid was not able to survive on bean.

After hatching, larvae were placed individually into 5-cm Petri dishes and observed every day to determine survival and appearance of nymphs until the pupal stage. Percent mortality at each development stage was analyzed by ANOVA after $\arcsin\sqrt{x}$ transformation (Dagnelie, 1973) and followed by mean comparisons by the Newman and Keuls method.

Glucosinolate Analysis. Glucosinolates were analyzed by high-perfor-

mance liquid chromatography (HPLC L-6200 and L-4000 UV detector, Merck-Hitachi) according to the ISO 9167-1 method, slightly modified. Plant, insect body, and honeydew samples were weighed and crushed in a hot methanol–water mixture (70 : 30 v/v). Glucosinolate enzymatic desulfatation was carried out on a DEAE column (Sephadex A-25) using commercial sulfatase. Desulfoglucosinolates were then analyzed by HPLC.

The *M. persicae* honeydew was collected on 20-cm × 20-cm glass plates placed just under the plants. After 48 hr of exposure, the plates were removed and rinsed with 10 ml of distilled water. To assess the occurrence of GLS in honeydew, samples were submitted to HPLC analysis as previously described. Sinigrin was used as the internal standard when *B. napus* and *S. alba* were involved whereas glucotropaeolin was added for *S. nigra* or aphid-related samples. Three replicates were considered for each analysis.

Glucosinolate Hydrolysis Product Analysis. White and black mustards contain high amounts of one major GLS (sinalbin and sinigrin, respectively), whereas the double zero variety of rape includes a mixture of low concentration GLS. We considered *B. nigra* and *S. alba* as two models, knowing that the low GLS content in rape would not allow easy detection of volatiles from aphids reared on it. That is why only aphids reared on both mustards were analyzed for the GLS hydrolysis products.

The volatile GLS metabolites from both aphid species (250 mg) reared on *B. nigra* were analyzed by use of a purge and trap unit (P&T Hewlett Packard) connected to a Hewlett Packard GC-MS system (HP5972 mass spectrometer coupled to a HP5890 series II gas chromatograph). Crushed samples were first maintained for 1 hr at 30 ± 0.2°C in thermostated glass tubes adapted to the P&T unit. They were purged with He at 4 ml/min for 11 min, the volatiles concentrated at room temperature on a Vocarb 3000, and finally desorbed by flash-heating to 250°C. The molecules of interest were automatically transferred to the GC-MS through a split-splitless injector at 250°C (splitless mode). They were separated on a HP5-MS (5% phenyl-dimethylpolysiloxane) column (30 m × 0.25 mm, *df* = 1 μm). The temperature program was from 40°C (1 min hold) to 180°C at 6°C/min then to 280°C at 15°C/min. MS spectra were obtained in the EI mode at 70 eV (scanned mass range from 30 to 300 amu). Analytes were identified on the basis of their retention times and by interpretation of MS fragmentation patterns. The recorded spectra were finally compared to those of the Wiley 238.L spectral library.

Nonvolatile GLS degradation products from both aphid species (samples each of ~250 mg) reared on *S. alba* were extracted with 500 μl pure diethyl ether for 12 hr. Solutions were injected as such in the splitless injector using the aforementioned GC-MS conditions. Quantification was performed by GLC with exactly the same chromatographic parameters on a Hewlett Packard (HP6890) apparatus equipped with a flame ionization detector maintained at

250°C. Two hundred micrograms of phenethyl-ITC was used as internal standard. The response factor was fixed to 1. The measurement of allyl-ITC was not performed due to its high volatility and the potential losses which could occur during crushing and transfer of the samples to the P&T system.

RESULTS

Ladybird Mortality. When total mortality was considered (Figure 1), significant differences were observed according to the aphid species ($F = 60.46$, $P < 0.001$). There was no difference in ladybird mortality when fed with *M. persicae* reared on the three Brassicaceae species and broad bean used as control ($F = 0.87$, $P = 0.494$). When *B. brassicae* was used as ladybird prey, highly significant differences in total mortality were observed depending on the host plant ($F = 447.97$, $P < 0.001$). Mortality of ladybirds fed *B. brassicae* reared on *B. napus* was lower than when they were fed aphids from *B. nigra* or *S. alba* ($q = 0.141$; $P < 0.01$). However, mortality rates on both mustard species reared aphids were not different ($q = 0.097$; $P > 0.05$).

Mortality of each larval stage of *A. bipunctata* fed a combination of aphid species and host plant was assessed (Figures 2a and b). Change of *M. persicae*

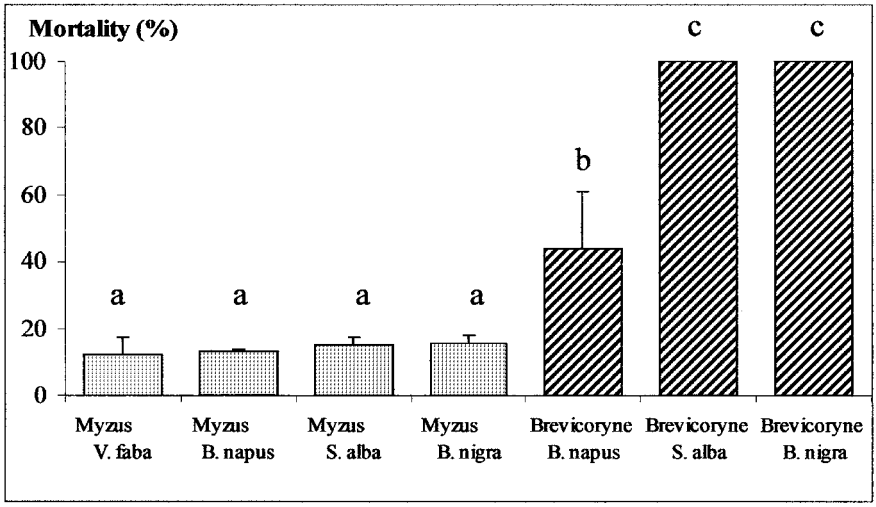


FIG. 1. Total mortality rates of *Adalia bipunctata* fed with different combinations of host plant and aphid species ($N = 60$). Error bars represent standard deviation from the mean and letters indicate significant differences at $P = 0.05$.

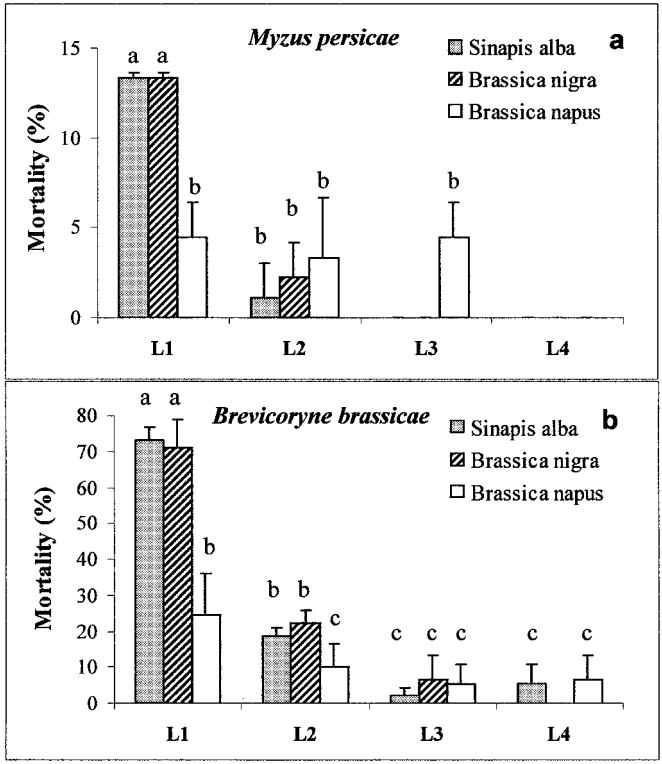


FIG. 2. Effect of aphid prey and host plant on larval mortality rates of *Adalia bipunctata* fed with *Myzus persicae* (a) and *Brevicoryne brassicae* (b) reared on *Brassica napus*, *Brassica nigra*, or *Sinapis alba* ($N = 60$ for each one). Error bars represent standard deviation from the mean and letters indicate significant differences at $P = 0.05$.

host plant displayed difference of mortality rates for first and third larval stages ($F = 40.41$ and $P < 0.001$; $F = 65.65$ and $P < 0.001$, respectively). Whether *B. napus* host plant induced lower mortality than the two mustard species ($q = 0.094$ and $P < 0.01$ for both mean tests), no difference was observed between the two latter plants ($q = 0.064$ and $P > 0.05$). Ladybird mortality rates observed for the three *M. persicae* host plants at the second larval stage were not different ($F = 0.60$ and $P = 0.579$).

A change of *B. brassicae* host plant induced a significant difference in mortality rates at the first and second larval stages ($F = 27.42$ and $P = 0.001$; $F = 5.16$ and $P = 0.048$, respectively). Mortality rates of *A. bipunctata* fed *B. brassicae* reared on both mustard species were higher than when fed aphids reared on rape ($q = 0.354$ and $P < 0.01$; $q = 0.132$ and $P < 0.05$ for first and second

TABLE 1. GLUCOSINOLATE CONTENTS IN PLANT AND APHID SPECIES[a]

Glucosinolate	Host plant leaf	*Myzus persicae*		*Brevicoryne brassicae* total aphid
		Total aphid	Honeydew	
Brassica napus				
Glucobrassicin	0.16 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.10 ± 0.00
Gluconasturtin	0.49 ± 0.04	0.11 ± 0.01	0.06 ± 0.01	2.80 ± 0.77
4-OH-Glucobrassicin	<0.01	<0.01	ND	ND
Progoitrin	0.57 ± 0.00	<0.01	ND	<0.01
Glucoraphanin	0.37 ± 0.01	0.33 ± 0.06	0.26 ± 0.12	2.91 ± 1.07
Total	1.59 ± 0.04	0.47 ± 0.05	0.34 ± 0.09	5.81 ± 1.13
Sinapis alba				
Sinalbin	8.83 ± 0.15	0.72 ± 0.10	1.12 ± 0.29	180.96 ± 15.83
Gluconasturtin	1.82 ± 0.01	0.28 ± 0.02	0.35 ± 0.04	ND
4-OH-Glucobrassicin	<0.01	ND	ND	1.92 ± 0.37
Brassicanapin	<0.01	ND	ND	<0.01
Progoitrin	0.28 ± 0.06	0.65 ± 0.11	0.03 ± 0.00	3.28 ± 0.16
Total	10.93 ± 0.13	1.65 ± 0.09	1.50 ± 0.43	185.16 ± 14.36
Brassica nigra				
Sinigrin	8.45 ± 0.54	1.75 ± 0.39	1.92 ± 0.17	132.88 ± 17.12
Sinalbin	0.23 ± 0.04	<0.01	ND	4.89 ± 0.37
4-OH-Glucobrassicin	0.79 ± 0.03	<0.01	<0.01	8.32 ± 1.72
Progoitrin	0.07 ± 0.01	1.58 ± 0.48	<0.01	2.21 ± 0.41
Total	9.54 ± 0.42	2.33 ± 0.46	1.92 ± 0.17	148.20 ± 15.03

[a] Values are in μmol. g^{-1}FW. GLS amounts of *M. persicae* honeydew (in μmol) were determined from the glass plate rinsing solution. ND = glucosinolates were not detected.

larval stages, respectively). No difference was observed between aphids reared on *B. nigra* or *S. alba* ($q = 0.243$ and $P > 0.05$). *B. brassicae* host plant did not induce differences in mortality rates of third and fourth larval stage ladybirds ($F = 0.26$ and $P = 0.779$; $F = 1.88$ and $P = 0.232$, respectively).

Glucosinolate Analysis. The glucosinolates found in aphid samples and in host plant leaves are presented in Table 1 and 2. Small quantities of glucobrassicin, brassicanapin, and glucoraphanin were detected in both aphid species reared on the low-GLS-content rape. Larger quantities of sinalbin and sinigrin were found in aphid samples collected on *S. alba* and *B. nigra*, respectively. Larger differences in GLS content were observed between the aphid species. Higher GLS amounts were found in *B. brassicae* than in *M. persicae* samples. Complementary analysis of honeydew from the latter reared on each Brassicaceae species revealed traces of sinalbin and sinigrin in *S. alba* and *B. nigra*, respectively (Table 1). Honeydew production by *B. brassicae* was so small that analysis was not possible.

Glucosinolate Hydrolysis Product Analysis. Regardless of the host plant

TABLE 2. GLUCOSINOLATES FOUND IN HOST PLANT AND APHID SPECIES ANALYZED

Common name	Glucosinolate side chain
Sinigrin	allyl
Sinalbin	*p*-hydroxybenzyl
4-OH-glucobrassicin	4-OH-3-indolylmethyl
Progoitrin	2-OH-3-butenyl
Brassicanapin	4-pentenyl
Gluconasturtin	2-phenylethyl
Glucoraphanin	4-methylsulfinylbutyl
Glucobrassicin	3-indolylmethyl

(*B. napus*, *B. nigra*, and *S. alba*), the GC-MS chromatograms of volatiles from *M. persicae* only displayed β-farnesene, a well known aphid alarm pheromone (Pickett et al., 1992). No glucosinolate degradation product was detected for *M. persicae* (Table 3). *B. brassicae* samples displayed substantial amounts of ITC(s) and nitrile(s) according to the GLS present in the aphid host plant (Table 3). Allyl-ITC and allylnitrile were found in aphids reared on *B. nigra*, which biosynthesizes sinigrin. Analyses of aphids collected on *S. alba*, which contains sinalbin (4-hydroxybenzyl-GLS) by the purge-and-trap method, did not reveal the occurrence of ITC. As no peak was detected by this method, the unsuccessful recovery of 4-hydroxybenzyl-ITC was attributed to its low volatility. Diethyl ether extraction was then undertaken. Diethyl ether extracts of *B. brassicae* reared on *S. alba* had two peaks: benzyl-ITC and benzyl-nitrile. The occurrence of benzyl-ITC was surprising as this compound is characteristic of glucotropaeolin (benzyl-glucosinolate) degradation. Further analysis was carried out to be sure that this was not an analytical artifact. Commercial thioglucosidase was added to an aqueous

TABLE 3. VOLATILE CONSTITUENT ANALYSIS OF *Brevicoryne brassicae* AND *Myzus persicae* EXTRACTS BY GC-MS (TOTAL ION CURRENT)[a]

Aphid and host plant species	GLS hydrolysis products	Others
Brevicoryne brassicae		
On *Sinapis alba*	Benzyl-ITC (R_t = 22.87)	
	Benzylnitrile (R_t = 17.27)	
On *Brassica nigra*	Allyl-ITC (R_t = 10.53)	
	Allylnitrile (R_t = 4.70)	
Myzus persicae		
On *Sinapis alba*		β-farnesene (R_t = 24.60)
On *Brassica nigra*		β-farnesene (R_t = 24.63)

[a] Names of identified compounds are followed by their retention time (R_t).

solution of sinalbin (purified from *S. alba*; 2 mg/ml); 4-hydroxy benzyl-ITC was identified by GC-MS. This result indicates that sinalbin metabolism in *B. brassicae* is more complex than suspected. We hypothesize that the loss of an hydroxy group is linked to the insect extract and not to the analytical method. This observation needs further investigation.

Quantitative evaluation of the benzyl-ITC production from specialist aphids was assessed and revealed a constant ITC/associated nitrile ratio (5.59 ± 0.37; benzyl-ITC/benzonitrile) for all replicates. The benzyl-ITC that was produced from the *B. brassicae* samples was 3.0 ± 0.2 μmol/g fresh material ($N = 4$ replicates).

DISCUSSION

In association with visual cues, chemical composition plays an important role in host plant location by herbivorous insects (Prokopy and Owens, 1983). Fraenkel (1959) suggested that secondary plant metabolites were directly involved in food searching by insects. The impact of allelochemicals is not limited to the second trophic level, interactions are observed when entomophagous insects use synomones emitted by plants as cues for prey location. Volatile chemical cues employed by natural enemies may originate from the plant upon which the host is feeding, the host itself, other organisms associated with the host, or chemicals released as a result of interactions between the host and its food plant (Lewis and Martin, 1990). Release of herbivore-induced plant volatiles that act as reliable and detectable host location cues for parasitoids and predators has previously been demonstrated for several tritrophic systems (Dicke et al., 1990; Turlings et al., 1990).

The chemical traces of aphids, even in the absence of insects themselves, induce changes in ladybird oviposition behavior (Carter and Dixon, 1984). In this work, GLS were found in the honeydew of *M. persicae* reared on the three Brassicaceae host plants Weber et al. (1986) detected 3.6–28.7 μmol/g (dry weight) of GLS in the peach-potato aphid depending on the rape variety (low or high GLS content). Isothiocyanates and nitriles were detected by GC-MS in *B. brassicae* infesting either *B. nigra* or *S. alba*. The presence of benzyl-ITC was quite surprising; 4-hydroxy benzyl-ITC, the normal product of sinalbin degradation was not found. Loss of the hydroxy group seems to be due to the *B. brassicae* enzymatic extract but needs to be more extensively studied from a biochemical point of view. Benzyl-ITC, a typical glucotropaeolin degradation product, is toxic to some insects (Bartlet and Mikolajczak, 1989). These molecules emitted by the Brassicaceae specialist aphid could be used as infochemicals by entomophagous insects. Assays on the *B. brassicae* parasitoid *Diaeretiella rapae* demonstrated the role of volatiles from aphids as kairomones. Indeed,

they responded to odors of infested cabbage leaves or aphids alone but not plants uninfested by aphids, suggesting an innate odor preference for crucifer-feeding aphids (Reed et al., 1995). If Brassicaceae pest natural enemies use these infochemicals for their own benefit, generalist predators could use the ITC produced by *B. brassicae* as allomones to avoid this aphid species. When two aphid species were offered to *A. bipunctata* (*M. persicae* and *B. brassicae* or the latter and *A. pisum*), the generalist predator first consumed the other, then the Brassicaceae specialist species (Francis, personal communication). This rejection is directly linked to the food suitability represented by the aphid species (Hodek and Honek, 1996). No ladybird was able to reach the adult stage when fed with the specialist aphid reared on both mustard species. When prey was collected from rape, twofold lighter adults emerged (compared to control food, *M. persicae* on bean) and no reproduction was observed (Francis, 1999b).

Isothiocyanates, GLS degradation compounds, stimulate olfactory receptors of generalist herbivores such as *Aphis fabae*. Both pentenyl- and butyl-ITC are repellent to this aphid species (Isaacs et al., 1993). Alkenyl-ITCs are characteristic defensive chemicals of Brassicaceae. Chemical precursors that break down to release ITC have shown biological activity against pathogens and insect pests of Brassicaceae (Lamb, 1989; Dawson et al., 1993). The higher toxicity of *B. brassicae* prey to generalist predators can be objectively explained by the presence of these ITC compounds. Indeed, allyl-ITC and methyl-ITC are toxic to *A. bipunctata* (0.189 ppm $< LD_{50} <$ 0.308 ppm and 0.218 ppm $< LD_{50} <$ 0.551 ppm, respectively) after a 24-hr exposure (Francis et al., 1999). Moreover, myrosinase, the enzyme that catalyzes GLS degradation, was detected in *B. brassicae* but not in *M. persicae* (Francis, 1999a). ITC or nitriles were not found in the latter species reared on Brassicaceae species. Only intact GLS were observed in this aphid species reared on mustards or rape (0.47–2.33 μmol/g fresh aphid weight). On the other hand, high amounts of GLS were found in *B. brassicae* reared on both mustard species while lower allelochemical levels were observed on rape (25–30 times less). Weber et al. (1986) detected more than 60 μmol/g (when expressed on a fresh weight basis) of GLS in *B. brassicae* reared on high GLS content rapes.

Glucosinolates are known to display biological properties towards insects. While they are oviposition stimulants or phagostimulants (Hicks, 1974; Reed et al., 1989; Traynier and Truscott, 1991) for many Brassicaceae herbivore specialists, they also serve as repellents for most generalist crop pests (Blau et al., 1978; Erickson and Feeny, 1974). Foliar concentrations of GLS found in both mustard species were much higher than in rape (around sixfold more) and were in accordance with the results of Hopkins et al. (1998). Total GLS content in white mustard leaves were 50–70 μmol/g of dry weight, which corresponded to a concentration of 10–15 μmol/g GLS when expressed in fresh weight. Whereas 12.5 μmol/g (in dry weight) of total GLS was found in 00 rape leaves, more than

60 μmol/g was detected in a high-GLS variety of rape (Weber et al., 1986). Similar GLS concentrations (expressed in fresh weight), were reported in this study. Variations in GLS contents of cultivated species were tested to obtain resistant varieties of plant against herbivores. *Sinapis alba*, containing high amounts of sinalbin, was shown to be less susceptible to damage by insect pests than *B. napus* with low GLS content. The specialist aphid, *B. brassicae*, was found mainly on young tissues containing the highest total GLS levels, while the generalist aphid, *M. persicae*, was found predominantly on older plant parts (Hopkins et al., 1998). According to these authors, aphid distribution is directly linked to GLS concentration in plant.

Other secondary plant compounds are toxic to nonspecialist herbivores and to natural enemies of herbivores. Toxicity of *Aphis sambuci* seemed to be assumed by the passage of the glycoside sambunigrin from the plant *Sambuscus nigra* into the aphid. Several other species of aphids also seem to be poisonous to coccinellids, including *A. bipunctata*, and are rejected by ladybirds due to presence of allelochemicals, including *Macrosiphum aconitum* feeding on *Aconitum* (including aconitin), *Aphis nerii* infesting oleanders (including oleandrin and neriin), and *Aphis jacobaeae* feeding on *Senecio* sp. (including pyrrolizidine alkaloids) (Hodek and Honek, 1996).

Allelochemicals from plants are partly responsible for host specificity of herbivorous insects (Schoonhoven, 1981). As specialized organisms, insects developed many defense strategies. Their responses towards secondary metabolites vary considerably. Some insects have evolved adaptations to resist these allelochemicals, including stocking them as defense substances against natural enemies (Harborne, 1993). Others evolved biochemical systems to metabolize and assimilate secondary plant substances. Detoxification mechanisms can contribute to neutralizing the toxic potential of some compounds (Yu, 1984; Yu and Hsu, 1993). These adaptations range from chemical modification of toxin, its transformation into a nontoxic conjugated compound, or its sequestration. Allyl- and benzyl-ITC were metabolized by glutathione *S*-transferase (GST) from two lepidopterous generalist species (*Spodoptera frugiperda* and *Trichoplusa ni*), but no activity was detected with the specialist *Anticarsia gemmatalis* (Wadleigh and Yu, 1988). While the generalist aphid *M. persicae* displayed induction of the GST system when reared on Brassicaceae species (*S. alba*, *B. nigra*, *B. napus*), induction was not observed in the *B. brassicae* specialist herbivore whatever the host plant (Francis, 1999a).

Adaptation of the aphid predator, *A. bipunctata*, to crucifer allelochemicals is facilitated by induction of GST (Francis, 1999a). Indeed, the increase of GST activity was linked to the plant GLS levels predominantly with *B. brassicae* aphid. We hypothesize that the latter accumulates the plant secondary substances, constituting an aphid defense against their natural predators. More than the volatile emission as chemical cues, this phenomenon of bioaccumulation of

high GLS amounts in prey would imply an aphid limited consumption by entomophagous insects.

In conclusion, studies on integrated pest management must include both herbivore and plant trophic levels to determine the plant allelochemical impact on the third trophic level, the beneficial entomophagous insects. To obtain effective biological control, determination of the involved aphid species is not sufficient. Semiochemicals from plants, directly or through herbivore prey, must be taken into account as a potential toxin or reliable infochemical in relation to the efficacy of pest control by natural enemies. Effectiveness of biological control by coccinellids is influenced by many factors, among them the host plant species of the pest. If natural enemies attack a wide variety of herbivores that occur on the same host species, information from the plant may become very important (Vet and Dicke, 1992). The use of generalist predators is not always a general solution to control aphid infestations. This work confirms the need for a case-by-case consideration when setting an integrated pest management program, including chemical ecology approaches.

REFERENCES

BARTLET, R. J., and MIKOLAJCZAK, K. L. 1989. Toxicity of compounds derived from *Limnanthes alba* seed to fall armyworm (Lepidoptera: Noctuidae) and European corn borer (Lepidoptera: Pyralidae) larvae. *J. Econ. Entomol.* 82:1054–1060.

BIRCH, A. N. E., GRIFFITHS, D. W., and MACFARLANE SMITH, W. H. 1990. Changes in forage and oilseed rape (*Brassica napus*). Root glucosinolates in response to attack by turnip root fly (*Delia floralis*). *J. Sci. Food Agric.* 51:309–320.

BLAU, P. A., FEENY, P., CONTARDO, L., and ROBSON, D. S. 1978. Allyl-glucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. *Science* 200:1296–1298.

CARTER, M. C., and DIXON, A. F. G. 1984. Honeydew: An arrestant stimulus for coccinellids. *Ecol. Entomol.* 9:383–387.

DAGNELIE, P. 1973. Théories et méthodes statistiques, tome 2, Presses agronomiques, Gembloux, Belgium.

DAWSON, G. W., DOUGHTY, K. J., HICK, A. J., PICKETT, J. A., PYE, B. J., SMART, L. E., and WADHAMS, L. J. 1993. Chemical precursors for studying the effects of glucosinolate catabolites on diseases and pests of oilseed rape (*Brassica napus*) or related plants. *Pestic. Sci.* 39:271–278.

DICKE, M., SABELIES, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990. Plant strategies of manipulating predator–prey interactions through allelochemicals: Prospects for application in pest control. *J. Chem. Ecol.* 16:3091–3118.

ERICKSON, J. M., and FEENY, P. 1974. Sinigrin: A chemical barrier to the black swallowtail butterfly, *Papilio polyxenes. Ecology* 55:103–111.

FAO. 1995. Annuaire Commerce, Vol. 50. Statistics series no. 138.

FAO. 1997. Annuaire Production, Vol. 51. Statistics series no. 142.

FRAENKEL, G. S. 1959. The raison d'être of secondary plant substances. *Science* 129:1234–1237.

FRANCIS, F. 1999a. Conséquences évolutives des relations entre le puceron et son prédateur en présence de substances allélochimiques chez les Brassicaceae, 5ème Conférence Internationale sur les Ravageurs en Agriculture, Annales ANPP, Tome II, pp. 503–510.

FRANCIS, F. 1999b. Effects des glucosinolates sur les interactions du modèle plante–puceron–coccinelle. DEA dissertation. Gembloux Agricultural University, Gembloux.

FRANCIS, F., HAUBRUGE, E., and GASPAR, C. 1999. Effects of isothiocyanates on the glutathione S-transferases activity from *Adalia bipunctata* L. (Coleoptera: Coccinellidae). *Med. Fac. Landbouw. Univ. Gent* 64(3a):297–303.

GROB, K., and MATILE, P. 1979. Vacuolar location of glucosinolate in horseradish root cells. *Plant Sci. Lett.* 14:327–335.

HARBORNE, J. B. 1993. Introduction to Chemical Ecology, 4th ed. Academic Press, London.

HEANEY, R. K., and FENWICK, G. R. 1995. Natural toxins and protective factors in *Brassica* species, including rapeseed. *Natural Toxins* 3:233–237.

HICKS, K. L. 1974. Mustard oil glucosinolates: feeding stimulants for adult cabbage flea beetles, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 67:261–264.

HODEK, I. 1959. The influence of aphid species as food for the ladybirds *Coccinella 7-punctata* L. and *Adalia bipunctata* L. *In* The Ontogeny of Insects, Prague.

HODEK, I., and HONEK, A. 1996. Ecology of Coccinellidae, Kluwer Academic, The Netherlands.

HOPKINS, R. J., EKBOM, B., and HENKOW, L. 1998. Glucosinolate content and susceptibility for insect attack of three populations of *Sinapis alba. J. Chem. Ecol.* 24(7):1203–1216.

ISAACS, R., HARDIE, J., HICK, A. J., PYE, B. J., SMART, L. E., WADHAMS, L. J., and WOODOCK, C. M. 1993. Behavioural responses of *Aphis fabae* to isothiocyanates in the laboratory and field. *Pestic. Sci.* 39:349–355.

LAMB, R. J. 1989. Entomology of oilseed Brassica crops. *Annu. Rev. Entomol.* 34:211–229.

LEWIS, W. J., and MARTIN, W. R. 1990. Semiochemicals for use with parasitoids: Status and future. *J. Chem. Ecol.* 90:415–421.

MACGIBBON, D. B., and ALLISON, R. M. 1968. Glucosinolate system in the aphid *Brevicoryne brassicae. N.Z. J. Sci.* 11:444–446.

NAULT, L. R., and STAYER, W. E. 1972. Cited in J. A. Pickett, L. J. Wadhams, and Woodcock. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

PROKOPY, R. J., and OWENS, E. D. 1983. Visual detection of plants by herbivorous insects. *Annu. Rev. Entomol.* 28:337–364.

QUAGLIA, F., ROSSI, E., PETACCHI, R., and TAYLOR, C. E. 1993. Observations on an infestation by green peach aphids (Homoptera: Aphididae) on greenhouse tomatoes in Italy. *J. Econ. Entomol.* 86(4):1019–1025.

REED, D. W., PIVNICK, K. A., and UNDERHILL, E. W. 1989. Identification of oviposition stimulants for the diamondback moth, *Plutella xylostella*, present in three species of Brassicaceae. *Entomol. Exp. Appl.* 53:277–286.

REED, H. C., TAN, S. H., HAAPANEN, K., KILLMON, M., REED, D. K., and ELLIOT, N. C. 1995. Olfactory responses of the parasitoid *Diaeretiella rapae* (Hymenoptera: Aphidiidae) to odor plants, aphids and plant–aphid complexes. *J. Chem. Ecol.* 21(4):407–415.

SAULS, C. E., NORDLUND, D. A., and LEWIS, W. J. 1979. Kairomones and their use for management of entomophagous insects. VIII. Effect of diet on the kairomonal activity of frass from *Heliothis zea* (Boddie) larvae for *Micropolitis croceipes* (Cresson). *J. Chem. Ecol.* 5:363–369.

SCHOONHOVEN, L. M. 1981. Chemical mediators between plants and phytophagous insects, pp. 31–50, in D. A. Nordlund, R. L. Jones, and W. J. Lewis (eds.). Semiochemicals, Their Role in Pest Control, John Wiley & Sons, New York.

TRAYNIER, R. M. M., and TRUSCOTT, R. J. W. 1991. Potent natural egg-laying stimulant for cabbage butterfly *Pieris rapae. J. Chem. Ecol.* 17(7):1371–1379.

TURLINGS, T. C. J., TUMLINSON, J. H., ELLER, F. J., and LEWIS, W. J. 1990. Exploitation of herbivore induced plant odors by host seeking parasitic wasps. *Science* 250:1251–1253.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

WADLEIGH, R. W., and YU, S. J. 1988. Metabolism of an organothiocyanate allelochemical by glutathione transferase in three lepidopterous insects. *J. Econ. Entomol.* 81(3):776–780.

WEBER, G., OSWALD, S., and ZOLLNER, U. 1986. Suitability of rape cultivars with different glucosinolate content for *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sultzer) (Hemiptera, Aphididae). *J. Plant Dis. Prot.* 93:113–124.

YU, S. J. 1984. Interactions of allelochemicals with detoxification enzymes of insect-susceptible and resistant armyworm. *Pestic. Biochem. Physiol.* 22:60–68.

YU, S. J., and HSU, E. L. 1993. Induction of detoxification enzymes in phytophagous insects: Roles of insecticide synergists, larval age and species. *Arch Insect Biochem. Physiol.* 24:21–32.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

SYNTHESIS AND ANTIALGAL ACTIVITY OF DIHYDROPHENANTHRENES AND PHENANTHRENES II: MIMICS OF NATURALLY OCCURRING COMPOUNDS IN *Juncus effusus*

M. DELLAGRECA,[1] A. FIORENTINO,[2] P. MONACO,[1] G. PINTO,[3] L. PREVITERA,[1,*] and A. ZARRELLI[1]

[1]*Dipartimento di Chimica Organica e Biologica*
Università Federico II
Via Mezzocannone 16, I-80134 Naples, Italy

[2]*Dipartimento di Scienze della Vita*
II Università di Napoli
Via Arena, I-84100 Caserta, Italy

[3]*Dipartimento di Biologia Vegetale*
Università Federico II
Via Foria 223, I-80139 Naples, Italy

(Received November 15, 1999; accepted September 30, 2000)

Abstract—9,10-Dihydrophenanthrenes and phenanthrenes, mimics of natural compounds with strong antialgal activity, have been synthesized through cross-coupling by zerovalent Ni of 1-(2-iodo-5-methoxy)-phenylethanol or 2-iodo-5-methoxyacetophenone with iodoxylenes. The synthetic compounds had a hydroxyl or a methoxyl group at C-2 and two methyls in the C ring. Assays on the green alga *Selenastrum capricornutum* showed that all the compounds, except 2-methoxy-5,7-dimethylphenanthrene, caused strong inhibition of algal growth at 10^{-4} M. 2-Hydroxy-7,8-dimethyl-9,10-dihydrophenanthrene and 2-methoxy-5,6-dimethylphenanthrene fully inhibited growth at 10^{-5} M.

Key Words—*Juncus effusus*, phenanthrenes, 9,10-dihydrophenanthrenes, cross-coupling algicides, *Selenastrum capricornutum*.

INTRODUCTION

We reported in a previous paper (DellaGreca et al., 2000) the synthesis of 9,10-dihydrophenanthrenes 1a–1l and phenanthrenes 2a–2l, mimics of natural com-

*To whom correspondence should be addressed.

0098-0331/01/0200-0257$19.50/0

pounds isolated from the wetland plant, *Jancus effusus* (DellaGreca et al., 1992, 1993, 1995a,b, 1997). The compounds have the common feature of a hydroxyl or a methoxyl group at the C-2 position of the A ring, while a methyl group is located at different positions of the C ring. Synthesis was based on cross-coupling of 1-(2-iodo-5-methoxy)-phenylethanol (**3**) with the three isomeric iodotoluenes. The compounds, except 7-methyl-2-methoxyphenanthrene (**2g**), showed strong antialgal activity in assays of the freshwater green alga *Selenastrum capricornutum* at 10^{-4} M concentration. Most of them also retained strong activity at 10^{-5} M.

In pursuing the study of the structure–activity relationship, we have now synthesized all the isomeric phenanthrenes and 9,10-dihydrophenanthrenes with a hydroxyl or a methoxyl at C-2 bearing two methyls in the C ring. The synthesis was based on the cross-coupling of iodoarenes by zerovalent nickel (Semmelhack et al., 1981).

METHODS AND MATERIALS

Chemicals. 3-Iodo-4-methylbenzoic acid and iodobenzenes were obtained commercially (Aldrich). 1-(2-Iodo-5-methoxy)-phenylethanol (**3**) was prepared as reported by DellaGreca et al. (2000).

General Experimental Procedures. NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker AC 400 spectrometer in $CDCl_3$ solutions. One-bond and long-range H-C COSY experiments were performed with the XHCORR microprogram with delays corresponding to $J_{C,H}$ = 160 Hz and 8 Hz, respectively. HPLC was performed on a Varian Vista 5500 with a UV detector.

*Synthesis of 2-Hydroxy-6,7-dimethyl-9,10-dihydrophenanthrene (**1t**), 2-Hydroxy-7,8-dimethyl-9,10-dihydrophenanthrene (**1x**), 2-Hydroxy-6,7-dimethylphenanthrene (**2t**), and 2-Hydroxy-7,8-dimethylphenanthrene (**2x**).* To a 50-ml three-necked flask, zinc dust (392 mg, 6 mmol washed with 2 N HCl, H_2O, EtOH, Me_2CO, Et_2O, and dried in an oven at 120°C), $NiCl_2$ (784 mg, 6 mmol), PPh_3 (6.3 g, 24 mmol), and dry DMF (10 ml) were added. The mixture was warmed at 40°C under N_2 and stirred for 1 hr. A solution of **3** (834 mg, 3 mmol) and iodo-3,4-dimethylbenzene (696 mg, 3 mmol) in dry DMF (5 ml) was added at once, and the reaction mixture was kept at 40°C for 6 hr. Then 2 N NH_4OH was added and the mixture was extracted with Et_2O. After evaporation the residue was chromatographed on silica gel (benzene–Et_2O 19 : 1) to afford biphenyl **5b** (360 mg, 1.4 mmol, 47%): ^{1}H NMR δ 7.21 (1H, d, J = 2.3 Hz, H-3), 6.84 (1H, dd, J = 2.3 and 8.4 Hz, H-5), 7.15 (1H, d, J = 8.4 Hz, H-6), 7.05 (1H, d, J = 2.3 Hz, H-2′), 7.16 (1H, d, J = 8.5 Hz, H-5′), 7.01 (1H, dd, J = 2.3 and 8.5 Hz, H-6′), 5.00 (1H, q, J = 6.4 Hz, H-7), 1.39 (3H, d, J = 6.4 Hz, H-8), 3.86 (3H, s, OMe), 2.30 (3H, s, Me), 2.31 (3H, s, Me).

To a solution of **5b** (360 mg, 1.4 mmol) in dry xylene (1 ml), I_2 (20 mg, 0.08 mol) was added and the mixture was kept at 140°C for 6 hr. Addition of 10% aq $NaHSO_3$ and extraction with Et_2O gave a residue that was chromatographed on silica gel (hexane) to give **6b** (265 mg, 1.1 mmol, 80%): ^{1}H NMR δ 7.24 (1H, d, J = 2.2 Hz, H-3), 6.94 (1H, dd, J = 2.2 and 8.4 Hz, H-5), 7.19 (1H, d, J = 8.4 Hz, H-6), 7.21 (1H, d, J = 2.4 Hz, H-2′), 7.28 (1H, d, J = 8.4 Hz, H-5′), 7.16 (1H, dd, J = 2.4 and 8.5 Hz, H-6′), 6.76 (1H, dd, J = 10.5 and 17.6 Hz, H-7), 5.24 (1H, dd, J = 1.8 and 10.5 Hz, H-8), 5.75 (1H, dd, J = 1.8 and 17.6 Hz, H-8), 3.91 (3H, s, OMe), 2.37 (6H, s, Me).

A sample of **6b** (130 mg, 0.55 mmol) in dry benzene (6.5 ml) in a Pyrex flask was irradiated under an air atmosphere with a 450-W Hanovia lamp at room temperature for 30 min with magnetic stirring to give a mixture of **2s** and **2w** (63 mg, 0.30 mmol, 54%) along with unreacted **6b** (52 mg). **2s** and **2w** were separated by HPLC-NH_2 (hexane–isopropyl ether 98.5 : 1.5) **2s** (28 mg) had: ^{1}H NMR δ 7.22–7.36 (2H, m, H-1 and H-3), 8.60 (1H, d, J = 9.1 Hz, H-4), 8.36 (1H, s, H-5), 7.63 (1H, s, H-8), 7.63 (1H, d, J = 7.4 Hz, H-9), 7.48 (1H, d, J = 7.4 Hz, H-10), 3.97 (3H, s, OMe), 2.55 (3H, s, Me), 2.49 (3H, s, Me); ^{13}C NMR δ 117.0 (C-1), 157.2 (C-2), 108.9 (C-3), 124.6 (C-4), 122.8 (C-5), 134.9 (C-6), 133.8 (C-7), 129.5 (C-8), 125.8 (C-9), 127.3 (C-10), 132.6 (C-1a), 125.9 (C-4a), 127.9 (C-5a), 133.9 (C-8a) 55.6 (OMe), 20.8 (Me), 22.9 (Me). Compound **2w** (27 mg) had: ^{1}H NMR δ 7.22–7.36 (2H, m, H-1 and H-3), 8.62 (1H, d, J = 9.3 Hz, H-4), 8.39 (1H, d, J = 9.3 Hz, H-5), 7.71 (1H, d, J = 9.3 Hz, H-6), 8.02 (1H, d, J = 8.4 Hz, H-9), 7.57 (1H, d, J = 8.4 Hz, H-10), 3.98 (3H, s, OMe), 2.55 (3H, s, Me), 2.83 (3H, s, Me); ^{13}C NMR δ 117.3 (C-1), 157.3 (C-2), 108.5 (C-3), 124.3 (C-4), 120.0 (C-5), 128.9 (C-6), 133.9 (C-7), 136.1 (C-8), 123.8 (C-9), 126.4 (C-10), 132.7 (C-1a), 126.6 (C-4a), 128.1 (C-5a), 129.8 (C-8a), 55.6 (OMe), 20.1 (Me), 21.0 (Me).

To a solution of **2s** (26 mg, 0.12 mmol) in dry CH_2Cl_2 (1.5 ml) cooled at −70°C, BBr_3 (100 μl) was added. Addition of 1 N $NaHCO_3$ after 3 hr and extraction with $CHCl_3$ gave a residue that was purified by preparative TLC (hexane–Et_2O 1 : 1) to give **2t** (18 mg, 0.08 mmol, 70%): ^{1}H NMR δ 7.21 (1H, d, J = 2.1 Hz, H-1), 7.19 (1H, dd, J = 2, 1 and 9.2 Hz, H-3), 8.56 (1H, d, J = 9.2 Hz, H-4), 8.34 (1H, s, H-5), 7.60 (1H, s, H-8), 7.62 (1H, d, J = 7.4 Hz, H-9), 7.46 (1H, d, J = 7.4 Hz, H-10), 2.52 (3H, s, Me), 2.45 (3H, s, Me); ^{13}C NMR δ 116.6 (C-1), 153.8 (C-2), 111.7 (C-3), 124.9 (C-4), 122.7 (C-5), 135.0 (C-6), 133.8 (C-7), 129.5 (C-8), 125.4 (C-9), 127.5 (C-10), 132.7 (C-1a), 125.8 (C-4a), 128.0 (C-5a), 133.5 (C-8a) 20.8 (Me), 21.1 (Me).

To a solution of **2w** (25 mg, 0.12 mmol) in dry CH_2Cl_2 (1.5 ml) cooled at −70°C, BBr_3 (100 μl) was added. Addition of 1 N $NaHCO_3$ after 3 hr and extraction with $CHCl_3$ gave a residue that was purified by preparative TLC (hexane–Et_2O 1 : 1) to give **2x** (17 mg, 0.09 mmol, 70%): ^{1}H NMR δ 7.21 (1H, d, J = 2.3 Hz, H-1), 7.19 (1H, dd J = 2.3 and 9.2 Hz), 8.58 (1H, d, J = 9.2 Hz,

H-4), 8.37 (1H, d, J = 9.3 Hz, H-5), 7.70 (1H, d, J = 9.3 Hz, H-6), 8.00 (1H, d, J = 8.4 Hz, H-9), 7.52 (1H, d, J = 8.4 Hz, H-10), 2.52 (3H, s, Me), 2.65 (3H, s, Me); ^{13}C NMR δ 116.9 (C-1), 154.0 (C-2), 112.0 (C-3), 124.6 (C-4), 119.9 (C-5), 128.9 (C-6), 133.9 (C-7), 136.2 (C-8), 124.0 (C-9), 126.1 (C-10), 133.1 (C-1a), 126.4 (C-4a), 128.1 (C-5a), 129.8 (C-8a), 20.1 (Me), 20.8 (Me).

To an EtOH solution (4.8 ml) of **2s** (26 mg, 0.12 mmol), $NaBH_4$ (0.3 mmol) and Et_3N (1.2 mmol) were added. The reaction mixture was irradiated for 2.5 hr with a Hanovia 450-W lamp and then HCl 10% was added. $CHCl_3$ extraction gave a crude product, which was chromatographed by preparative TLC (hexane–benzene 9 : 1) to give **1s** (13 mg, 0.06 mmol, 50%): ^{1}H NMR δ 6.72 (1H, d, J = 2.3 Hz, H-1), 6.88 (1H, dd, J = 2.3 and 8.4 Hz, H-3), 7.72 (1H, d, J = 8.2 Hz, H-4), 7.03 (1H, s, H-5), 7.49 (1H, s, H-8), 2.83 (4H, brs, H-9 and H-10), 3.85 (3H, s, OMe) 2.33 (3H, s, Me), 2.28 (3H, s, Me); ^{13}C NMR δ 112.2 (C-1), 159.1 (C-2), 113.5 (C-3), 124.5 (C-4), 124.3 (C-5), 134.9 (C-6), 134.9 (C-7), 129.4 (C-8), 28.6 (C-9), 29.8 (C-10), 137.9 (C-1a), 127.9 (C-4a), 138.9 (C-5a), 132.0 (C-8a), 55.3 (OMe), 19.7 (Me), 19.4 (Me).

BBr_3 (50 μl) treatment of **1s** (10 mg, 0.05 mmol) in dry CH_2Cl_2 (1 ml) at -70°C for 3 hr gave **1t** (7 mg, 0.03 mmol): ^{1}H NMR δ 6.74 (1H, d, J = 2.2 Hz, H-1), 6.78 (1H, dd, J = 2.2 and 8.4 Hz, H-3), 7.63 (2H, d, J = 8.4 Hz, H-4), 7.03 (1H, s, H-5), 7.47 (1H, s, H-8), 2.81 (4H, brs, H-9 and H-10), 2.32 (3H, s, Me), 2.28 (3H, s, Me); ^{13}C NMR δ 113.7 (C-1), 154.1 (C-2), 114.9 (C-3), 124.8 (C-4), 124.3 (C-5), 135.0 (C-6), 135.0 (C-7), 129.4 (C-8), 28.5 (C-9), 29.5 (C-10), 137.8 (C-1a), 127.7 (C-4a), 139.2 (C-5a), 132.0 (C-8a), 19.7 (Me), 19.4 (Me).

Photoreduction of **2w** (25 mg, 0.12 mmol), as described for **2s**, gave **1w** (11 mg, 0.06 mmol, 50%): ^{1}H NMR δ 6.79 (1H, d, J = 2.4 Hz, H-1), 6.84 (1H, dd, J = 2.4 and 8.5 Hz, H-3), 7.66 (1H, d, J = 8.5 Hz, H-4), 7.49 (1H, d, J = 8.1 Hz, H-5), 7.09 (1H, d, J = 8.1 Hz, H-6), 2.85 (4H, brs, H-9 and H-10), 3.84 (3H, s, OMe) 2.34 (3H, s, Me), 2.26 (3H, s, Me); ^{13}C NMR δ 112.3 (C-1), 158.6 (C-2), 113.0 (C-3), 125.0 (C-4), 120.6 (C-5), 128.1 (C-6), 134.9 (C-7), 134.8 (C-8), 25.3 (C-9), 29.4 (C-10), 135.1 (C-1a), 128.1 (C-4a), 138.6 (C-5a), 132.2 (C-8a), 55.3 (OMe), 15.4 (Me), 20.8 (Me).

Demethylation by BBr_3 of **1w** (10 mg, 0.06 mmol) gave **1x** (6 mg, 0.04 mmol, 67%): ^{1}H NMR δ 6.72 (1H, d, J = 2.4 Hz, H-1), 6.77 (1H, dd, J = 2.4 and 8.5 Hz, H-3), 7.61 (1H, d, J = 8.5 Hz, H-4), 7.49 (1H, d, J = 8.1 Hz, H-5), 7.11 (1H, d, J = 8.1 Hz, H-6), 2.84 (4H, brs, H-9 and H-10), 2.36 (3H, s, Me), 2.28 (3H, s, Me); ^{13}C NMR δ 113.7 (C-1), 154.6 (C-2), 114.4 (C-3), 125.3 (C-4), 120.6 (C-5), 128.1 (C-6), 134.9 (C-7), 135.2 (C-8), 25.2 (C-9), 29.5 (C-10), 135.2 (C-1a), 128.4 (C-4a), 138.7 (C-5a), 132.3 (C-8a), 15.4 (Me), 20.8 (Me).

*Synthesis of 2-Hydroxy-6,8-dimethyl-9,10-dihydrophenanthrene (**1v**) and 2-Hydroxy-6,8-dimethylphenanthrene (**2v**).* A solution of **3** (3 mmol) and iodo-3,5-dimethylbenzene (3 mmol) in dry DMF (5 ml) was added to the mixture of the active nickel complex prepared as reported above. Work-up after 9 hr at 35°C

and chromatography on silica gel (benzene–Et_2O 19 : 1) gave biaryl **5a** (273 mg, 1.1 mmol, 37%): ^{1}H NMR δ 7.24 (1H, d, J = 2.2 Hz, H-3), 6.86 (1H, dd, J = 2.2 and 8.3 Hz, H-5), 7.08 (1H, d, J = 8.3 Hz, H-6), 6.92 (3H, d, J = 2.1 Hz, H-2′ and H-4′) 7.01 (1H, brs, H-6′), 5.01 (1H, q, J = 6.2 Hz, H-7), 1.41 (3H, d, J = 6.2 Hz, H-8), 3.86 (3H, s, OMe), 2.38 (6H, s, Me).

To a solution of **5a** (270 mg, 1.1 mmol) in dry xylene (1 ml), I_2 (20 mg, 0.08 mol) was added and the mixture was kept at 140°C for 6 hr. Work-up as for **6b** gave **6a** (212 mg, 0.88 mmol, 80%): ^{1}H NMR δ 7.30 (1H, d, J = 2.2 Hz, H-3), 6.98 (1H, dd, J = 2.2 and 8.4 Hz, H-5), 7.32 (1H, d, J = 8.4 Hz, H-6), 7.09 (2H, brs, H-2′ and H-6′), 7.18 (1H, brs, H-4′), 6.88 (1H, dd, J = 10.6 and 17.5 Hz, H-7), 5.32 (1H, dd, J = 1.8 and 10.6 Hz, H-8), 5.83 (1H, dd, J-1.8 and 17.5 Hz, H-8), 3.98 (3H, s, OMe), 2.49 (6H, s, Me).

Photocyclization of **6a** (100 mg, 0.4 mmol) in an Ar atmosphere for 1 hr gave 2-methoxy-6,8-dimethyl-9,10-dihydrophenanthrene (**1u**) (74 mg, 0.3 mmol, 75%): ^{1}H NMR δ 6.81 (1H, d, J = 2.4 Hz, H-1), 6.88 (1H, dd, J = 2.4 and 8.5 Hz, H-3), 7.71 (1H, d, J = 8.5 Hz, H-4), 6.95 (1H, d, J = 2.3 Hz, H-5), 7.41 (1H, d, J = 2.3 Hz, H-7), 2.82 (4H, brs, H-9 and H-10), 3.86 (3H, s, OMe) 2.37 (3H, s, Me), 2.33 (3H, s, Me); ^{13}C NMR δ 112.3 (C-1), 158.9 (C-2), 113.2 (C-3), 125.3 (C-4), 121.8 (C-5), 135.2 (C-6), 129.4 (C-7), 134.4 (C-8), 24.5 (C-9), 29.4 (C-10), 135.6 (C-1a), 128.0 (C-4a), 138.9 (C-5a), 132.0 (C-8a), 55.3 (OMe), 21.3 (Me), 19.7 (Me). BBr_3 demethylation of **1u** (30 mg, 0.18 mmol) gave **1v** (20 mg, 0.12 mmol, 68%): ^{1}H NMR δ 6.72 (1H, d, J = 2.5 Hz, H-1), 6.78 (1H, dd, J = 2.5 and 8.3 Hz, H-3), 7.64 (1H, d, J = 8.3 Hz, H-4), 6.93 (1H, d, J = 2.3 Hz, H-5), 7.41 (1H, d, J = 2.3 Hz, H-7), 2.80 (4H, brs, H-9 and H-10), 2.39 (3H, s, Me), 2.33 (3H, s, Me); ^{13}C NMR δ 113.7 (C-1), 154.7 (C-2), 114.6 (C-3), 125.4 (C-4), 121.6 (C-5), 135.1 (C-6), 129.4 (C-7), 134.8 (C-8), 24.3 (C-9), 29.1 (C-10), 135.5 (C-1a), 128.1 (C-4a), 139.2 (C-5a), 131.9 (C-8a), 21.2 (Me), 19.7 (Me).

Photocyclization of **6a** (100 mg, 0.4 mmol) in the presence of atmospheric oxygen gave **2u** (70 mg, 0.3 mmol, 75%): ^{1}H NMR δ 7.22–7.36 (2H, m, H-1 and H-3), 8.52 (1H, d, J = 9.2 Hz, H-4), 8.30 (1H, d, J = 1.9 Hz, H-5), 7.22 (1H, d, J = 1.9 Hz, H-7), 7.91 (1H, d, J = 7.4 Hz, H-9), 7.57 (1H, d, J = 7.4 Hz, H-10), 3.98 (3H, s, OMe), 2.72 (3H, s, Me), 2.52 (3H, s, Me); ^{13}C NMR δ 117.0 (C-1), 158.2 (C-2), 108.3 (C-3), 124.6 (C-4), 120.1 (C-5), 134.7 (C-6), 123.5 (C-7), 135.9 (C-8), 125.3 (C-9), 128.7 (C-10), 133.3 (C-1a), 124.8 (C-4a), 127.8 (C-5a), 130.7 (C-8a), 55.4 (OMe), 22.0 (Me), 19.8 (Me). Demethylation of **2u** (35 mg, 0.2 mmol) by BBr_3 gave **2v** (26 mg, 0.14 mmol, 70%): ^{1}H NMR δ 7.22–7.36 (2H, m, H-1 and H-3), 8.61 (1H, d, J = 9.2 Hz, H-4), 8.28 (1H, d, J = 1.9 Hz, H-5), 7.22 (1H, d, J = 1.9 Hz, H-7), 7.91 (1H, d, J = 7.4 Hz, H-9), 7.58 (1H, d, J = 7.4 Hz, H-10), 2.71 (3H, s, Me), 2.57 (3H, s, Me); ^{13}C NMR δ 117.1 (C-1), 153.0 (C-2), 111.5 (C-3), 124.9 (C-4), 120.2 (C-5), 134.9 (C-6), 123.5 (C-7), 136.0 (C-8), 125.3 (C-9), 128.7 (C-10), 133.7 (C-1a), 124.6 (C-4a), 127.8 (C-5a), 130.4 (C-8a), 22.2 (Me), 20.0 (Me).

*Synthesis of 2-Hydroxy-5,6-dimethyl-9,10-dihydrophenanthrene (**1n**) and 2-Hydroxy-5,6-dimethylphenanthrene (**2n**).* 2-Iodo-5-methoxyacetophenone (830 mg, 3 mmol), obtained by reaction of equimolar amounts of 3-methoxyacetophenone, I_2, CF_3COOAg, and iodo-2,3-dimethylbenzene (696 mg, 3 mmol) were added to a flask containing active Ni, prepared as described for **1t**. The reaction mixture was kept under N_2 at 40°C for 50 hr with magnetic stirring. Addition of 2 N NH_4OH and extraction with Et_2O gave crude **4c**, which was purified by silica gel column chromatography (hexane–Et_2O 19 : 1). Pure **4c** (300 mg, 1.3 mmol, 43%) gave the following data: ^{1}H NMR δ 7.20 (1H, d, J = 2.3 Hz, H-3), 6.97 (1H, dd, J = 2.3 and 8.3 Hz, H-5), 7.16 (1H, d, J = 8.3 Hz, H-6), 7.02–7.18 (3H, m, H-4′–H-6′), 3.89 (3H, s, OMe), 2.33 (3H, s, H-8), 2.04 (3H, s, Me), 1.92 (3H, s, Me). A pure sample of **4c** (200 mg), dissolved in MeOH (10 ml), was treated with $NaBH_4$ excess at 40°C for 20 min. The reaction mixture was added to 10% HCl and extracted with Et_2O. Preparative TLC (benzene–Et_2O, 47 : 3) gave pure **5c** (180 mg): ^{1}H NMR δ 7.18 (1H, d, J = 2.1 Hz, H-3), 6.97 (1H, dd, J = 2.1 and 8.4 Hz, H-5), 7.14 (1H, d, J = 8.4 Hz, H-6), 7.00–7.23 (3H, m H-4′–H-6′), 4.60 and 4.72 (1H, q, J = 6.2 Hz, H-7), 1.29 (3H, d, J = 6.2 Hz, H-8, 3.89 (3H, s, OMe), 2.01 and 1.96 (3H, s, Me), 2.36 (3H, s, Me).

To a solution of **5c** (180 mg, 0.7 mmol) in dry xylene (1 ml), I_2 (20 mg, 0.08 mol) was added, and the mixture was kept at 140°C for 6 hr. Work-up as for **6b** gave **6c** (143 mg, 0.6 mmol, 67%): ^{1}H NMR δ 6.90 (1H, dd, J = 2.3 and 8.3 Hz, H-5), 6.95–7.20 (5H, m), 6.41 (1H, dd, J = 11.0 and 17.1 Hz, H-7), 5.64 (1H, dd, J = 1.8 and 17.1 Hz, H-8), 5.11 (1H, dd, J = 1.8 and 11.0 Hz, H-8), 3.90 (3H, s, OMe), 2.35 (3H, s, Me), 1.98 (3H, s, Me). Photocyclization of **6c** (73 mg, 0.3 mmol) in an Ar atmosphere for 1 hr gave 2-methoxy-5,6-dimethyl-9,10-dihydrophenanthrene (**1m**) (55 mg, 0.2 mmol, 67%): ^{1}H NMR δ 6.86 (1H, d, J = 2.1 Hz, H-1), 6.84 (1H, dd, J = 2.1 and 8.5 Hz, H-3), 7.55 (1H, d, J = 8.5 Hz, H-4), 7.03 (2H, brs, H-7 and H-8), 2.73 (4H, brs, H-9 and H-10), 3.87 (3H, s, OMe) 2.50 (3H, s, Me), 2.35 (3H, s, Me); ^{13}C NMR δ 111.2 (C-1), 159.0 (C-2), 113.4 (C-3), 130.4 (C-4), 135.1 (C-5), 136.7 (C-6), 128.1 (C-7), 125.0 (C-8), 31.0 (C-9), 31.0 (C-10), 137.8 (C-1a), 128.2 (C-4a), 141.8 (C-5a), 132.8 (C-8a), 55.6 (OMe), 21.4 (Me), 19.4 (Me). BBr_3 demethylation of **1m** (45 mg, 0.17 mmol) gave **1n** (30 mg, 0.12 mmol, 66%): ^{1}H NMR δ 6.78 (1H, d, J = 2.4 Hz, H-1), 6.75 (1H, dd, J = 2.4 and 8.3 Hz, H-3), 7.48 (1H, d, J = 8.3 Hz, H-4), 7.03 (2H, brs, H-7 and H-8), 2.71 (4H, brs, H-9 and H-10), 2.49 (3H, s, Me), 2.35 (3H, s, Me); ^{13}C NMR δ 112.3 (C-1), 154.1 (C-2), 114.4 (C-3), 130.2 (C-4), 135.0 (C-5), 136.3 (C-6), 127.7 (C-7), 124.7 (C-8), 30.4 (C-9), 30.6 (C-10), 136.8 (C-1a), 127.9 (C-4a), 142.3 (C-5a), 132.6 (C-8a), 21.0 (Me), 19.0 (Me).

Photocyclization of **6c** (70 mg, 0.3 mmol) in the presence of atmospheric oxygen gave **2m** (50 mg, 0.18 mmol, 65%): ^{1}H NMR δ 7.28 (1H, d, J = 2.3 Hz, H-1), 7.21 (1H, dd, J = 2.3 and 9.2 Hz, H-3), 8.68 (1H, d, J = 9.2 Hz, H-4), 7.64 (1H, d, J = 8.7 Hz, H-7), 7.65 (1H, d, J = 8.7 Hz, H-8), 7.57 (1H, d, J = 7.9 Hz,

H-9), 7.38 (1H, d, J = 7.9 Hz, H-10), 3.98 (3H, s, OMe), 2.94 (3H, s, Me), 2.57 (3H, s, Me); ^{13}C NMR δ 114.8 (C-1), 157.4 (C-2), 108.8 (C-3), 129.8 (C-4), 135.4 (C-5), 136.4 (C-6), 128.2 (C-7), 125.8 (C-8), 128.0 (C-9), 126.3 (C-10), 132.6 (C-1a), 125.1 (C-4a), 129.7 (C-5a), 131.1 (C-8a), 55.4 (OMe), 22.0 (Me), 21.7 (Me). Demethylation of **2m** (50 mg, 0.18 mmol) by BBr_3 gave **2n** (30 mg, 0.12 mmol, 63%): ^{1}H NMR δ 7.27 (1H, d, J = 2.3 Hz, H-1), 7.15 (1H, dd, J = 2.3 and 9.2, H-3), 8.65 (1H, d, J = 9.2 Hz, H-4), 7.52 (1H, d, J = 8.6 Hz, H-7), 7.63 (1H, d, J = 8.6 Hz, H-8), 7.63 (1H, d, J = 7.9 Hz, H-9), 7.39 (1H, d, J = 7.9 Hz, H-10), 2.92 (3H, s, Me), 2.55 (3H, s, Me); ^{13}C NMR δ 114.4 (C-1), 153.1 (C-2), 111.7 (C-3), 130.0 (C-4), 135.3 (C-5), 136.5 (C-6), 128.3 (C-7), 126.1 (C-8), 127.9 (C-9), 125.2 (C-10), 132.4 (C-1a), 125.4 (C-4a), 129.8 (C-5a), 130.9 (C-8a), 21.8 (Me), 21.6 (Me).

*Synthesis of 2-Hydroxy-5,7-dimethyl-9,10-dihydrophenanthrene (**1p**) and 2-Hydroxy-5,7-dimethylphenanthrene (**2p**).* 2-Iodo-5-methoxyacetophenone (830 mg, 3 mmol) and iodo-2,3-dimethylbenzene (690 mg, 3 mmol) were cross coupled as described for **1n**. The reaction mixture was kept under N_2 at 40°C for 45 hr under magnetic stirring. Work-up gave **4d**, which was purified by column chromatography (hexane–Et_2O 9 : 1). Pure **4d** (350 mg, 1.5 mmol, 50%) gave the following data: ^{1}H NMR δ 7.00–7.11 (4H, m, H-3–H-6, H-5′), 7.19 (1H, d, J = 2.4 Hz, H-3′), 7.16 (1H, dd, J = 2.4 and 8.3 Hz, H-6′), 3.89 (3H, s, OMe), 2.37 (3H, s, H-8), 2.11 (3H, s, Me), 1.94 (3H, s, Me). $NaBH_4$ excess reduction of **4d** (300 mg, 1.3 mmol) gave crude **5d**, purified by preparative TLC (benzene–Et_2O 4 : 1). **5d** (270 mg, 1.1 mmol, 90%) had: ^{1}H NMR δ 7.20 (1H, d, J = 2.7 Hz, H-3), 6.85 (1H, dd, J = 2.7 and 8.4 Hz, H-5), 6.96–7.12 (4H, m, H-6, H-4′–H-6′), 4.61 and 4.75 (1H, q, J = 6.5 Hz, H-7), 1.30 and 1.32 (3H, d, J = 6.5 Hz, H-8), 3.89 (3H, s, OMe), 2.06 and 2.02 (3H, s, Me), 2.38 (3H, s, Me).

To a solution of **5d** (270 mg, 1.1 mmol) in dry xylene (1 ml), I_2 (30 mg, 0.14 mmol) was added and the mixture was kept at 140°C for 5 hr. Work-up as for **6b** gave **6d**, purified by preparative TLC (hexane), (217 mg, 0.9 mmol, 81%): ^{1}H NMR δ 7.19 (1H, d, J = 2.3 Hz, H-3), 6.88 (1H, dd, J = 2.3 and 8.6, H-5), 7.00–7.10 (4H, m, H-6, H-3′–H-6′), 6.42 (1H, dd, J = 11.0 and 17.1 Hz, H-7), 5.65 (1H, dd, J = 1.8 and 17.1 Hz, H-8), 5.10 (1H, dd, J = 1.8 and 11.0 Hz, H-8), 3.89 (3H, s, OMe), 2.38 (3H, s, Me), 2.03 (3H, s, Me).

Photocyclization of **6d** (100 mg, 0.4 mmol) in an Ar atmosphere for 1 hr gave 2-methoxy-5,6-dimethyl-9,10-dihydrophenanthrene (**1o**) (75 mg, 0.3 mmol, 75%): ^{1}H NMR δ 6.87 (1H, d, J = 2.1 Hz, H-1), 6.83 (1H, dd, J = 2.1 and 8.5 Hz, H-3), 7.61 (1H, d, J = 8.5 Hz, H-4), 7.02 (1H, brs, H-6), 6.95 (1H, brs, H-8), 2.74 (4H, brs, H-9 and H-10), 3.87 (3H, s, OMe) 2.59 (3H, s, Me), 2.34 (3H, s, Me); ^{13}C NMR δ 110.8 (C-1), 158.2 (C-2), 113.2 (C-3), 131.2 (C-4), 134.0 (C-5), 129.0 (C-6), 136.0 (C-7), 126.3 (C-8), 30.5 (C-9), 30.7 (C-10), 138.8 (C-1a), 127.9 (C-4a), 141.3 (C-5a), 131.4 (C-8a), 55.2 (OMe), 22.9 (Me), 20.9 (Me). BBr_3 demethylation of **1o** (45 mg, 0.17 mmol) gave **1p** (30 mg, 0.12 mmol,

66%): ^{1}H NMR δ 6.77 (1H, d, J = 2.4 Hz, H-1), 6.74 (1H, dd, J = 2.4 and 8.3 Hz, H-3), 7.52 (1H, d, J = 8.3 Hz, H-4), 6.99 (1H, brs, H-6), 6.93 (1H, brs, H-8), 2.71 (4H, brs, H-9 and H-10), 2.57 (3H, s, Me), 2.33 (3H, s, Me); ^{13}C NMR δ 1124 (C-1), 153.8 (C-2), 114.4 (C-3), 131.1 (C-4), 133.7 (C-5), 129.2 (C-6), 135.6 (C-7), 126.3 (C-8), 30.3 (C-9), 30.6 (C-10), 139.0 (C-1a), 127.9 (C-4a), 141.8 (C-5a), 131.1 (C-8a), 22.9 (Me), 20.9 (Me).

Photocyclization of **6c** (100 mg, 0.4 mmol) in the presence of atmospheric oxygen gave **2o** (73 mg, 0.3 mmol, 75%): ^{1}H NMR δ 7.22–7.38 (3H, m, H-1, H-3 and H-10), 8.89 (1H, d, J = 9.0 Hz, H-4), 7.67 (2H, s, H-6 and H-8), 7.64 (1H, d, J = 8.4 Hz, H-9), 4.00 (3H, s, OMe), 3.13 (3H, s, Me), 2.55 (3H, s, Me); ^{13}C NMR δ 115.5 (C-1), 157.1 (C-2), 109.3 (C-3), 134.6 (C-4), 134.9 (C-5), 128.5 (C-6), 134.6 (C-7), 127.3 (C-8), 128.2 (C-9), 126.8 (C-10), 133.0 (C-1a), 126.2 (C-4a), 128.1 (C-5a), 131.4 (C-8a), 55.4 (OMe), 27.3 (Me), 21.1 (Me). Demethylation of **2o** (36 mg, 0.2 mmol) by BBr_3 gave **2p** (27 mg, 0.14 mmol, 70%): ^{1}H NMR δ 7.26 (1H, d, J = 1.9 Hz, H-1), 7.21 (1H, dd, J = 1.9 and 9.2, H-3), 8.81 (1H, d, J = 9.2 Hz, H-4), 7.68 (2H, s, H-6 and H-8), 7.58 (1H, d, J = 8.4 Hz, H-9), 7.30 (1H, d, J = 8.4 Hz, H-10), 3.01 (3H, s, Me), 2.51 (3H, s, Me); ^{13}C NMR δ 115.2 (C-1), 152.9 (C-2), 112.1 (C-3), 132.9 (C-4), 134.9 (C-5), 128.6 (C-6), 134.5 (C-7), 127.2 (C-8), 128.1 (C-9), 126.0 (C-10), 133.1 (C-1a), 126.2 (C-4a), 127.5 (C-5a), 130.2 (C-8a), 27.2 (Me), 20.9 (Me).

*Synthesis of 2-Hydroxy-5,8-dimethyl-9,10-dihydrophenanthrene (**1r**) and 2-Hydroxy-5,8-dimethyl-phenanthrene (**2r**).* Equimolar amounts of methyl 3-iodo-4-methylbenzoate (830 mg, 3 mmol), obtained from 3-iodo-4-methylbenzoic acid by CH_2N_2 treatment in MeOH, and **3** (830 mg, 3 mmol) were added to the flask containing the active Ni complex. After 35 hr at 40°C in N_2 atmosphere, the reaction mixture was worked up as previously described and chromatography on a silica gel column (hexane–Et_2O, 7 : 3) gave diastereoisomeric biphenyls **5e** (245 mg, 1 mmol 34%): ^{1}H NMR δ 7.31 (1H, d, J = 2.3 Hz, H-3), 6.91 (1H, dd, J = 2.3 and 8.4 Hz, H-5), 7.08 (1H, d, J = 8.4 Hz, H-6), 7.31 (1H, d, J = 8.4 Hz, H-3′), 7.92 (1H, dd, J = 2.1 and 8.4 Hz, H-4′), 7.85 (1H, d, J = 2.1 Hz, H-6′), 4.51 and 4.69 (1H, q, J = 6.5 Hz, H-7), 1.26 and 1.28 (3H, d, J = 6.5 Hz, H-8), 3.87 (6H, s, OMe), 2.10 and 2.14 (3H, s, Me). Dehydration of **5e** (245 mg, 1 mmol) as reported gave **6e**, purified by silica gel column chromatography (hexane–Et_2O, 4 : 1) (205 mg, 0.8 mmol, 80%): ^{1}H NMR δ 7.20 (1H, d, J = 2.3 Hz, H-3), 6.89 (1H, dd, J = 2.3 and 8.4 Hz, H-5), 7.07 (1H, d, J = 8.4 Hz, H-6), 7.31 (1H, d, J = 8.4 Hz, H-3′), 7.99 (1H, dd, J = 2.1 and 8.4 Hz, H-4′), 7.81 (1H, d, J = 2.1 Hz, H-6′), 6.32 (1H, dd, J = 11.0 and 17.1 Hz, H-7), 5.68 (1H, dd, J = 1.8 and 17.1 Hz, H-8), 5.13 (1H, dd, J = 1.8 and 11.0 Hz, H-8), 3.89 (6H, s, OMe), 2.11 (3H, s, Me).

A pure sample of **6e** (200 mg, 0.8 mmol) in dry benzene (6.5 ml) in a Pyrex flask was irradiated under air atmosphere with a 450-W Hanovia lamp at room temperature for 5 hr with magnetic stirring to give **2y** (150 mg, 0.6 mmol,

75%): ^{1}H NMR δ 7.20–7.34 (2H, m, H-1 and H-3), 8.67 (1H, d, J = 8.7, H-4), 7.51 (1H, d, J = 8.5 Hz, H-6), 7.99 (1H, d, J = 8.5 Hz, H-7), 8.78 (1H, d, J = 8.0 Hz, H-9), 7.77 (1H, d, J = 8.0 Hz, H-10), 4.00 (6H, s, OMe), 3.16 (3H, s, Me). A sample of **2y** (115 mg, 0.6 mmol) dissolved in dry toluene (3 ml) was treated with Red-Al solution (3 mmol) for 2 hr. NaOH (2 N) was added and the crude product was extracted with Et_2O and chromatographed on silica gel (hexane–Et_2O 9 : 1) to give **2q** (90 mg, 0.45 mmol, 75%): ^{1}H NMR δ 7.22–7.38 (3H, m, H-1, H-3 and H-7), 8.87 (1H, d, J = 9.0 Hz, H-4), 7.40 (1H, d, J = 8.8 Hz, H-6), 8.00 (1H, d, J = 7.6 Hz, H-9), 7.58 (1H, d, J = 7.6 Hz, H-10), 4.00 (3H, s, OMe), 3.11 (3H, s, Me), 2.75 (3H, s, Me); ^{13}C NMR δ 115.3 (C-1), 157.2 (C-2), 108.7 (C-3), 130.7 (C-4), 134.8 (C-5), 129.2 (C-6), 127.2 (C-7), 134.3 (C-8), 124.0 (C-9), 126.4 (C-10), 132.7 (C-1a), 125.8 (C-4a), 128.5 (C-5a), 132.9 (C-8a), 55.3 (OMe), 274 (Me), 20.3 (Me). Demethylation of **2q** (40 mg, 0.2 mmol) by BBr_3 gave **2r** (35 mg, 0.18 mmol, 90%): ^{1}H NMR δ 7.28 (1H, d, J = 1.9 Hz, H-1), 7.18 (1H, dd, J = 1.9 and 9.2, H-3), 8.81 (1H, d, J = 9.2 Hz, H-4), 7.65 (1H, d, J = 8.5 Hz, H-6), 7.30 (1H, d, J = 8.5 Hz, H-7), 7.95 (1H, d, J = 7.7 Hz, H-9), 7.35 (1H, d, J = 7.7 Hz, H-10), 3.09 (3H, s, Me), 2.73 (3H, s, Me); ^{13}C NMR δ 115.1 (C-1), 153.1 (C-2), 111.7 (C-3), 130.8 (C-4), 134.8 (C-5), 129.6 (C-6), 126.5 (C-7), 134.8 (C-8), 124.3 (C-9), 126.0 (C-10), 132.5 (C-1a), 126.2 (C-4a), 128.1 (C-5a), 132.9 (C-8a), 27.4 (Me), 20.3 (Me).

A sample of **2q** (40 mg, 0.2 mmol) was photoreduced as reported for **2s** to give **1q** (30 mg, 0.15 mmol, 75%): ^{1}H NMR δ 6.85 (1H, d, J = 2.1 Hz, H-1), 6.83 (1H, dd, J = 2.1 and 8.5 Hz, H-3), 7.58 (1H, d, J = 8.5 Hz, H-4), 7.11 (1H, d, J = 8.1 Hz, H-6), 7.01 (1H, d, J = 8.1 Hz, H-7), 2.72 (4H, brs, H-9 and H-10), 3.87 (3H, s, OMe) 2.58 (3H, s, Me), 2.33 (3H, s, Me); ^{13}C NMR δ 110.7 (C-1), 158.1 (C-2), 112.7 (C-3), 129.5 (C-4), 134.3 (C-5), 129.5 (C-6), 127.9 (C-7), 131.3 (C-8), 26.0 (C-9), 30.0 (C-10), 137.4 (C-1a), 127.9 (C-4a), 141.4 (C-5a), 132.1 (C-8a), 55.2 (OMe), 22.9 (Me), 19.9 (Me). BBr_3 demethylation of **1q** (20 mg, 0.1 mmol) gave **1r** (15 mg, 0.09 mmol, 90%): ^{1}H NMR δ 6.76 (1H, d, J = 2.4 Hz, H-1), 6.74 (1H, dd, J = 2.4 and 8.3 Hz, H-3), 7.51 (1H, d, J = 8.3 Hz, H-4), 7.10 (1H, d, J = 8.1 Hz, H-6), 7.00 (1H, d, J = 8.1 Hz, H-7), 2.71 (4H, brs, H-9 and H-10), 2.57 (3H, s, Me), 2.34 (3H, s, Me); ^{13}C NMR δ 112.3 (C-1), 153.7 (C-2), 114.2 (C-3), 129.4 (C-4), 134.1 (C-5), 129.3 (C-6), 128.2 (C-7), 131.6 (C-8), 26.2 (C-9), 30.2 (C-10), 137.6 (C-1a), 127.9 (C-4a), 141.8 (C-5a), 132.2 (C-8a), 22.8 (Me), 20.1 (Me).

Bioassay. The strain UTEX 1648 *Selenastrum capricornutum* was maintained on Bold basal medium (BBM) solidified with agar 1.5% in continuous light at 23°C. Fresh axenic cultures for the experiments were grown in 100-ml cylinders on the same culture medium. For growth tests, the compounds were dissolved in acetone. Each soluion (20 μl) was added to test tubes containing 6 ml of inoculated medium, giving final concentrations of 10^{-4}, 5×10^{-5}, and 10^{-5} M. Controls containing only acetone also were tested. The test tubes were

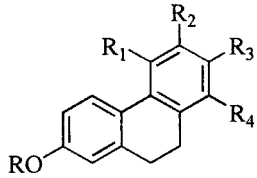
incubated at 23°C on a shaking apparatus previously described (Aliotta et al., 1991). Total irradiation of 150 μE/sec/m^2 was provided by daylight fluorescent lamps (Philips TLD 30 w/55) from below the apparatus. The photoperiod was 16 hr light and 8 hr dark. Growth of cultures was followed daily, either by measuring the absorbance increase at 550 nm with a Bausch & Lomb Spectronic 20 colorimeter or by counting the cell numbers with a Thoma blood-counting chamber. The cell numbers of the initial inocula ranged from 10^6 to 1.5×10^6/ml, corresponding to 0.05–0.06 units of absorbance. Growth experiments were carried out in triplicate. To test statistical significance of results, one-way ANOVA was performed with $P = 0.05$. For each compound, a comparison among means was performed by Student-Newman-Keuls test (SNK), with $P = 0.05$. The statistical package SPSS was used.

The index of inhibition for compounds was calculated as $[1 - (X_a/Y_a)] \times 100$ (Blankley, 1973), where X_a is the growth rate of the alga in the presence of the compound tested and Y_a is the growth rate of the control.

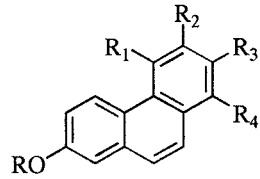
RESULTS AND DISCUSSION

The basic formulas and substitutions of groups for the compounds synthesized are shown in Figure 1. 1-(2-Iodo-5-methoxy)-phenylethanol (**3**), synthesized as reported in the previous paper (DellaGreca et al., 1999), was used as a starting block in the synthesis of 6,7-, 6,8-, and 7,8-dimethyl compounds. In all the reactions, equimolar amounts of **3** and iodoxylene were added at once to the reaction mixture containing the active nickel complex generated in situ by action of dust zinc on $NiCl_2(PPh_3)_2$ (Tiecco et al., 1984). Coupling of **3** with iodo-3,4-dimetilbenzene gave biphenyl **5b**, which was converted into **6b** by dehydration with I_2 in dry xylene at 140°C. All attempts to obtain 2-methoxy-6,7-dimethyl-9,10-dihydrophenanthrene (**1s**) and 2-methoxy-7,8-dimethyl-9,10-dihydrophenanthrene (**1w**) by UV irradiation of **6b** in an Ar atmosphere (Padwa et al., 1977) failed, while photocyclization of **6b** by UV irradiation in an air atmosphere easily gave a mixture of 2-methoxy-6,7-dimethylphenanthrene (**2s**) and 2-methoxy-7,8-dimethylphenanthrene (**2w**), which were separated by HPLC-NH_2. Demethylation by BBr_3 in CH_2Cl_2 for 3 hr at −70°C converted **2s** and **2w** into 2-hydroxy-6,7-dimethylphenanthrene (**2t**) and 2-hydroxy-7,8-dimethylphenanthrene (**2x**).

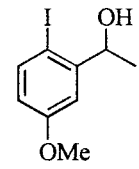
2-Methoxy-6,7-dimethyl-9,10-dihydrophenanthrene (**1s**) and 2-methoxy-7,8-dimethyl-9,10-dihydrophenanthrene (**1w**) were obtained from **2s** and **2w** by photoreduction with $NaBH_4$ in EtOH and Et_3N and UV irradiation (Nien-chu et al., 1984). Subsequent demethylation as above reported gave 2-hydroxy-6,7-dimethyl-9,10-dihydrophenanthrene (**1t**) and 2-hydroxy-7,8-dimethyl-9,10-dihydrophenanthrene (**1x**).



1

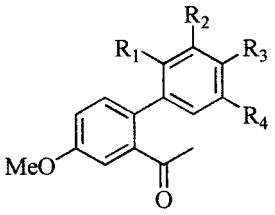


2

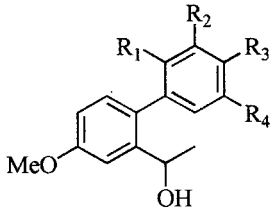


3

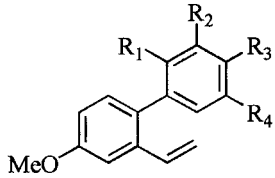
	R	R_1	R_2	R_3	R_4		R	R_1	R_2	R_3	R_4
a	Me	H	H	H	H	o	Me	Me	H	Me	H
b	H	H	H	H	H	p	H	Me	H	Me	H
c	Me	Me	H	H	H	q	Me	Me	H	H	Me
d	H	Me	H	H	H	r	H	Me	H	H	Me
e	Me	H	Me	H	H	s	Me	H	Me	Me	H
f	H	H	Me	H	H	t	H	H	Me	Me	H
g	Me	H	H	Me	H	u	Me	H	Me	H	Me
h	H	H	H	Me	H	v	H	H	Me	H	Me
i	Me	H	H	H	Me	w	Me	H	H	Me	Me
l	H	H	H	H	Me	x	H	H	H	Me	Me
m	Me	Me	Me	H	H	y	Me	Me	H	H	CO_2Me
n	H	Me	Me	H	H						



4



5



6

FIG. 1. The basic structural skeletons of compounds synthesized, and the various groups that were substituted at the positions marked by R_1, R_2, R_3, and R_4.

Coupling of **3** with iodo-3,5-dimethylbenzene gave **5a**, which, through the styrene derivative **5b** was converted by UV irradiation into 2-methoxy-6,8-dimethyl-9,10-dihydrophenanthrene (**1u**) in an argon atmosphere and into 2-methoxy-6,8-dimethylphenanthrene (**2u**) in the presence of atmospheric oxygen. Demethylation of **1u** and **2u** afforded 2-hydroxy-6,8-dimethyl-9,10-dihydrophenanthrene (**1v**) and 2-hydroxy-6,8-dimethylphenanthrene (**2v**).

The synthesis of dimethyl compounds with a methyl at C-5 by cross-coupling of **3** with the right iodoxylene failed as the steric hindrance of

the methyl caused long reaction times, and in such conditions the primary process was replacement of iodine by alcoholic hydrogen. The same problem was overcome in the synthesis of 2-hydroxy-5-methyl-9,10-dihydrophenanthrene (**1d**) and 2-hydroxy-5-methylphenanthrene (**2d**) by cross-coupling ortho iodotoluene with 2-iodo-5-methoxyacetophenone. In the same way, the synthesis of 2-hydroxy-5,6-dimethyl-9,10-dihydrophenanthrene (**1n**), 2-hydroxy-5,7-dimethyl-9,10-dihydrophenanthrene (**1p**), 2-hydroxy-5,6-dimethylphenanthrene (**2n**), and 2-hydroxy-5,7-dimethylphenanthrene (**2p**) was performed. Couplings of 2-iodo-5-methoxyacetophenone with iodo-2,3-dimethylbenzene and iodo-2,4-dimethylbenzene gave ketones **4c** and **4d**, respectively. $NaBH_4$ reduction afforded carbinols **5c** and **5d**. These compounds, obtained as diastereoisomeric mixtures because free rotation of the rings about each other is hindered for the presence of the methyl at C-2′, were dehydrated directly to **6c** and **6d**. Photocyclization of **6c** in the absence and in the presence of atmospheric oxygen gave 2-methoxy-5,6-dimethyl-9,10-dihydrophenanthrene (**1m**) and 2-methoxy-5,6-dimethylphenanthrene (**2m**), respectively, which were converted into **1n** and **2n** by BBr_3 demethylation. Irradiation in both the conditions of **6d** gave 2-methoxy-5,7-dimethyl-9,10-dihydrophenanthrene (**1o**) and 2-methoxy-5,7-dimethylphenanthrene (**2o**), which were demethylated to **1p** and **2p**. All attempts to synthesize 2-hydroxy-5,8-dimethyl-9,10-dihydrophenanthrene (**1r**) and 2-hydroxy-5,8-dimethylphenanthrene (**2r**) this way failed because of very long reaction times in which no coupling between 2-iodo-5-methoxyacetophenone and iodo-2,5-dimethylbenzene was obtained.

Semmelhack (1981) observed an efficient coupling of aryl halides bearing aldehyde, ketone, ester, and nitrile groups. On this basis, **3** was cross-coupled with methyl 3-iodo-4-methylbenzoate to give the corresponding diastereoisomeric biaryls **5e** that were dehydrated to **6e**. Photocyclization of **6e** in both the reported conditions gave 9,10-dihydrophenanthrene (**1y**) and phenanthrene (**2y**), which were converted into 2-methoxy-5,8-dimethyl-9,10-dihydrophenanthrene (**1q**) and 2-methoxy-5,8-dimethylphenanthrene (**2q**) by sodium bis(2-methoxyethoxy)aluminum hydride. Final demethylation of **1q** and **2q** gave **1r** and **2r**.

The 9,10-dihydrophenanthrenes and phenanthrenes purified by HPLC were assayed on *Selenastrum capricornutum* at concentrations of 10^{-5}–10^{-4} M. The compounds were stable in the assay conditions and the results had statistical significance (Tables 1 and 2). All dimethyl-9,10-dihydrophenanthrenes cause full inhibition of algal growth at 10^{-4} M concentration and no differences were observed between the hydroxy and the methoxy derivatives. High activity is maintained at 5×10^{-5} M, while at 10^{-5} M only 2-methoxy-5,6-dimethyl-9,10-dihydrophenanthrene (**1m**), 2-methoxy-5,7-dimethyl-9,10-dihydrophenanthrene (**1o**), 2-methoxy-6,8-dimethyl-9,10-dihydrophenanthrene (**1u**), and 2-methoxy-7,8-dimethyl-9,10-dihydrophenan-threne (**1w**) are still active. These compounds

TABLE 1. INHIBITION (%) OF GROWTH OF *S. capricornutum* BY SYNTHETIC 9,10-DIHYDROPHENANTHRENES[a]

	1m	**1n**	**1o**	**1p**	**1q**	**1r**	**1s**	**1t**	**1u**	**1v**	**1w**	**1x**
10^{-4} M	100 d	100 b	100 b	98 b	100 b	100 b	100 b	100 b	100 d	100 c	100 b	100 b
5×10^{-5} M	60 c	100 b	100 b	98 b	100 b	100 b	100 b	100 b	89 c	71 b	100 b	100 b
10^{-5} M	40 b	0 a	100 b	0 a	0 a	0 a	0 a	0 a	60 b	0 a	100 b	0 a
Control	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a

[a] In each column values followed by different letters are statistically significant. Student-Newman-Keuls test; $P = 0.05$.

TABLE 2. INHIBITION (%) OF GROWTH OF *S. capricornutum* BY SYNTHETIC PHENANTHRENES

	2m	**2n**	**2o**	**2p**	**2q**	**2r**	**2s**	**2t**	**2u**	**2v**	**2w**	**2x**
10^{-4} M	100 b	98 b	0 a	100 c	98 b	100 b	70 c	100 c	100 b	70 c	75 c	100 c
5×10^{-5} M	100 b	98 b	0 a	100 c	98 b	100 b	70 c	95 b	100 b	50 b	75 c	95 b
10^{-5} M	0 a	98 b	0 a	50 b	0 a	0 a	22 b	95 b	0 a	50 b	20 b	95 b
Control	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a

[a] In each column values followed by different letters are statistically significant. Student-Newman-Keuls test; $P = 0.05$.

have a methyl at C-5 or at C-8, and when the other methyl is at C-7, 100% activity is observed. Except for 2-methoxy-5,7-dimethylphenanthrene (**2o**), all the dimethylphenanthrenes have high activity at 10^{-4} and 5×10^{-5} M concentrations with no differences among hydroxy and methoxy derivatives. At the lowest concentration, only 2-hydroxy-5,6-dimethylphenanthrene (**2n**), 2-hydroxy-5,7-dimethylphenanthrene (**2p**), 2-hydroxy-6,7-dimethylphenanthrene (**2t**), 2-hydroxy-6,8-dimethylphenanthrene (**2v**), and 2-hydroxy-7,8-dimethyl-phenanthrene (**2x**) are active, and **2n**, **2t**, and **2x** still cause full inhibition.

In conclusion, all the synthetic phenanthrenes and 9,10-dihydrophenanthrenes cause inhibition of algal growth in the examined concentration range. A comparison among monomethyl and dimethyl compounds shows that at the highest concentration the monomethyl derivatives are less active than the dimethyl derivatives, while at the lowest concentration the activity goes in the opposite direction.

REFERENCES

ALIOTTA, G., MONACO, P., PINTO, G., POLLIO, A., and PREVITERA, L. 1991. Potential allelochemicals from *Pistia stratiotes* L. *J. Chem. Ecol.* 17:2223–2234.

BLANKLEY, W. F. 1973. Toxic and inhibitory materials associated with culturing, pp. 207–223, *in* J. R. Stein (ed.). Handbook of Phycological Methods. Cambridge University Press, Cambridge, UK.

DELLAGRECA, M., FIORENTINO, A., MANGONI, L., MOLINARO, A., MONACO, P., and PREVITERA, L. 1992. 9,10-Dihydrophenanthrenes metabolites from *Juncus effusus* L. *Tetrahedron Lett.* 33:5257–5260.

DELLAGRECA, M., FIORENTINO, A., MANGONI, L., MOLINARO, A., MONACO, P., and PREVITERA, L. Cytotoxic 9,10-dihydrophenanthrenes from *Juncus effusus* L. 1993. *Tetrahedron* 49:3425–3432.

DELLAGRECA, M., FIORENTINO, A., MOLINARO, A., MONACO, P., and PREVITERA, L. 1995a. 9,10-Dihydrophenanthrenes glucosides from *Juncus effusus. Nat. Prod. Lett.* 6:111–117.

DELLAGRECA, M., FIORENTINO, A., MONACO, P., PREVITERA, L., and ZARRELLI, A. 1995b. Effusides I–V: 9,10-Dihydrophenanthrenes glucosides from *Juncus effusus. Phytochemistry* 40:533–535.

DELLAGRECA, M., MONACO, P., PREVITERA, L., ZARRELLI, A., PINTO, G., POLLIO, A., and FIORENTINO, A. 1997. Minor bioactive dihydro-phenanthrenes from *Juncus effusus* L. *J. Nat. Prod.* 60:1265–1268.

DELLAGRECA, M., FIORENTINO, A. MONACO, P., POLLIO, A., PREVITERA, L., and ZARRELLI, A. 2000. Dihydrophenanthrenes and phenanthrenes mimics of natural compounds. Synthesis and antialgal activity. *J. Chem. Ecol.* 26:587–600.

PADWA, A., DOUBLEDAY, C., and MAZZU, A. 1977. Photocyclization reaction of substituted 2,2′-divinylbifenyl derivatives. *J. Org. Chem.* 42:3271–3279.

SEMMELHACK, M. F., HELQUIST, P., JONES, L. D., KELLER, L., MENDELSON, L., RYONO, L. S., SMITH, J. G., and STAUFFER, R. D. 1981. Reaction of aryl and vinyl halides with zerovalent nickel—preparative aspects and synthesis of Alnusone. *J. Am. Chem. Soc.* 21:6460–6470.

TIECCO, M., TESTAFERRI, L., TINGOLI, M., CHIANELLI, D., and MONTANUCCI, M. 1984. A convenient synthesis of bipyridines by nickel phosphine complex-mediated homocoupling of halopyridines. *Synthesis* 9:736–738.

YANG, N. C., CHIANG, W., and LANGAN, Y. 1984. Photoreduction of aromatic compounds via amine exciplexes. *Tetrahedron Lett.* 2855–2858.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

FEEDING BY THE APHID *Sipha flava* PRODUCES A REDDISH SPOT ON LEAVES OF *Sorghum halepense*: AN INDUCED DEFENSE?

CÉSAR COSTA-ARBULÚ, ERNESTO GIANOLI,*
WILFREDO L. GONZÁLES, and HERMANN M. NIEMEYER

Departamento de Ciencias Ecológicas, Facultad de Ciencias,
Universidad de Chile, Casilla 653, Santiago, Chile

(Received April 26, 2000; accepted October 4, 2000)

Abstract—Feeding by the aphid *Sipha flava* produces a reddish spot on mature leaves of *Sorghum halepense*. The present work is aimed at determining whether this plant response entails induced resistance against the aphid. Old and young leaves showed the same response to aphid feeding (reddish coloration). Water-stressed plants displayed a similar reddish coloration to aphid-infested plants. This was verified by evaluation of absorbance peaks of the respective leaf extracts. Aphid fecundity was reduced on previously infested (and hence reddish colored) leaves. However, aphid fecundity was not affected on water stressed plants. Furthermore, aphid survival was not different on artificial diets containing increasing concentrations of the reddish pigment. It is concluded that the reddish spot is correlated with, but is not itself responsible for, the observed induced resistance of *S. halepense* against *S. flava*.

Key Words—Induced responses, induced resistance, *Sipha flava*, *Sorghum halepense*, aphids, anthocyanins.

INTRODUCTION

Induced responses of plants to herbivory are frequently found in plant–insect interactions (Karban and Baldwin, 1997). When such responses have a negative effect on insect fitness, they are termed induced resistance (Karban and Baldwin, 1997). The magnitude of induced responses, and consequently of their effect on herbivores, may be affected by environmental conditions (Gianoli and Niemeyer,

* To whom correspondence should be addressed. e-mail: aletheia@abulafia.ciencias.uchile.cl

0098-0331/01/0200-0273$19.50/0

1996; Karban, 1987) as well as by the particular plant tissue that is attacked (Gianoli and Niemeyer, 1998; Gianoli, 1999).

Sipha flava (Forbes) is a cereal aphid that uses different cultivated and wild Poaceae (Young and Teetes, 1977; Breen and Teetes, 1986a; Blackman and Eastop, 1984). It has been recently reported in Chile, colonizing *Sorghum halepense* L. (Gonzáles et al., 1998), a common weed in cereal fields (Monaghan, 1979; McWhorther, 1989; Matthei, 1995). *S. flava* has a particular pattern of attack on host plants. Aphids initially colonize lower (older) leaves (Long and Hensley, 1972; Holman, 1974), producing a characteristic damage: a reddish spot appears on infested leaves (Breen and Teetes, 1986a, 1990; Webster, 1990). The spot spreads and eventually covers the entire leaf, accelerating its senescence (Breen and Teetes, 1986a; Webster, 1990). Aphids then move upward to colonize the next leaf. *S. flava* infestation may significantly decrease plant fitness. Thus, infested sorghum (*Sorghum bicolor* L.) plants show a reduction in biomass (Breen and Teetes, 1986b) and seed production (Breen and Teetes, 1986a). Likewise, aphid infestation reduces biomass of *S. halepense* plants (Gonzáles et al., unpublished).

The appearance of reddish coloration on tissues of sorghum plants, presumably involving anthocyanins, has also been related to the attack of the aphid *Schizaphis graminum* (Young and Teetes, 1977), to fungal infection (Nicholson et al., 1987), as well as to irradiation and low temperature (Shichijo et al., 1993, 1996). Moreover, the induction of anthocyanins by water stress is considered a widespread phenomenon in plants (Chalker-Scott, 1999).

The major aim of the present work is to determine whether the reddish coloration on leaves of *S. halepense* induced by feeding of *S. flava* is involved with the induced resistance. For this purpose, the fecundity of *S. flava* on previously infested (and hence reddish colored) and on uninfested leaves is compared. Given the particular pattern of occupation of plants of this aphid, these comparisons are performed on the natural site of attack, i.e., an old leaf, and on a young leaf. In addition, bioassays are conducted on artificial diets with different concentrations of plant extracts containing the reddish pigment. Complementary experiments evaluate aphid fecundity on plants where reddish coloration is induced by water stress. The qualitative similarity of the reddish coloration in water-stressed plants and in aphid-infested plants is assessed by their absorbance spectrum. Finally, some spatial and temporal characteristics of the induced response of *S. halepense* to *S. flava* infestation are addressed, and the effect on them of temperature and leaf age is evaluated.

METHODS AND MATERIALS

Organisms. Cultures of *S. flava* originally collected on *S. halepense* in the area of wheat fields in Santiago, Chile, were maintained on barley seedlings.

Seeds of *S. halepense* were collected in the same area. Aphids and *S. halepense* seedlings were kept in rooms at 20 ± 2°C and a 14L : 10D photoperiod. Aphids reproduce parthenogenetically under these conditions.

Leaf Age and Effect of Induced Response on Aphid Fecundity. Seedlings at the eight-leaf stage were infested with 15 adult aphids confined inside a clip cage attached to the leaf that bore the treatment. Two groups of infested plants were used: one received the aphid infestation on the third leaf (an old leaf); the other on the seventh leaf (a young leaf). Two groups of uninfested plants were used as controls, bearing empty clip cages on the respective leaves ($N = 15$ plants per treatment). Plants were then placed in growth chambers at constant temperature (20°C) and a 12L : 12D photoperiod. Aphids were removed after 24 hr of infestation, and plants were left for 92 hr, a time period after which a reddish spot appears on the infested leaves (data not shown). Immediately afterwards, aphid fecundity was evaluated in the four treatments (infested/uninfested; young/old leaf). An 8-day-old nymph was placed on the same leaves and on the same leaf part where clip cages were previously attached. Aphids were confined inside clip cages for eight days. At the end of this period, the number of nymphs produced was recorded. Data on aphid fecundity were analyzed by using a two-way ANOVA, with leaf age (young/old) and treatment (infested/uninfested) as main factors.

Bioassay and Extraction of Reddish Pigment. Aphid survival on artificial diets containing increasing concentrations of the reddish pigment, previously extracted from leaves of *S. halepense*, was evaluated. Ten aphids were confined in a vertically placed Plexiglas cylinder (3.0 cm high × 2.5 cm ID). A plastic net covered one end of the cylinder, and the other end was closed with a sachet made of two Parafilm M membranes. The sachet contained 200 μl of a sucrose solution (25% w/v, prepared in phosphate buffer pH 5.5) and the extract to be tested at concentrations of 0 (control) 0.4, 4, 10, 20, and 40 mg of reddish extract/ml of solution. The cylinders containing the aphids were kept at 20°C and in the dark for 48 hr. At the end of this period the number of aphids still alive was recorded. Five replicates were performed for each concentration of the extract tested.

The extract of the reddish pigment was obtained as follows. One hundred grams of leaves, which had been infested by aphids and showed the reddish coloration, were extracted for 24 hr at 4°C with 500 ml of HCl–MeOH (0.1% v/v) solution. The solution was vacuum concentrated to 100 ml, adjusted to pH 5 with $NaCO_3$, and maintained at −20°C for 5 hr. One milliliter of Me_2CO was then added and the suspension centrifuged for 20 min (−20°C, 20,000 rpm); the precipitate was discarded. Vacuum concentration was continued until the final reddish solid extract was obtained.

Aphid Fecundity on Plants Under Water Stress. Greenhouse observations indicated that plants under water stress develop a similar response to that

observed after aphid infestation, i.e., the appearance of a reddish spot. This led us to evaluate whether such a response could affect aphid fecundity, as previous aphid infestation did. Plants at the four-leaf stage were used in the experiment. A group of plants was deprived of watering for five days, whereas control plants were watered daily during that period ($N = 15$ per treatment, 20°C). At the end of the five-day period, aphid fecundity was measured exactly as in the first experiment (see above).

Comparison of Spectra of Reddish Spots of Plants Subjected to Aphid Infestation or Water Stress. To compare spectra, a 3-cm section was excised from a pigmented zone of a leaf (one per treatment). The leaf sections were extracted for 24 hr at 4°C in 5 ml of HCl–MeOH solution, as was described above (see procedure of extraction of the pigment). A Shimadzu UV–visible spectrophotometer (model UV-240) was used. The spectrum was evaluated at wavelengths of 400–700 nm.

Characterization of Induced Response. Experiments were conducted to determine the minimum duration of aphid infestation required to produce the induced response, the time until the appearance of the reddish spot, and its spatial extension. These evaluations were performed at 20°C and 28°C. *S. halepense* plants at the four-leaf stage were infested with 15 adults of *S. flava* inside a clip cage attached to the third leaf in the following treatments: 8, 10, 12, and 16 hr. At the end of the respective periods aphids were removed. Observations on the infested leaf were performed every 2 hr, and a positive response was considered when evidence of a reddish spot was apparent ($N = 10$ per treatment). In addition to this qualitative evaluation, the timing of the induced response, i.e., the time elapsed from the removal of aphids to the appearance of the spot, was recorded. The area of the reddish spot on leaves of plants of the same age, but infested during 24 hr, was assessed measuring its length (L) and width (W) and assuming a rectangular shape (Area = L × W). The percentage of leaf area covered by the spot was calculated after assessing the area of the whole leaf ($N = 10$). This was done by using image analysis software (Sigmascan). Evaluations with plants at the eight-leaf stage also were performed to determine the effect of leaf age on the timing of the induced response. The same protocol of aphid infestation (for 24 hr) was followed on the third leaf (an old leaf) and on the seventh leaf (a young leaf) ($N = 10$ for each treatment). Timing of the induced response was evaluated as described above.

RESULTS

Leaf Age and Induced Responses. The fecundity of *S. flava* was significantly reduced by previous aphid infestation on the leaf. Neither leaf age nor the interaction of factors had a significant effect on *S. flava* fecundity (Table 1).

TABLE 1. EFFECT OF LEAF AGE AND PREVIOUS INFESTATION OF *S. halepense* ON *S. flava* FECUNDITY

	Nymphs produced (*N*, mean ± SE)	
Leaf age	Control	Infested
Young	24.00 ± 2.20	20.36 ± 1.46
Old	21.55 ± 1.15	18.38 ± 1.44

	Analysis of variance			
Source	*df*	Mean square	*F* ratio	*P*
Leaf age (L)	1	61.98	1.80	NS[a]
Pre infestation (I)	1	146.40	4.25	<0.05
L × I	1	0.73	0.02	NS[a]
Error	47	34.40		

[a] NS = not significant ($P > 0.05$, two-way ANOVA test).

Bioassay of Reddish Pigment. The survival of *S. flava* was not significantly different on diets with increasing concentrations of the extract containing the reddish pigment ($F = 0.63$, $P > 0.05$, one-way ANOVA) (Table 2).

Aphid Fecundity on Water-Stressed Plants. The fecundity of *S. flava* (number of nymphs, mean ±SE) on *S. halepense* was not significantly different on plants under water stress (15.33 ± 1.3) and on control plants (18.45 ± 1.73; $t = 1.38$, $P > 0.1$).

Comparison of Spectra of Reddish Spots. The spectra of the reddish spot of plants under water stress and plants infested with *S. flava* showed a remarkable similarity in qualitative terms (Figure 1).

Characterization of Induced Response. The minimum duration of aphid

TABLE 2. SURVIVAL OF *S. flava* FEEDING ON ARTIFICIAL DIETS WITH DIFFERENT CONCENTRATIONS OF AN EXTRACT CONTAINING REDDISH PIGMENT

Extract conc. (mg/ml)	Survival (%, mean ± SE)[a]
0.0	78 ± 9.69
0.4	92 ± 5.83
4.0	88 ± 5.83
10.0	86 ± 4.00
20.0	90 ± 3.16
40.0	88 ± 5.83

[a] Means are not significantly different ($P > 0.05$, one-way ANOVA).

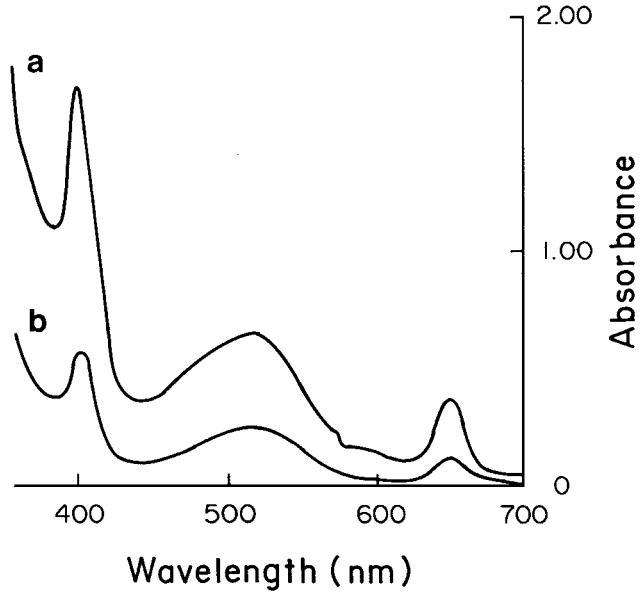


FIG. 1. Visible spectra of *S. halepense* leaf extracts showing reddish coloration caused by (a) infestation by *S. flava*, and (b) water stress.

infestation able to induce the reddish spot at 20°C was 16 hr, whereas at 28°C this time was reduced to 10 hr. After 16 hr of aphid infestation, the time (hours, mean ± SE) to the production of the reddish spot was similar ($t = 1.12$, $P > 0.1$) at 20°C (96.3 ± 1.02) and at 28°C (93.8 ± 1.05). The reddish spot was localized at 28°C, whereas it was spatially spread at 20°C ($t = 26.79$, $P < 0.001$) (Table 3). The reddish spot appeared on old leaves 92.4 ± 1.03 hr after the end of aphid infestation, whereas this time was significantly shorter on young leaves (72.15 ± 1.96 hr; $t = -8.43$, $P < 0.001$).

TABLE 3. CHARACTERISTICS OF THE RESPONSE (REDDISH SPOT) PRODUCED BY *S. halepense* AFTER *S. flava* FEEDING AT DIFFERENT TEMPERATURES

	20°C	28°C
Minimum time of infestation (hr)	16	10
Time to response (hr)	96.3 ± 0.02	100.3 ± 0.47
Leaf area covered by the spot (%)	37.61 ± 1.37	<1.0

DISCUSSION

Results showed a reduction in the fecundity of *S. flava* feeding on a previously infested leaf, which exhibited the reddish spot as induced response. The reddish spot, or another induced response correlated with it, could be the cause of the decrease in fecundity of the aphids. Negative effects on aphid fitness after infestation have been reported earlier (Cabrera et al., 1995; Gianoli, 1999; but see Formusoh et al., 1992). It has been shown that aphid infestation on other species of Poaceae may affect the nitrogen content of leaves (Dorschner et al., 1987) and change the concentration of secondary metabolites (Leszczynski, 1985; Gianoli and Niemeyer, 1997). The mechanism underlying the aphid-induced reddish spot in *S. halepense* is not clear at this point. Studies addressing the reddish spots on wheat plants caused by the aphid *S. graminum* have ascribed the leaf discoloration to an abnormal increase in volume of plastoglobuli, which in turn has a deleterious effect on chloroplasts producing chlorophyll loss (Ryan et al., 1990). Recent work involving sorghum and a nonpathogenic fungus suggests that anthocyanin accumulation is a consequence of the deactivation of related phytoalexins (3-deoxyanthocyanidins), which are induced immediately after fungal attack (Lo and Nicholson, 1998). It is considered that the induction of phytoalexins by microbial infections and by aphid feeding is very similar in nature, the common elicitors being oligosaccharides produced by plant cell wall degrading enzymes (Campbell and Dreyer, 1990).

Regardless of the particular mechanism involved in its production, the reddish spot in *S. halepense* is associated with induced resistance against *S. flava*. Thus, the plant response decreases aphid reproduction. The biological activity of anthocyanins against insects (Hedin et al., 1983), fungi (Schutt and Netzly, 1991), and bacteria (Stonecipher et al., 1993) has been demonstrated. In the present work, the negative effect on aphid fitness is achieved, but the infested leaf loses its photosynthetic capacity. This resembles hypersensitive responses found in other plant–insect systems (Fernándes, 1990), particularly those in which the insects have poor mobility, as is the case of *S. flava*.

Leaf age did not affect fitness of *S. flava*. This result is surprising given the usual difference in quality of leaves of different age for aphids (Merritt, 1996; Dixon, 1998) and the marked preference of *S. flava* for older leaves in the field (Long and Hensley, 1972; Gonzáles et al., unpublished). This suggests that ecological factors other than plant tissue quality may explain the within-plant distribution of the aphid (e.g., natural enemies) (Gonzáles et al., 2001). The interaction of aphid infestation and leaf age did not have a significant effect on aphid fecundity, which means that the occurrence of induced resistance was not related to leaf age. Two nonmutually exclusive explanations, from both sides of the insect–plant interaction, may be advanced. Thus, it may be thought that aphid feeding, and hence elicitation, is similar on usual (old leaves) and unusual (young

leaves) feeding substrates. Interestingly, differences in feeding behavior have been reported for aphids developing on young and mature leaves of the same host plant (Gabrys et al., 1997). From the side of the plant, the physiological events that take place following aphid feeding may not be different in young and old leaves. Similar results have been observed in aphid-infested wheat seedlings (Gianoli and Niemeyer, 1997).

The induction of anthocyanins by water stress is a common phenomenon (Chalker-Scott, 1999). In the present work, water stressed plants developed a response similar to that induced by aphid infestation, i.e., the appearance of a reddish spot on the leaves, which is likely to correspond to anthocyanin accumulation (Snyder and Nicholson, 1990; Shichijo et al., 1993, 1996; Chalker-Scott, 1999). Moreover, the reddish spot was shown to be qualitatively the same in terms of absorbance peaks for plants subjected to either treatment. Unlike aphid infestation, water stress did not produce a decrease in plant quality for aphids, suggesting that the induced resistance observed in *S. halepense* is specifically triggered by aphid attack. Thus, the reddish spot would be a symptom associated with induced resistance, but not a symptom of induced resistance itself. This is supported by the bioassay results that showed no deleterious effects of the reddish pigment on aphids. Interestingly, previous reports describe an increase in plant resistance conferred by anthocyanin induction (Snyder and Nicholson, 1990; Schutt and Netzly, 1991; Stonecipher et al., 1993). Caution is needed before ascribing a particular role to anthocyanin induction in insect–plant interactions.

Environmental variables affect the expression of both constitutive (Larsson et al., 1986) and induced (Coleman and Jones, 1991) levels of plant resistance. We evaluated some spatial and temporal aspects of the aphid-induced reddish spot under two different temperatures (20°C and 28°C). These evaluations are of interest because the main distribution of *S. flava* is in the tropics (Medina-Gaud et al., 1965; Smith and Cermeli, 1979), and hence the temperate climate of Chile could mediate or constrain some issues of the aphid–plant relationship. At 28°C, a shorter infestation time was needed to produce the reddish spots; they took slightly more time to appear, and were considerably smaller than at 20°C. The spots were notably more spread at 20°C, suggesting that the induced response may be constrained at higher temperatures. A similar pattern was found for aphid-infested wheat seedlings (Gianoli and Niemeyer, 1996). Provided that the spatial extension of the spot is correlated with the magnitude of its associated induced response (see above), these results suggest that *S. flava* would be favored at higher temperatures. This agrees with earlier findings of better performance of this aphid at 30°C than at lower temperatures (Starks and Mirkes, 1979).

Finally, young leaves reacted more readily than old leaves in terms of the appearance of the reddish spot. This result does not match the initial observation that the reduction in aphid fecundity did not vary with leaf age. Therefore, it

provides additional support to the main conclusion of this paper, i.e., that the reddish spot is correlated with, but is not itself, the induced response responsible for the observed induced resistance of *S. halepense* against *S. flava*.

Several lines of research are open after the preliminary findings of the present work. In terms of the plant, identifying the compound(s) responsible for the induced resistance and elucidating how the physiological mechanism of aphid resistance compares with that described for fungal infections need to be examined further. In terms of the insect, determining the factors that promote or constrain the pattern of distribution within the plant and relating this to the quality and responsiveness of the tissues should be studied. Other ecological factors, such as natural enemies (e.g., Gonzáles et al., 2001) and interspecific aphid competition (e.g., Gianoli, 2000) should not be overlooked given the fact that *S. flava* usually coexists with other aphid species (*Rhopalosiphum maidis*, *Schizaphis graminum*) on its host plants.

Acknowledgments—This work was supported by IPICS (International Program in the Chemical Sciences at Uppsala University), and the Presidential Chair in Sciences awarded to H.M.N. C.C.-A. was supported by a LANBIO (Latin American Network for Research on Bioactive Natural Compounds) fellowship during the development of this work. This work is part of the activities of the Center for Advanced Studies in Ecology and Research in Biodiversity funded by the Millennium Scientific Iniciative (P99-103-F ICM).

REFERENCES

BLACKMAN, R. L., and EASTOP, V. P. 1984. Aphids on the World's Crops. An Identification Guide. John Wiley & Sons, New York.

BREEN, J. P., and TEETES, G. L. 1986a. Relationships of yellow sugarcane aphid (Homoptera: Aphididae) density to sorghum damage. *J. Econ. Entomol.* 79:1106–1110.

BREEN, J. P., and TEETES, G. L. 1986b. Yellow sugarcane aphid (Homoptera: Aphididae) infestation duration effect on sorghum. *J. Econ. Entomol.* 79:1603–1605.

BREEN, J. P., and TEETES, G. L. 1990. Economic injury levels for yellow sugarcane aphid (Homoptera: Aphididae) on seedling sorghum. *J. Econ. Entomol.* 83:1008–1014.

CABRERA, H. M., ARGANDOÑA, V. H., ZÚÑIGA, G. E., and CORCUERA, L. J. 1995. Effect of infestation by aphids on the water status of barley and insect development. *Phytochemistry* 40:1083–1088.

CAMPBELL, B. C., and DREYER, D. L. 1990. The role of plant matrix polysaccharides in aphid–plant interactions, pp. 149–170 *in*. R. K. Campbell and R. D. Eikenbary (eds.). Aphid-Plant Genotype Interactions. Elsevier, Amsterdam.

CHALKER-SCOTT, L. 1999. Environmental significance of anthocyanins in plant stress response. *Photochem. Photobiol.* 70:1–9.

COLEMAN, J. S., and JONES, C. G. 1991. A phytocentric perspective of phytochemical induction by herbivores, pp. 3–46. *in* D. W. Tallamy and M. J. Raupp (eds.). Phytochemical Induction by Herbivores. Wiley, New York.

DIXON, A. F. G. 1998. Aphid Ecology. Chapman & Hall, London, p. 38.

DORSCHNER, K. W., RYAN, J. D., JOHNSON, R. C., and EIKENBARY, R. D. 1987. Modification of host nitrogen levels by the greenbug (Homoptera: Aphididae): Its role in resistance of winter wheat to aphids. *Environ. Entomol.* 16:1007–1011.

FERNÁNDES, G. W. 1990. Hypersensitivity: A neglected plant resistance mechanism against insect herbivores. *Environ. Entomol.* 19:1173–1182.

FORMUSOH, E. S., WILDE, G. E., and REESE, J. C. 1992. Reproduction and feeding behavior of greenbug biotype E (Homoptera, Aphididae) on wheat previously fed upon by aphids. *J. Econ. Entomol.* 85:789–793.

GABRYS, B., TJALLINGII, W. F., and VAN BEEK, T. A. 1997. Analysis of EPG recorded probing by cabbage aphid on host plant parts with different glucosinolate contents. *J. Chem. Ecol.* 23:1661–1674.

GIANOLI, E. 1999. Within-plant distribution of *Rhopalosiphum padi* on wheat seedlings is affected by induced responses. *Entomol. Exp. Appl.* 93:227–230.

GIANOLI, E. 2000. Competition in cereal aphids on wheat plants. *Environ. Entomol.* 29:213–219.

GIANOLI, E., and NIEMEYER, H. M. 1996. Environmental effects on the induction of wheat chemical defences by aphid infestation. *Oecologia* 107:549–552.

GIANOLI, E., and NIEMEYER, H. M. 1997. Characteristics of hydroxamic acid induction in wheat triggered by aphid infestation. *J. Chem. Ecol.* 23:2695–2705.

GIANOLI, E., and NIEMEYER, H. M. 1998. Allocation of herbivory-induced hydroxamic acids in the wild wheat *Triticum uniaristatum. Chemoecology* 8:19–23.

GONZÁLES, W. L., FUENTES-CONTRERAS, E., and NIEMEYER, H. M. 1998. Una nueva especie de áfido (Hemiptera: Aphididae) detectada en Chile: *Sipha flava* (Forbes). *Rev. Chil. Entomol.* 25:87–90.

GONZÁLES, W. L., GIANOLI, E., and NIEMEYER, H. M. 2001. Plant quality versus risk of parasitism: Within-plant distribution and performance of the corn leaf aphid, *Rhopalosiphum maidis* (Fitch) (Hemiptera: Aphididae). *Agric. For. Entomol.* In press.

HEDIN, P. A., JENKINS, J. N., COLLUM, D. H., WHITE, W. H., and PARROTT, W. L. 1983. Multiple factors in cotton contributing to resistance to the tobacco budworm, *Heliothis virescens* F., pp. 347–365. *in* P. A. Hedin (ed.). Plant Resistance to Insects. American Chemical Society, Washington, D.C.

HOLMAN, J. 1974. Los áfidos de Cuba. Instituto Cubano del Libro, La Habana.

KARBAN, R. 1987. Environmental conditions affecting the strength of induced resistance against mites in cotton. *Oecologia* 73:414–419.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago.

LARSSON, S., WIRÉN, A., LUNDGREN, L., and ERICSSON, T. 1986. Effects of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (Coleoptera). *Oikos* 47:205–210.

LESZCZYNSKI, B. 1985. Changes in phenols content and metabolism in leaves of susceptible and resistant winter wheat cultivars infested by *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). *Z. Angew. Entomol.* 100:343–348.

LO, S. C. C., and NICHOLSON, R. L. 1998. Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. *Plant Physiol.* 116:979–989.

LONG, W. H., and HENSLEY, S. D. 1972. Insect pest of sugar cane. *Annu. Rev. Entomol.* 17:149–176.

MATTHEI, O. J. 1995. Manual de las malezas que crecen en Chile. Alfabeta Impresores, Santiago.

MCWHORTHER, C. G. 1989. History, biology, and control of Johnsongrass. *Rev. Weed Sci.* 4:85–121.

MEDINA-GAUD, S., MARTORELL, L. F., and BONILLA, R. R. 1965. Notes on the biology and control of the yellow sugarcane aphid of sugarcane, *Sipha flava* (Forbes) in Puerto Rico, pp. 1307–1320. *in* J. Bague (ed.). Proceedings, 12th Congress of the International Society Sugar-Cane Technologists. San Juan, Puerto Rico.

MERRITT, S. Z. 1996. Within-plant variation in concentration of amino acids, sugar, and sinigrin in phloem sap of black mustard, *Brasicca nigra* (L.) Korch (Cruciferae). *J. Chem. Ecol.* 22:1133–1145.

MONAGHAN, N. 1979. The biology of Johnson grass (*Sorghum halepense*). *Weed Res.* 19:261–267.

NICHOLSON, R. L., KOLLIPARA, S. S., VINCENT, J. R., LYONS, P. C., and CADENA-GÓMEZ, G. 1987. Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. *Proc. Natl. Acad. Sci. U.S.A.* 84:5520–5524.

RYAN, J. D., MORGHAM, A. T., RICHARDSON, P. E., JOHNSON, R. C., MORT, A. J., and EIKENBARY, R. D. 1990. Greenbugs and wheat: A model system for the study of phytotoxic Homoptera, pp. 171–186. *in* R. K. Campbell and R. D. Eikenbary (eds.). Aphid-Plant Genotype Interactions, Elsevier, Amsterdam.

SCHUTT, C., and NETZLY, D. 1991. Effect of apiforol and apigeninidin on the growth of selected fungi. *J. Chem. Ecol.* 17:1123–1125.

SHICHIJO, C., HAMADA, T., HIRAOKA, M., JOHNSON, C. B., and HASHIMOTO, T. 1993. Enhancement of red light-induced anthocyanin synthesis in sorghum first internodes by moderate low temperature given in the pre-irradiation culture period. *Planta* 191:238–245.

SHICHIJO, C., HAMADA, T., JOHNSON, C. B., and HASHIMOTO, T. 1996. Effects of moderately low temperature (20°C) on phytochrome response during preirradiation: Anthocyanin synthesis in *Sorghum bicolor* at high- and low-P_{fr}/P_{tot} ratios. *Photochem. Photobiol.* 63:328–335.

SMITH, C. F., and CERMELI, M. M. 1979. An annotated list of Aphididae (Homoptera) of the Caribbean islands and South and Central America. *N.C. Agric. Res. Serv. Tech. Bull.* 259:1–131.

SNYDER, B. A., and NICHOLSON, R. L. 1990. Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science* 248:1637–1639.

STARKS, K. J., and MIRKES, K. A. 1979. Yellow sugarcane aphid: Plant resistance in cereal crops. *J. Econ. Entomol.* 72:486–488.

STONECIPHER, L. L., HURLEY, P. S., and NETZLY, D. H. 1993. Effect of apigeninidin on the growth of selected bacteria. *J. Chem. Ecol.* 19:1021–1027.

WEBSTER, J. A. 1990. Yellow sugarcane aphid (Homoptera: Aphididae): Detection and mechanisms of resistance among Ethiopian sorghum lines. *J. Econ. Entomol.* 83:1053–1057.

YOUNG, W. R., and TEETES, G. L. 1977. Sorghum entomology. *Annu. Rev. Entomol.* 22:193–218.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

MINERALOGICAL AND CHEMICAL INTERACTIONS OF SOILS EATEN BY CHIMPANZEES OF THE MAHALE MOUNTAINS AND GOMBE STREAM NATIONAL PARKS, TANZANIA

SUSANNE AUFREITER,* WILLIAM C. MAHANEY,[2] MICHAEL W. MILNER,[2] MICHAEL A. HUFFMAN,[3] RONALD G. V. HANCOCK,[4] MICHAEL WINK,[5] and MATTHIAS REICH

[1]*SLOWPOKE Reactor Facility and*
Department of Chemical Engineering and Applied Chemistry
University of Toronto
Toronto, Canada M5S 3E5

[2]*Geomorphology and Pedology Laboratory*
York University, Atkinson College
Toronto, Canada, M3J 1P3

[3]*Kyoto University, Primate Research Institute*
Inuyama, Japan

[4]*SLOWPOKE-2 Facility*
Department of Chemistry and Chemical Engineering
Royal Military College of Canada
Kingston, Canada, K7K 7B4

[5]*Universität Heidelberg*
Institut für Pharmazeutische Biologieim
Neuenheimer Feld 364, D-69120 Heidelberg, Germany

[6]*University of Toronto Institute for Biomaterials and Biomedical Engineering*
Toronto, Canada M5S 3E5

(Received July 5, 2000; accepted October 12, 2000)

Abstract—Termite mound soils eaten by chimpanzees of the Mahale Mountains and Gombe National Parks, Tanzania, have mineralogical and geochemical compositions similar to many soils eaten by higher primates, but release very low levels of either toxic or nutritional inorganic elements to solution at acid pH. Comparison with control (uneaten) soils from the same areas showed lower levels of carbon and nitrogen in the eaten soils,

* To whom correspondence should be addressed. e-mail: susanne.aufreiter@utoronto.ca

0098-0331/01/0200-0285$19.50/0

a relationship confirmed by surface analysis. Surface analysis also revealed lower levels of iron on particle surfaces versus interiors, and higher levels of iron on ingested versus control soil particle surfaces. The soils can adsorb dietary toxins, present in the plant diet or those produced by microorganisms. Taking the toxic alkaloids quinine, atropine, sparteine, and lupanine as examples, it is evident that soils from Mahale have a very good adsorptive capacity. A new adaptive advantage of geophagy is proposed, based on the prevention of iron uptake. The behavior of the soils in vitro is consistent with the theory that geophagy has a therapeutic value for these chimpanzees.

Key Words—Adsorption, geophagy, health maintenance, iron, surface chemistry, termite mounds, XPS.

INTRODUCTION

Studies of soil consumption (geophagy) among nonhuman primates have focused on its potential value as a source of supplementary dietary minerals and as a vehicle for adsorption and hence removal of toxins with passage through the gut (Bolton et al., 1998; Muller et al., 1997; Mahaney et al., 1995; Izawa, 1993; Davies and Baillie, 1988; Fossey, 1974, 1983; Uehara, 1982; Oates, 1977, 1978; Hladik, 1977; Hladik and Gueguen, 1974). The possibility that geophagy is used by animals for seld-medication in disease and parasite infestation (as an adsorbent of harmful bacteria and toxins), as it is used by humans both traditionally (Johns, 1990) and in modern pharmaceuticals (Brouillard and Rateau, 1989), has also been suggested (Mahaney et al., 1996, 1997; Stambolic-Robb, 1997; Johns, 1990; Hladik and Gueguen, 1974).

Among higher primates, humans of many societies also have engaged in geophagy (Aufreiter et al., 1997; Johns and Duquette, 1991; Johns, 1990; Hunter, 1973; Laufer, 1930) for various reported reasons, e.g., to alleviate starvation in time of famine and to counter deficiencies of inorganic nutrients during pregnancy. A correlation was established between consumption of highly calcium-enriched termite-mount clay by pregnant women of various African ethnic groups and the absence of milk and other dairy products in their diet. Wiley and Katz (1998) found that soil consumption was absent in pasturalist societies with daily access to milk-producing livestock and used this to implicate geophagy soils as dietary calcium supplements. Geophagy has also been seen as a cultural tradition sponsored and/or advocated by religion. Clear reasons to justify the behavior are frequently unobtainable; possibly human geophagy was retained over evolutionary time due to survival benefits conferred by the behavior (Johns, 1990), and experimentation occurred with highly weathered natural earths in different environments (Mahaney, 1999).

Studies of geophagy soils from different geographical locations have shown that they are often similar in mineralogy and in chemical elemental content

(Browman and Gunderson, 1993). These similarities lead to the inference that soils of a particular chemical environment are selected for ingestion, from the mounds of termites of the subfamily Macrotermitinae. Reports of the ingestion of bioturbation (insect-selected) soils of termites or ants by primates are not unusual (Julliot and Sabatier, 1993; Aufreiter et al., 1997), and ants and termites themselves are often a source of food (Uehara, 1982; Watts, 1984). The chimpanzees of Mahale may be aware of key characteristics associated with termite mound soils. Uehara (1982) reported chimpanzees there sampling the soils of termite mounds through the year, apparently monitoring by taste the reproductive condition of the termites, for feeding on the termites.

The adaptive value of the soils is likely linked to their composition. Most of the analyzed soils have been found to contain a high proportion of $Al : Si = 1 : 1$ secondary clay minerals, with a lesser amount of $Al : Si = 2 : 1$ minerals, some quartz, and, often, high amounts of iron. The clay fraction of the soils is a physically and chemically active material that may absorb or adsorb (or conversely release) water, inorganic elements, or organic compounds to varying degrees, depending on the amounts of specific clay minerals and depending on variations of such conditions as pH. Many studies have considered a benefit of geophagy to be the adsorption of toxic organic components such as alkaloids in plant foods, as shown in vivo for parrots, by Gilardi et al. (1999), and for geese by Wink et al. (1993). Clay minerals are also capable of adsorbing bacterial or parasite-generated toxins (Said et al., 1980). This may be a key benefit of geophagy for the chimpanzees of the Mahale Mountains. Beneficial compounds or organisms could also be obtained from ingestion of the high-clay soils and provide sensory cues of smell and taste used by animals in choosing appropriate soils.

Behavioral research on chimpanzee groups in Mahale has been carried out since 1965 (Nishida, 1990). Investigation into the health and self-medicative behavior of chimpanzees at Mahale with a focus on plants has been carried out by one of the authors (M.A.H.) since 1985. Recently geophagy has also been studied with the objective of determining if this behavior can be considered another form of self-medication (Huffman, 1997). Early behavioral observations have shown that chimpanzees of Mahale sometimes consume termite mound soils during periods of gastrointestinal distress and when beset by intestinal parasites, and thus a preliminary reconnaissance was made of such soils in 1994 (Mahaney et al., 1996). A larger set of samples of eaten soils and uneaten control soils collected in 1995 (Mahaney et al., 1999) confirmed that the chimpanzees chose soils characterized both by combinations of halloysite and kaolinite, similar to those in formulations used by humans to reduce gastrointestinal afflictions, and by high concentrations of iron, for which a nutrient role was considered. Further observations showed that the chimpanzees frequently consume small quantities of these soils (~3 cc per feeding) (Figure 1).

FIG. 1. A chimpanzee of Mahale Mountains National Park, Tanzania, sampling termite mound soil. Photo by Michael Huffman.

Here, a new, larger set of samples of ingested termite mound earths from the Mahale sites was examined along with controls chosen at several meters' greater distance from the mounds than for collection of previous control samples. Additional analytical foci were placed on the potential of the soils to adsorb certain toxins and to release nutrients at acid pH and on an analysis of the soil particle surfaces. We also include information on two samples from the nearby Gombe

National Park research site where chimpanzees have similarly been observed engaging in geophagy. Results from a study of the microbiology of these soils by Ketch (1998) will be published separately. This paper concerns the chemistry, geochemistry, mineralogy, and adsorptive capacities of ingested soils, compared with control soils from the same areas, in order to isolate parameters that warrant further study.

METHODS AND MATERIALS

Behavioral Observations. Geophagy has been observed at Mahale and Gombe in both dry and rainy season months. In the course of ad libitum and focal-animal observations, the locations of chimpanzee geophagy were noted by researchers and field assistants at Mahale and Gombe. During the 15-day collection trip by Huffman in 1996, these sites were visited and collections made. No systematic observations covering all seasons have been made at Mahale, making systematic analysis of the seasonality of geophagy difficult. However, in 59 days of focal-animal observations made by Huffman in the 1991 rainy season, five incidences were recorded (Mahaney et al., 1996).

The Sites. The Mahale Mountains National Park is located on the eastern shore of Lake Tanganyika approximately 100 km south of the Gombe Stream National Park (6°S, 30°E; Figure 2). The climate of the area is influenced by north- and southeasterly (trade) winds. Local wind from the mountains has a drying effect, whereas wind from the lake brings moisture. The mountains range up to 2500 m asl, lake level is about 773 m asl. The underlying bedrock is primarily granite along with gneiss and schist (McConnell, 1950). Soils formed from this crystalline rock are relatively thin, stony, and porous (Collins and McGrew, 1988) with thin organic (Ah) horizons less than 18 cm thick. Soils were collected in mid-October 1996 over a period of five days during the end of the dry season at Mahale and during one day the same month at Gombe. Each termite mound was sampled by Huffman at those spots where chimpanzees were observed to consume the mound soil. All sites were located above ground at the top of a mound. Each control sample was collected approximately 4–6 m away from a mound, avoiding possible contamination down the slope of runoff mound material. Each sample came from the surrounding Ah horizons and had a darker tone than the mound soils.

Laboratory Analyses. Each sample was analyzed for water content and particle size of the <2 mm fraction. Water retention was determined by heating samples overnight at 110°C. The air-dried equivalent of 50 g oven dried soil was used to calculate the particle size. The sands (63–2000 μm) were separated by wet sieving. The silt and clay fractions were determined by pipet and hydrometer (Day, 1965). The particle size curves were drawn from the dry weight of sand and from density measurements made by hydrometer. Particle size distributions

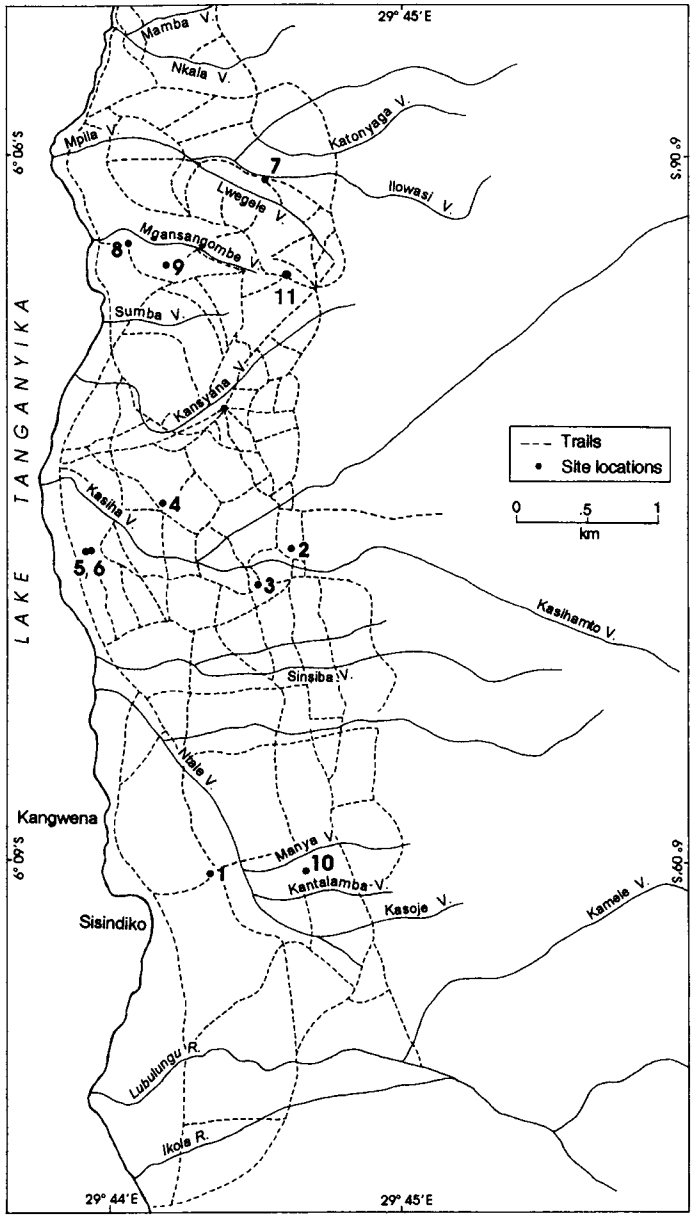


FIG. 2. Map showing the location of sample sites in Mahale Mountains National Park, Tanzania.

were analyzed as curves to determine the relative ratios of sand and silt to clay in each termite mound soil and each control soil.

Mineralogy was determined by X-ray diffraction (XRD) (Whittig, 1965). Major, minor, and trace elements were measured using instrumental neutron activation analysis (INAA). Five-hundred-milligram subsamples of the bulk materials were weighed into polyethylene vials and analyzed at the SLOWPOKE Reactor Facility of the University of Toronto, following procedures established by Hancock (1978, 1984). Two neutron irradiations and four counts were required to produce a database of 23 elements for each sample. Extracts of selected samples were prepared by shaking approximately 1 g of soil in 10 ml of pH 2.0 ammonium oxalate solution for 30 min, followed by gravity filtration through Whatman 42 ashless filter paper. The filtrates were allowed to stand for 48 hr to allow very fine particles to settle; approximately 1-ml volumes of the filtrates were weighed into polyethylene vials, which were then heat-sealed. The sealed samples were analyzed by INAA for short-lived, isotope-producing elements, and some selected samples were also analyzed for long-lived isotope-producing elements, following the procedures of Hancock (1978, 1984).

Selected soil samples were analyzed by scanning electron microscope (SEM) and energy dispersive spectrometry (EDS), including the light and heavy mineral fractions. For the light fraction, sands were randomly selected for analysis by SEM and EDS. All sands were analyzed for grain mineralogy and coatings that might reveal the chemistry of weathering products and coatings including clay minerals. The SEM-EDS analyses followed procedures outlined by Mahaney (1990) and Vortisch et al. (1987).

X-ray photoelectron spectroscopy (XPS) was used to analyze elements at the surface layers of individual particles of soil. The spectra were obtained on a Leybold MAX 200 XPS system based at the Institute for Biomaterials and Biomedical Engineering at the University of Toronto. Samples were prepared for XPS analysis by pressing the dried powdery <2-mm fraction of the soils onto adhesive copper tape, removing loose particles with light gas pressure, and mounting the tapes on a sample holder. Unmonochromatized Al K_α X-ray radiation was used as the excitation source. The source was run at 15 keV and 25 mA. Large-area analysis was performed (4×7 mm^2) to minimize collection time while maximizing signal. Relative atomic percentages were obtained from survey spectra (0–1000 eV, step size 800 meV) run in a low-resolution mode (pass energy = 192 eV). Details of the techniques are described in Sodhi et al. (1992). The data were normalized to unit transmission of the spectrometer by means of a routine provided by the manufacturer (Berresheim et al., 1991). The sensitivity factors (see Table 6 below) used to obtain these values were empirically derived by Leybold for the normalized spectra. Actual integration of the peaks was performed using Escatools (Surface Interface Inc., Mountainview, California). The surface specific nature of XPS limited depth analysis to 7–10 nm.

To measure the adsorptive capacity of soils, we employed the following design: About 500 mg of soil was added to 5 ml of water in 15-ml centrifugation tubes; 0.35 mmol of each of the alkaloids sparteine, lupanine, quinine, and atropine (adjusted to pH 6–7) were added. After 20 min of incubation, samples were vortexed and centrifuged for 20 min at 4000*g*. The separated supernatants were brought to pH 12 with 1 M KOH and subjected to solid–liquid extraction using ChemElute columns. Methylene chloride (3 × 15 ml) was used as eluent. The crude alkaloid extract obtained was analyzed by capillary gas–liquid chromatography, using a Carlo Erba gas chromatograph with a flame ionization detector (FID) and OV-1 (Ohio Valley) capillary column (15 m × 0.25 mm × 0.25 μm) (for details see Wink et al., 1993, 1995; Wink, 1993b). Authentic alkaloids were employed as external standards for quantification. Appropriate controls (samples with water and alkaloids) were used to define the amount of nonadsorbed alkaloids. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Particle Size. The data show little variation in sand + silt, but large variations in clay contents. Figure 3 shows results for ingested samples from sites in the Mahale Mountains. To read the data it is necessary to refer to the boundaries for sand (63–2000 μm), silt (2–63 μm) and clay (<2 μm). The *y* axis is cumulative, so the percentage at 4 phi is the sand and the percentage at 9 phi is the sand + silt. The percentage at 100 less 9 phi is the percent clay. The data for ingested soils show higher proportions of <2-μm material over control soils. There are clear textural differences between the ingested and control samples, showing that there is a preference for chimpanzees to select clay-rich material for ingestion.

Mineralogy. The X-ray diffraction data shown in Table 1 include both clay and primary minerals. Within the 1 : 1 group (Si : Al = 1 : 1), metahalloysite is predominant but is not confined to the ingested group. Kaolinite is dominant at site 3 and site 6 in moderate to abundant amounts and is present in eaten and control soils. Kaolinite and metahalloysite have similar chemistries (although metahalloysite has variable water content) and among the 1 : 1 sample group chimpanzees may be able to achieve the same benefit consuming both the control and ingested soils.

Within the 2 : 1 (Si : Al = 2 : 1) sample group, illite is present in both the ingested and control soils. Randomly interstratified illite–smectite is present in trace to moderate quantities in all sites; smectite is present in five sites including four where ingestion occurred. Smectite is often present in small amounts in geophagic soils and may have some value in geophagy, although the ingestion of Turkish soils high in smectite was shown in vivo in humans to pre-

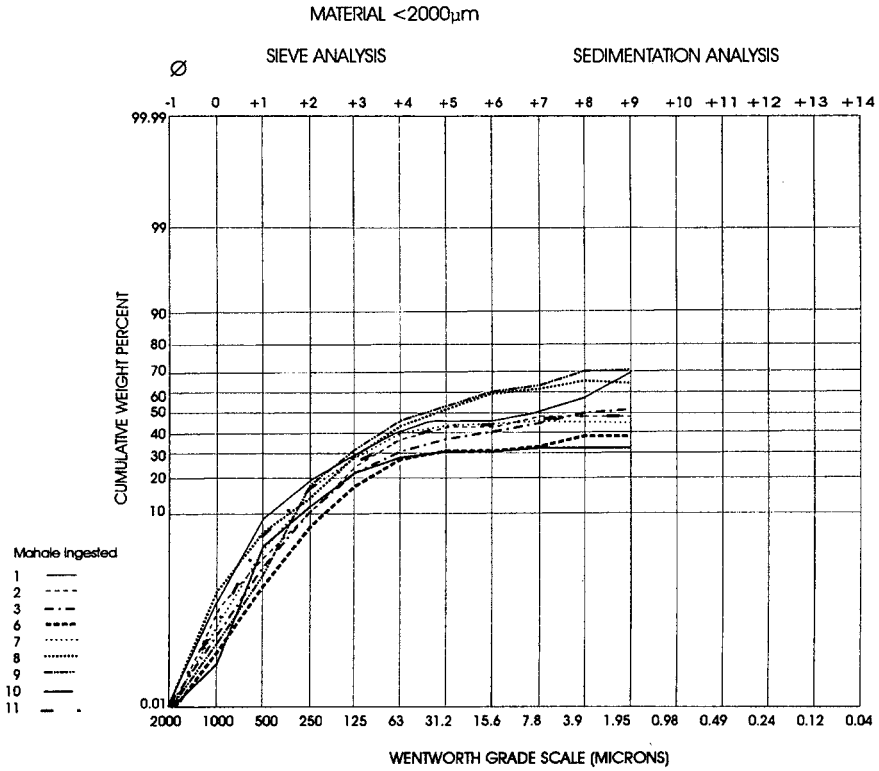


FIG. 3. Results for particle size analysis of soil samples ingested by chimpanzees.

vent uptake of Fe, whereas some soils of predominantly 1 : 1 clay mineral content, used by Native Americans, did not (Minnich et al., 1968). Vermiculite is absent in all soils; chlorite is also absent. Within the primary minerals, quartz, muscovite, orthoclase, biotite, and plagioclase feldspar are present in varying amounts. Quartz is somewhat more prevalent in the ingested samples. It is most abundant in weathered crystalline terrane and here may play a role in armoring the termite mound surface, by rain wash and surface hardening, against animal disturbance. With higher amounts of quartz, orthoclase and the micas are also seen to increase, indicating either younger soils (a sorting phenomenon) or a stronger granitic influence. Plagioclase occurred infrequently among the samples studied, and since it is generally one of the first minerals to weather by hydrolysis, this is not unexpected. Muscovite and its secondary cousins, on the other hand, are inert.

Metahalloysite and illite are common clay minerals in the Mahale soils. The

TABLE 1. MINERALOGY OF <2-μm FRACTION OF INGESTED AND CONTROL SAMPLES[a]

Sample	K	MH	I	I-S	S	C	V	Q	O	M	P
Mahale											
1E		X	XX	XX	tr			X	X	X	tr
1C		XX	XX	XX	tr			XX		XX	
7E		X	X	X				X			X
8E		XXX	XXX	X	XX			XXX	XXX	XX	
9E		XX	XXX	X	X			X	X	X	
11E		XX	X	X				X	X	X	
11C		XX	XX	XX				X	X	X	X
2E	X	XXX	X	X				X	X	X	
2C		XXX	X	X				X	X	X	
3E	XXX	X	XX	X	tr			XX	XX	X	
6E	X	XXX	X	tr				X	X	tr	
6C	XX	X	X	tr				X	X	X	
10E		XX	X	tr				tr	tr	tr	
10C		XXX	X	tr				tr	X	tr	
Gombe											
E		XX	XX	X				XX	X	X	X
C	X	X	XX	X					X		X

[a] The minerals identified include kaolinite (K), metahalloysite (MH), illite (I), illite-smectite (I-S), smectite (S), quartz (Q), orthoclase (O), mica (M), and plagioclase feldspar (P). Chlorite and vermiculite are not present even in trace quantities. Semiquantitative amounts of each mineral are given as trace (tr), small (X), moderate (XX) and abundant (XXX) quantities. E= eaten; C = control.

XRD traces show sharp, abundant reflections indicating a strong crystallinity and a mineralogy of a relatively high purity, similar to pharmaceutical clays marketed as digestive remedies. Data for sample 8E show that it contains somewhat more smectite than is common throughout the sample group. This profile is also similar to that from earth consumed by mountain gorillas in northwestern Rwanda (Mahaney et al., 1995). The chimpanzees do not always select soils with smectite present; the presence of chiefly kaolinite or metahalloysite is consistent.

Carbon and Nitrogen. The carbon and nitrogen contents (Table 2) of the control soils are consistently higher than those of eaten soils, as was also seen in the data from analysis of an earlier sample set from this area (Mahaney et al., 1999). This is probably a reflection of higher levels of organic matter in the surface soils of the area, as compared to the termite mounds that are derived from depths of up to 100 m. The lower levels of carbon and nitrogen of the termite mound soils are also seen in carbon values obtained for the XPS data (discussed below). This is likely due to the depth of acquisition as the insects build their mounds particle by soil particle from deep, wet soils. The resulting lower organic content may reflect lower levels of pathogens, a benefit of choos-

TABLE 2. TOTAL CARBON, HYDROGEN, NITROGEN, pH, ELECTRICAL CONDUCTIVITY, AND SOIL COLOR IN MAHALE SAMPLE GROUP[a]

Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)	pH	EC (1 : 5)	Color (1 : 5)
Mahale						
1E[b]	1.2	1.1	0.14	6.41	54	10YR 6/3
1C	2.1	0.79	0.20	5.36	52	10YR 5/3
7E	1.1	0.93	0.15	6.95	104	10YR 5/4
8E	0.9	0.72	0.12	7.49	97	10YR 6/3
9E	0.7	0.64	0.09	6.81	187	10YR 6/3
11E	0.9	0.97	0.11	7.52	138	7.5YR 6/4
11C	4.7	1.0	0.38	5.93	117	7.5YR 4/3
2E	1.0	0.87	0.14	6.72	161	10YR 6/6
2C	3.4	1.1	0.30	5.97	136	10YR 5/3
3E	1.0	1.1	0.14	7.41	130	10YR 7/4
6E	0.9	1.1	0.13	6.21	62	7.5YR 6/6
6C	4.1	1.1	0.38	5.01	137	10YR 4/4
10E	1.2	1.4	0.16	6.09	80	7.5YR 6/6
10C	2.5	1.2	0.26	5.25	98	7.5YR 5/4
Gombe						
E	1.0	0.70	0.14	4.66	62	7.5YR 5/6
C	2.6	0.59	0.24	4.44	48	7.5YR 4/4

[a] Color follows Oyama and Takehara (1970). E = eaten; C = control.

ing termite mound material for ingestion over the surrounding soils, although the composition is very similar in many other respects.

Acidity. pH measurements of the soils (Table 2) show that the eaten soils have consistently higher pH than the control soils, as did the data from Mahale geophagy soils of 1995 (Mahaney et al., 1999). An antacid function has been proposed for geophagy in some monkeys (Poirier, 1970; Oates, 1978; Davies and Baillie, 1988) to maintain a higher pH in the forestomach. Although primates such as chimpanzees and humans do not maintain a forestomach bacterial flora, these soils may play an analogous role. While the lower pH of the control soils may be related to their higher organic content, the higher pH of the termite mound soils may also be due to termite activities in creation and maintenance of suitable conditions for the cultivation of fungal gardens.

Electrical Conductivity. The electrical conductivities of pairs of eaten and control soils (Table 2) do not show any clear trends. Intersite variability is far greater than intrasite variability, likely a reflection of differences between the eaten and control soils seen in data presented here.

Soil Color and Texture. Soil colors of the eaten and control soils range from yellow–red hues of 10YR 5/4, 5/6 (bright to dull yellowish brown) to stronger

yellow–red 7.5YR 4/4, 4/6 (brown, bright brown to orange) hues (Oyama and Takehara, 1970). Control samples, collected approximately 6–8 m away from a mound, came from the surrounding Ah (organic) or subsurface (nonorganic) horizons, giving a mix of 10YR and 7.5YR colors (Oyama and Takehara, 1970) that were sometimes lighter and sometimes darker than the eaten soils (Table 2). In five of six cases in Table 2, the control soils are slightly darker by one color value and two chroma, indicating a slightly higher amount of organic matter. This is likely due to greater amounts of humus in the control soil compared to the termite mound soil. Of the eight eaten soils analyzed, four have 7.5YR colors, indicating higher amounts of iron. The change in yellow–reddish hues from 10YR to 7.5YR reflects increasing redness, and most probably reflects the form and relative amount of iron in these soils, either as goethite (yellow hues) or hematite (more reddish hues). Soil textures are clay to sandy clay loam (Mahaney et al., 1999), apparent as observed on well-worn animal paths in the area (M. A. Huffman, unpublished results). There are no dominant soil structures in the samples studied, but occasionally subangular blocks are present, which must play a minor role in soil aeration. The termite soil ingested by chimpanzees is considerably richer in clay-size material than the nearby control soils, which are closer in texture to those described by Collins and McGrew (1988).

Scanning Electron Microscopy. Sands in the eaten samples were studied to elicit information on their mineral composition, weathered state, and the composition of coatings they carry. Most grains are coated either with clay minerals or Fe oxides (Figure 4A) suggesting they serve as a source of fine particles or oxides which might play a role in nutrition or self-medication, or alternatively in increasing the adsorption of molecules on the soils. (Figures 4B, 4D). Quartz, which is present in all the samples studied, is either fairly fresh (10% of the samples studied; Figure 4C) or coated to varying degrees with 50% etching, sometimes as in Figure 4E. The remaining 40% are well etched, as in Figure 4E. The data suggest that sands carry coatings that could provide chemical elements important in nutrition. Iron is so prevalent in the suite of samples studied that we first assumed it would be available for absorption. The clay mineral coating on Figure 4F supports the clay mineralogy, which shows a preponderance of 1 : 1 clay minerals including halloysite and kaolinite. When the soils are ingested, these clays, similar to pharmaceutical clays, would be exposed to the gut surfaces as coatings on these sand grains. Organiogenic forms seen on some of the sands from local termite mounds may be related to the presence of micro-organisms.

Geochemistry. Data from analyses of whole soils (Table 3) show that they contain high amounts of iron (>2–6%) in combination with high aluminum (approximately 10%). Potassium occurs at 1–3%. Calcium values are low, with only one site (1C) showing >2%. Sodium levels are also low, at <1%, except for sites 8 and 9, which contain about 1.7%. The essential trace elements cobalt and

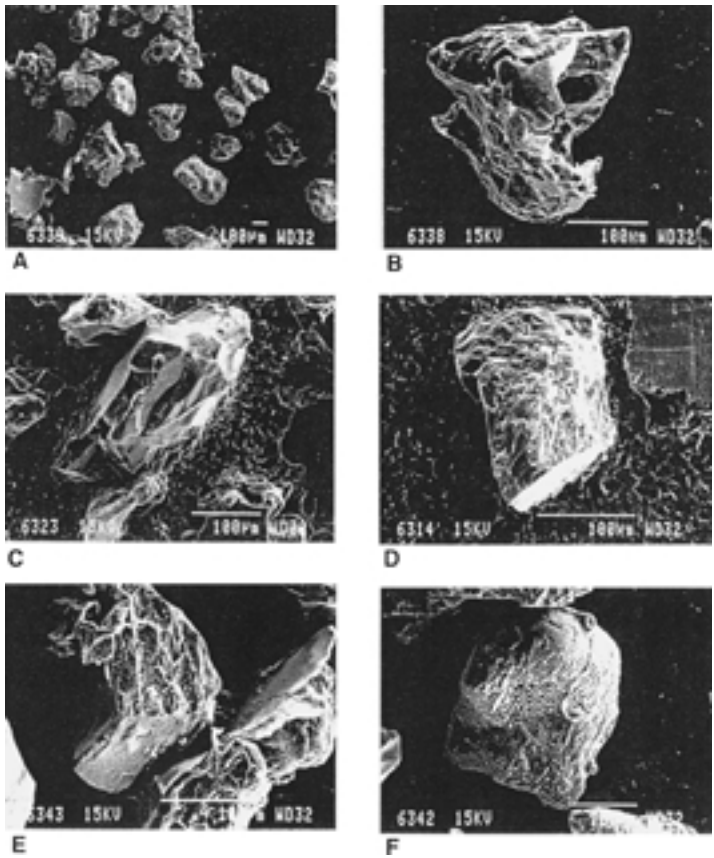


FIG. 4. Scanning electron microscopy from Mahale geophagy soils. (A) Group shot of mixed anhedral and euhedral grains of both lithic and mineral particles; most grains are coated with clay minerals or Fe oxides. Grain in center is enlarged in B. (B) Angular fragments from center of group. Flat, bent biotite mica on central surface, quartz above and amphibole (Fe, K, Mg, Al, Si) below. (C) Clean euhedral quartz showing polyhedral, grooved form with unworn, unetched surfaces—probably represents volcanic, pyroclastic particle. (D) Hornblende (Fe, Ca, Al, Si) showing compound morphology of etched, anhedral surfaces in contrast to cleavage-surface sides. Outcropping of amphibole cleavage on an anhedral surface form. (E) Quartz displaying a compound form with an organiogenic dendritic form on etched microstructural mineralic form, possibly a mineral other than quartz (calcite, feldspar or apatite), in contrast to fracture-surface sides. (F) Quartz displaying subround form, probably physically rounded. Etched clean upper surface is in sharp contrast to clay coating with Si : Al ratio of 1 : 1 indicating halloysite or kaolin group minerals. Linear and lobate-shaped clay ridges indicate downward movement of termite mount fluid on an overgrowth of possible crystalline, geopetal ornamentation.

TABLE 3. ELEMENT CONCENTRATIONS IN WHOLE SOILS DETERMINED BY INAA[a]

Sample	Al (%)	Ba	Ca (%)	Ce	Cl	Co	Cr	Cs	Eu	Fe (%)	Hf	I	K (%)	Lu	Mn	Na	Nd	Rb	Sc	Sr	Ta	Tb	Th	V	Yb
Mahale																									
1E	10.1	750	2.2	83	<120	12.6	18	1.8	2.5	5.77	7.3	<8	1.5	0.34	721	5050	53	72	19.6	260	0.4	0.83	10.3	135	3.2
1C	8.1	880	2.9	78	<150	8.4	16	1.1	2.2	4.44	12.0	<9	1.6	0.35	655	6360	39	58	23.0	360	1.1	0.65	16.2	144	3.0
7E	9.7	910	1.6	102	<160	11.7	19	1.7	2.0	4.14	8.4	<11	2.3	0.45	975	7360	44	113	15.9	270	1.0	0.76	14.2	95	3.7
8E	10.3	820	1.3	125	<200	10.1	16	1.5	2.6	4.28	8.5	<13	2.6	0.50	707	17500	69	110	13.6	320	0.8	1.01	14.5	83	4.1
9E	9.3	1280	1.4	98	<200	10.2	14	1.1	2.3	3.58	8.6	<10	2.5	0.49	798	17100	50	120	12.4	300	0.8	1.14	13.6	76	3.9
11E	11.6	580	0.5	112	<100	6.2	16	2.1	2.0	3.69	6.9	<8	2.9	0.72	438	5590	62	150	12.3	140	1.8	0.94	34.2	71	6.2
11C	7.9	510	0.6	80	<90	3.6	11	1.5	1.1	2.37	8.9	<4	3.4	0.40	362	7960	24	140	8.8	160	2.1	0.46	22.7	44	3.4
2E	9.6	680	0.4	92	<80	7.6	15	1.7	1.6	3.54	11.0	<8	2.6	0.36	617	2030	36	85	12.4	100	1.0	0.57	14.9	74	3.0
2C	9.0	680	0.4	77	<70	5.6	13	1.4	1.7	3.18	7.7	<7	2.7	0.34	519	2140	39	79	10.6	160	0.8	0.59	12.1	69	2.6
3E	10.9	700	0.5	114	<90	5.8	16	2.4	2.3	3.17	7.2	<8	2.8	0.52	464	1980	66	100	11.0	140	1.1	0.91	22.9	66	4.2
6E	12.0	680	0.1	108	<60	6.6	19	1.6	2.1	4.17	7.6	21	2.2	0.44	411	730	51	74	13.5	95	1.1	0.81	16.7	86	3.7
6C	8.7	680	0.2	94	<40	9.6	18	1.4	1.6	3.16	8.9	<4	2.3	0.36	413	920	40	79	10.6	160	1.1	0.57	14.0	62	2.7
10E	13.5	570	0.1	97	<60	5.8	20	2.4	1.6	5.64	7.9	22	1.5	0.37	254	700	37	65	17.5	51	1.0	0.63	17.4	121	3.3
10C	10.3	630	0.2	58	<40	4.9	17	2.1	0.9	4.15	12.0	16	1.7	0.27	220	1020	18	68	14.0	<45	1.0	0.30	13.9	92	2.2
Gombe																									
E	6.8	170	<0.1	72	190	3.6	136	3.4	1.5	4.15	6.9	15	1.7	0.29	304	330	31	80	13.0	120	1.8	0.50	12.5	155	2.5
C	3.4	91	<0.1	36	290	1.7	76	1.2	0.7	2.32	4.3	5	0.9	0.15	56	260	15	37	5.0	<32	1.0	0.20	5.9	99	1.3

[a] All data are in μg/g except those in marked %, as shown E = eaten; C = control.

chromium are present at measurable levels: cobalt is found in amounts of 2–13 μg/g and chromium at 10–20 μg/g, except for the Gombe site where roughly 100 μg/g was found. Several grams of soil are consumed daily.

Taking the north–south cline into consideration, calcium and manganese concentrations decrease in the sites from north to south, as does sodium (with some exceptions). Other chemical elements vary by up to a factor of 2 from site to site, indicating that we cannot use the whole data set as a coherent geochemical entity, but must restrict the geochemical evidence to a site-by-site (or microregion-to-microregion) basis. Electrical conductivity measurements (above) support this reservation.

In general, the levels of nutritionally important elements in these samples were similar to those reported by Hunter (1973) for soils consumed by humans in Africa. Hunter concluded they are a good source of mineral element supplementation. At Mahale, at each site for which we have both eaten and control material, the concentrations of most elements are highest in the eaten material. Enrichment of geophagy soils with essential elements has been noted previously. (Muller et al., 1997; Heymann and Hartmann, 1991; Mahaney et al., 1990; Davies and Baillie, 1988), and suggests that geophagy can be a strategy for obtaining mineral nutritional supplements. The presence of higher amounts of certain elements in soils is not, however, sufficient evidence of their availability for uptake, as shown below.

Acid Extractions. Acid extracts of the soils were prepared in order to simulate the dissolution of soil elements by the gastrointestinal tract. Although extraction using hydrochloric acid would have been a more realistic simulation of conditions in the stomach, the high chlorine levels thus produced would make analysis by INAA impossible for many elements. Thus, ammonium oxalate titrated to a pH of 1.99 was used to extract the soils. The elemental levels determined in these extracts are a measure of those available for absorption especially in the stomach. Changes of pH in the intestines may affect the element concentrations in solution at later points in passage of the soil through the digestive tract. Overall, low amounts of elements were found in the extracts. These levels were below those reported in a similar digestive experiment by Hunter (1973). The nutritionally important elements Ca, Co, Mn, Na, K, and V were determined in most of the samples analyzed. Cr, I, and Mg were below detection limits in many samples, although they were measured at site 10 (Tables 4 and 5).

Iron, an important nutritional element, although high in whole soils, was not detectable in extracts (Table 5). Aluminum, barium, and bromine, while generally not considered to be nutritionally important, may have some therapeutic effects and were present in most of the extracts tested. The enrichment, relative to control soils, of eaten samples with certain elements was not observed for these extracts. On the whole, the extracts contain only very low levels of inorganic nutrients, as shown by INAA. The results are not indicative of these soils as good sources of such nutrients.

TABLE 4. SHORT-LIVED ISOTOPE-PRODUCING ELEMENT CONTENT OF SOIL EXTRACTS[a]

Sample	Al	Ca	Cl	I	Mg	Mn	Na	V	K
Blank	0.02	—	0.25	—	—	—	0.49	<0.001	<0.4
Mahale									
1E	39	<16	59	<0.2	<12	0.51	25	0.11	24
1C	32	<17	71	<0.2	<10	0.18	35	0.07	110
7E	39	<16	59	<0.2	<12	0.57	25	0.11	17
8E	58	47	53	<0.2	<13	0.26	22	0.16	
9E	85	140	65	<0.2	<14	0.47	31	0.11	
11E	350	95	80	<0.4	<34	0.37	<26	<0.12	
11C	32	88	97	<0.1	<12	0.48	36	0.13	120
2E	26	95	76	<0.2	<11	0.27	34	0.09	88
2C	91	70	86	<0.2	<16	0.44	47	0.12	
3E	17	14	66	<0.1	<10	0.12	35	0.07	35
6E	54	<17	92	0.5	<13	0.76	37	0.13	41
6C	87	5	64	<0.2	<13	1.23	<13	0.15	65
10E	36	<19	62	0.9	28	0.54	25	0.13	54
10C	63	46	51	0.7	21	0.26	22	0.22	
Gombe									
E	61	<18	67	1.1	<13	1.82	26	0.17	34
C	130	54	98	0.2	13	0.55	36	0.17	54

[a] All data are in μg element/g dry soil extracted; blank values are in μg element/ml ammonium oxalate. E = eaten; C = control.

XPS Analyses. We hoped to obtain information on the surface characteristics of the soil particles by assaying inorganic elements on the soil surfaces using XPS analysis. These surfaces, the interface of soil particles and their environment, would, when ingested, interact directly with the digestive tract and its contents. The soil particles in these samples are likely to consist of mineral grains

TABLE 5. LONG-LIVED ISOTOPE-PRODUCING ELEMENT OF SOIL EXTRACTS[a]

Sample	Ba	Br	Co	Cr	Fe
Mahale					
7E	160	0.4	0.50	<2.3	<82
11C	350	3.1	0.75	<3.4	<92
10E	<50	<0.3	0.56	5.8	<65
Gombe					
E	330	0.5	1.0	<2.3	<130
C	<50	0.9	1.3	<2.0	<69

[a] Values are μg/g soil, determined in ammonium oxalate extracts of some geophagy. E = eaten; C = control.

TABLE 6. RESULTS OF ANALYSIS OF SOIL SAMPLES FROM MAHALE BY X-RAY PHOTOELECTRON SPECTROSCOPY REPRESENTING COMPOSITION OF APPROXIMATELY 3 SURFACE ATOMS DEPTH OF SAMPLE (RELATIVE ATOMIC PERCENT)

Sample	Fe	O	Ca	Mg	K	C	Si	Al	SiO_2/Al_2O_3
Mahale									
1	1.12	61.1	0.42	1.31	0.44	7.2	17.8	10.6	2.0
1C	0.67	56.8	0.45	1.30	0.43	13.9	16.7	9.8	2.1
7E	0.55	60.6	0.38	0.95	0.32	6.5	19.2	11.4	2.0
8E	1.05	58.6	1.08	0.65	0.23	10.1	18.9	9.5	2.4
9E	1.97	24.5	1.45	2.41	0.63	19.1	33.3	16.7	2.4
11E	0.57	30.9	0.19	0.45	0.08	7.7	39.0	21.1	2.2
11C	0.45	50.6	0.26	0.85	0.41	23.7	15.2	8.4	2.2
2E	1.62	60.6	0.22	0.51	0.33	7.3	18.4	11.7	1.9
2C	0.45	54.8	0.23	0.68	0.46	15.6	17.3	10.5	1.9
3E	0.63	60.3	0.24	0.54	0.29	8.4	17.9	11.7	1.8
6E	0.69	61.7	0.10	0.31	0.21	5.8	18.8	12.4	1.8
6C	0.46	56.1	0.13	0.36	0.25	14.9	16.9	11.0	1.8
10E	0.75	60.3	0.11	0.24	0.15	9.3	17.4	11.7	1.8
10C	0.64	56.4	0.10	0.32	0.18	13.3	17.2	11.7	1.8
Gombe[a]									
E	0.87	61.2	0.52	0.51	0.85	5.9	18.6	11.2	2.0
C	0.41	48.5	0.13	0.41	0.42	28.9	14.0	7.3	2.3

[a] E = eaten; C = control.

coated to varying degrees with clay minerals. The surfaces may thus represent the elemental composition of the clay minerals and molecules adsorbed onto the clay matrix, as well as some contribution from sharp or scraped-clean edges of hard mineral grain surfaces. The analytical technique allows for determination of all elements above a detection limit of about 1%. The results (Table 6) show that eight elements compose most of the soil particle surfaces.

Comparison with data from whole-soil analysis (Table 3) shows that Fe values, although relatively high (0.4–1.6%) in the surfaces, are more elevated in the interior of the particles. Although Fe is frequently found at high levels of several percent in soils ingested by primates, these soils do not seem to be good nutritional sources for the element, as Fe was not detectable in the extracts. However, without exception, the surfaces of the ingested soil particles were enriched with Fe compared to the controls. Whether iron plays a role in any physiological function of geophagy in this case is unknown, but charge relationships on the clay lattices could be altered by its presence, and hence adsorption properties of other molecules at these surfaces may be affected positively or negatively for different adsorbents. Fe may be present in a variety of forms in soils and clays, and further work would be useful to examine the form of iron in these soils which renders it unavailable for release at low pH. The interactions of iron in

aqueous media with clay surfaces are complex and alter the electrical properties of the clays (Henry et al., 1990), and the form of iron in these soils may play a role in their high adsorptive properties.

Magnesium was found to be enriched at the particle surfaces, relative to data for whole soil composition, but Ca was found at comparable levels in both the whole soils and at the soil particle surfaces, except in the Gombe samples, which were surface-enriched for Ca. Potassium was lower in the surfaces relative to whole-soil values. Aluminum was often slightly enriched, supporting the expectation that high-aluminum clay minerals coat the particles. Oxygen and silicon, however, were only measured by XPS. At all but one site, where both eaten and control samples were available, oxygen was found in higher concentrations in the eaten sample. This may be an indication of a higher oxide content and lower organic content in the eaten samples, as suggested by carbon concentration data for the whole soils (Table 2).

For each pair of samples, the carbon content was considerably higher in the controls, supporting the suggestion of Hladik and Gueguen (1974) that choosing insect-sorted soil reduces the ingestion of detrital organic material, including perhaps pathogens and parasites. The soil surfaces do not differ in carbon content between eaten and control samples except for the sample from Gombe, where the control soil is also enriched with carbon. Lower levels of carbon were found in the soil particle surface layers, compared to the whole soils. The surface carbon may include organic components or microorganisms peculiar to the chemistry of these soils and potentially beneficial to the chimpanzees. Organogenic forms on some surfaces were noted under SEM (see above).

Adsorption Capacity of Soils. Quinolizidine alkaloids (sparteine, lupanine), tropane alkaloids (atropine), and quinoline alkaloids (quinine) were employed as model compounds for toxic metabolites produced by plants (Wink, 1993a). The results, expressed as means ± SD, of triplicate analyses show that all the soil samples analyzed are capable of binding alkaloids, albeit to differing degrees (Table 7). Alkaloids remained unchanged in chemical terms, i.e., no degradation or conversion products were observed by GLC and GLC-MS. Soils from Mahale showed equally high adsorption rates for all four alkaloids. These binding properties are in general comparable to those of coal and charcoal, materials that are also used therapeutically to adsorb dietary and microbially produced toxins. However, usually the absolute capacities of soils were somewhat lower, depending on their chemical compositions. No differences were found between eaten and uneaten soils in most Mahale samples, an observation also made in earlier studies (Mahaney et al., 1999). However, in the Gombe samples, and in Mahale samples from sites 1 and 11, adsorption was lower in controls as compared to the eaten soils.

The chimpanzees of Mahale and Gombe National Parks eat soil with characteristic properties. Our results indicate certain features of the soils that should

TABLE 7. ALKALOID ADSORPTION OF SOILS FROM MAHALE AND GOMBE, TANZANIA[a]

Sample	Alkaloid adsorption (%)			
	Atropine	Lupanene	Quinine	Sparteine
Mahale				
1E	68.1 ± 0.1	49.6 ± 1.4	83.8 ± 0.6	54.7 ± 1.2
1C	26.7 ± 6.3	36.0 ± 2.5	60.3 ± 2.3	46.0 ± 1.8
7E	56.2 ± 4.8	32.5 ± 6.1	57.3 ± 1.1	34.4 ± 0.8
8E	84.4 ± 2.7	58.0 ± 1.1	88.8 ± 1.1	73.7 ± 3.1
9E	74.3 ± 0.0	445.9 ± 0.7	74.0 ± 1.0	60.8 ± 4.8
11E	59.6 ± 11.8	32.4 ± 2.9	74.4 ± 1.0	45.1 ± 2.8
11C	55.7 ± 12.3	22.3 ± 4.2	54.2 ± 1.3	43.1 ± 5.8
2E	56.8 ± 5.6	29.2 ± 2.3	54.1 ± 0.6	41.1 ± 6.9
2C	57.4 ± 6.3	38.1 ± 1.8	62.0 ± 3.2	47.5 ± 4.1
3E	65.2 ± 16.5	37.1 ± 0.0	76.9 ± 0.8	48.9 ± 2.9
6E	50.9 ± 2.8	21.4 ± 2.1	65.2 ± 2.8	34.9 ± 3.9
6C	54.1 ± 9.7	32.3 ± 2.9	50.3 ± 3.9	48.9 ± 3.7
10E	70.1 ± 0.2	39.3 ± 6.0	67.5 ± 5.5	46.6 ± 3.2
10C	63.1 ± 7.6	36.0 ± 3.1	54.3 ± 4.9	49.2 ± 1.1
Gombe				
E	57.9 ± 2.5	44.0 ± 5.5	46.8 ± 0.6	65.8 ± 3.6
C	46.0 ± 8.9	20.0 ± 1.0	6.5 ± 7.0	42.8 ± 5.4

[a] E = eaten; C = control.

be investigated further. One benefit of the ingestion of highly adsorptive high-clay soils has been considered to be the uptake by the soils of plant alkaloids and antinutrients ingested with a wild plant diet, shown to occur in vivo in birds (Wink et al., 1993; Gilardi et al., 1999), thereby preventing absorption of deleterious plant constituents via the gut. Such adsorptive properties are also known to be useful in preventing the absorption of bacterial toxins and in immobilizing and isolating pathogenic organisms in the gut by means of the adsorption of clay particles to surround the harmful organisms, to be eliminated with the feces (Said et al., 1980; Gardiner et al., 1993). Another possibility is the formation by clay particles of a mucoprotective barrier against intestinal pathogens, as shown in the lumen of *E. coli*-infected sections of rabbit ileum in vitro by Rateau et al. (1982). Knezevich (1998) cited low levels of diarrhea in geophagous rhesus macaques of Puerto Rico, in spite of high parasite loads, and thus geophagy may be a technique for tolerating the presence of some pathogens in the intestinal tract by blocking their action. Traditional human use of insect sorted soils in Zimbabwe for digestive upsets may be a parallel behavior to the use of termite mound soils by chimpanzees of Tanzania (Aufreiter et al., 1997). The mineral compositions of the Zimbabwean soils were comparable to that used in the pharmaceutical Kaopectate. However, such soils were reported to be recommended for collec-

tion from areas of the mound that had recently been disturbed by the insects. This suggests awareness of an influence on the soil resulting from insect contact, which could be the release of a functional compound or of beneficial microorganisms of insect origin applied as a remedy. Currie et al. (1999) demonstrated a mutualism between fungus-growing ants and a filamentous bacterium (an actinomycete) that produces antibiotics specific to a parasite of the insect-cultivated fungus. The ants studied by Currie et al. (1999) carry the bacterium on their ventral surfaces. The possibility that the chimpanzees ingesting termite mound soil are exploiting a similar insect-microbial relationship of termites is intriguing. Possibly some geophagy soils may also be a source of symbiotic organisms: Kortland (1984) pointed out the possible benefit to the health of zoo captives of a ciliate first observed and recognized by Reichenau (1920) as a potential symbiont of higher primates. The cellulose-digesting ciliate organisms, *Troglodytella abrasarti* and *T. gorillae*, were found attached to the mucous membranes of the cecum and large intestine, and although abundant in just-captured chimpanzees and gorillas, they disappeared soon from the gut of the captive animals, who also developed diarrhea in captivity. The source of these ciliates was not determined, and they were later dismissed as parasites, a conclusion disputed by Kortland (1984). These examples suggest the possibility of a relationship between intestinal microorganisms and the good health of higher primates that is maintained by geophagy. The soil is a possible source of beneficial gut microflora as well as of pathogens, and the gut flora of wild primates necessarily reflects their behavior and environment.

The role of geophagy among humans affected by iron depletion and anemia has long been a matter of debate. Geophagy in humans frequently has been associated with anemia and increased parasite loads, but it remains unclear whether geophagy of some soils is a causative factor or whether iron depletion or anemia may lead to geophagy. Geissler et al. (1997, 1998) found soils eaten by Kenyan children having a high prevalence of malnutrition to release iron at low pH sufficient to supplement their diet with 4.7 mg iron per day. They found, however, that there was more anemia and low serum ferritin among geophagous children, relative to those who did not eat soil. The soil eaten by chimpanzees of Tanzania did not release detectable iron at low pH. Iron depletion and anemia were associated with geophagy in Kenyan children (Geissler et al., 1998), whereas serum ferritin concentrations were found statistically to be dependent on geophagy, although no inference could be made on causality.

The possible prevention of iron absorption by geophagy of soils containing high iron concentrations may depend on the form of the iron. This has not been adequately investigated in geophagy studies but may help to predict the effect of iron on clay adsorptive and aggregation properties and on iron uptake or release by the clays. These are likely to be important in determining the consequences of geophagy. The effects of ingestion of several geophagy soils on iron uptake

in humans was tested by Minnich et al. (1968), who found that some soils, high in smectite, inhibited iron absorption while others, high in 1 : 1 clay minerals, did not. Iron may be associated with clay minerals in several forms. Ferric iron surface-complexed on mineral matter can be detrimental to health, in that it has been shown to play a role in increased lung inflammation related to inhalation of silicate dusts (Ghio et al., 1992), and silicate dusts can complex iron from biological sources. Structural iron, if in the form of Fe(III) on the other hand, may be reduced by soil bacteria to Fe(II). This reduction has also been shown to alter the swelling properties of smectites and also to affect particle and pore size (Gates et al., 1998). Acid may to some extent dissolve the edges of clay lattices and release such iron. Ferrous iron salts [Fe(II)] are readily absorbable, while ferric iron [Fe(III)], the rust-colored, oxidized form often found in reddish tropical and semitropical soils, is not considered bioavailable as a salt. However, it may be bioavailable but slowly released when complexed [for example, as in a polymaltose complex, as discussed in Tuomainen et al. (1999)]. The proportions of iron in different forms, the clay mineralogy of the soils, and the gut contents likely interact to confer particular properties on different soils when eaten. It is unknown if animals practicing geophagy can distinguish soils of different properties. The termite mound soils analyzed here, although shown to be high in iron, with slightly lower concentrations on the particle surfaces than the soil particle interiors (Tables 3 and 6) were found not to release detectable iron at low pH. The iron in these samples may be surface-complexed iron, with a limited availability for release from the clay, or it may be structural iron, since it was in a lower concentration at the surface of the soils particles. More extensive investigation would be necessary to clearly define the forms of iron present and the conditions for its bioavailability.

Minnich et al. (1968) described that high clay soils rich in iron and in smectites are habitually snacked on by women in Turkey, and they showed that their ingestion prevents iron uptake by the gut. The practice was associated with the occurrence of anemia among the women. The Turkish soils were also high in smectite. The termite mound soils of western Tanzania, however, contain significant amounts of illite and smectite–illite, 2 : 1 clay minerals comparable to smectite. Although they are rich in iron, they do not release it as low pH. The possible prevention of absorption of significant amounts of iron may have benefits for higher primates afflicted with pathogens: a wide spectrum of bacteria and fungi pathogenic to humans thrive in the presence of iron. Individuals with hyperferremia are more susceptible to pathogens: children with sickle cell disease commonly die of bacterial infection related to iron overload (Weinberg, 1974). Release of iron by the destruction of red blood cells in sickle cell anemia and thalassemia, and from the destruction of liver cells in malaria, increases susceptibility to pathogens as well as being damaging in its own right. Populations of humans practicing geophagy overlap with populations suffering from these

diseases, and it would be valuable to determine whether ingestion of high clay soils may be helpful in this respect.

Episodes of rapid absorption of iron, such as may be obtained from modern mineral supplements, may temporarily saturate the body's system for trapping free radical chemical species, leading to higher rates of lipid peroxidation (Tuomainen et al., 1999). Clay slows the movement of food through the gut. If very low levels of iron were continually released from some clays as they slowly move through the gastrointestinal tract, some iron could be provided for absorption, but the occurrence of peaks of iron absorption could be prevented. The already high concentration of iron in the soils may lend the soils particular, possibly useful aggregative and adsorptive properties, but, depending on its form, may also be a safety factor in limiting the rate of iron uptake by the gut.

However, an increased oxidative capacity in red blood cells has also been suggested to be beneficial in suppressing malarial infection. The Hausa of northern Nigeria, among whom geophagy is common (Wiley and Katz, 1998), utilize a variety of plants with antimalarial properties in their diet. The efficacy of these plants is related to rendering erythrocytes more sensitive to oxidative damage (Etkin and Ross, 1983). Excess oxidation is controlled by the erythrocyte by catalase, an enzyme that uses ferric iron as a cofactor. Sufficient iron in this case is therefore also necessary in controlling excessive oxidation.

In relation to the chimpanzees of Mahale and Gombe, the relationship between pathogenicity of *Entamoeba histolytica* and serum iron in humans, described by Meerovitch (1982), is particularly suggestive. Dysentery due to *E. histolytica* is more severe on an iron-rich diet, while cultures such as the Masai show a much lower incidence of this disease. Meerovitch (1982) related this to their drinking of milk, which supplies lactoferrin and transferrin, to compete with the bacteria for iron uptake in the gut. If adsorbent clay-rich soil also acts to take up iron in the gut, thereby inhibiting the pathogenicity of such bacteria, it would have considerable value in a semitropical environment.

Observations of ingestion of termite mound soils by chimpanzees apparently suffering from intestinal parasites and gastrointestinal symptoms were the impetus to this study (Mahaney et al., 1996, 1999), and ingestion of these soils may represent for the chimpanzees a means to limit the virulence of dysentery. Meerovitch (1982) noted that a high-starch diet, such as in herbivores, promotes the encystment of *E. histolytica*, so that such individuals may have high rates of infection, but high iron must be available for pathogenicity and virulence to rise. A change to meat diets would therefore promote virulent dysentery in such populations. Iron deficiency in human infants and young children is, however, associated with (negative) changes in cognitive function (Pollitt, 1993), and maintenance by geophagy of low iron stores to control pathogens may bring developmental risks. If humans use geophagy at a cost to cognitive abilities in order obtain resistance to endemic pathogens, geophagy is less likely

to have been part of the human behavioral repertoire throughout the course of evolution. The survival of geophagy as a prevalent behavior among pregnant women of nonindustrial cultures argues for its adaptive value (Geissler et al., 1999). We suggest that an adaptive benefit of geophagy of some soils is restriction or control of iron uptake, which helps to limit deleterious effects of some pathogens present in semitropical environments. An important area of investigation in understanding the effects of geophagy would be the routine application of methods that characterize iron chemistry and bioavailability in relation to clay mineralogy in geophagy soils and determination of the effects of iron speciation on clay aggregative behavior, on bioavailability of inorganics including iron, on microbial populations in the soil and in the gastrointestinal tract of geophagous creatures, and on adsorption of organic molecules onto the soils in physiological conditions.

In this context, the results of our analyses of termite mound soils eaten by wild chimpanzees of Mahale and Gombe are consistent with several mechanisms by which ingestion of high-clay soils help in maintaining health in a semitropical environment. In general, geophagy can be described as use of specific soil types, having particular clay components, which form in semitropical environments, as agents for the possible transfer of functional microorganisms and of molecules between the environment and the animals of that environment. They filter toxins and influence the movement of inorganic elements among plants, microorganisms, intestinal parasites, and the digestive tracts of animals.

Some geophagy soils have been shown to release significant amounts of nutrient elements at physiological pHs and are thought to contribute supplementary elements to the diet. It is unknown if geophagy soils with differing properties can be distinguished by sensory means. Although cultural memory may help primates to choose a location for obtaining appropriate soils, the mineralogy and possibly the compounds adsorbed by the clay minerals may also be a basis for sensing which soils are suitable for ingestion among those available in a complex environment. In relation to the particle surfaces, behavioral observations included that the chimpanzees often sucked on the soils, "like hard candy" (M. A. Huffman, field observations) and thus they were wetted in the mouth with saliva and likely broken down into small pieces before swallowing. Adsorbed material on the surface could be released by this behavior, which could be due to liking the taste of the soils, possibly because of the taste of kaolin materials or the taste of organics left by termite activity or of microorganisms. Here, it may be associated with chimpanzees monitoring the mounds for the presence of termites (Uehara, 1982). Occasionally, the chimps were observed to chew the soils, which would expose fresh mineral surfaces to the gut environment. Kaolinite, a 1 : 1 mineral often in high abundance in geophagic soils, is said to have a characteristic odor (and hence taste), and primates have been observed sniffing at soils before ingestion or rejection (Bolton et al., 1998; Uehara, 1982). Local children

at Mahale have said that they eat termite mound soil or clay from the walls of their homes in the rainy season because of the good smell. Women of Kenya distinguished between soils on the basis of taste (Geissler et al., 1999). Animals experienced in geophagy may also recognize the smell and taste of appropriate soils depending on their composition.

In summary, termite mound soils eaten by wild chimpanzees of the Mahale Mountains are rich in clay, particularly 1 : 1 clay minerals, with significant amounts of 2 : 1 clay minerals present. Soils selected for geophagy are relatively high in pH as compared to surrounding (control) soils. They contain elevated iron, which may affect binding affinities for other molecules. They release markedly low levels of elements to solution at low pH, show a relatively high capacity to adsorb alkaloids such as are found in the plants forming the diet of wild chimpanzees, and have a lower organic content than surrounding surface soils. We have described the health benefits of ingesting these soils that are consistent with behavioral observations of the wild chimpanzees of Tanzania, which may be relevant to human populations practicing geophagy.

Acknowledgments—M.A.H. sincerely thanks the people and the government of Tanzania for their long-term cooperation and commitment to his research, in particular the Tanzanian National Scientific Research Council, Tanzanian National Parks, Serengeti Wildlife Research Institute, Mahale Mountains Wildlife Research Centre, and the University of Dar es Salaam. Our deepest gratitude goes to all of our colleagues and friends for their generous efforts. Without their advice, support and understanding, the foundations of this work would not have been made. M.A.H., in particular, sincerely thanks (in alphabetical order) D. A. Collins, J. Goodall, M. S. Kalunde, H. Kayumbo, K. Koshimizu, H. Ohigashi, and Y. Sugiyama. Technical advice and support was provided to M.A.H. by staff of the Louis Pasteur Center for Medical Research, Kyoto. Field research and termite soil collection was funded by the Plant Science Research Foundation-Kyoto University, the Wellcome Trust, U.K., and the University of Toronto to M.A.H. Thanks also go to L. A. Ketch and D. W. Malloch of the Botany Department, University of Toronto, for their criticism of the early manuscript.

REFERENCES

AUFREITER, S., HANCOCK, R. G. V., MAHANEY, W. C., STAMBOLIC-ROBB, A., and SANMUGADAS, K. 1997. Geochemistry and mineralogy of soils eaten by humans. *Int. J. Food Sci. Nutr.* 48:229–242.

BERRESHEIM, K., MATTERN-KLOSSEN, N., and WILMERS, M. 1991. A standard form of spectra for quantitative ESCA analysis. *Fresenius' Z. Anal. Chem.* 341:121–124.

BOLTON, K. A., CAMPBELL, V. M., and BURTON, F. D. 1998. Chemical analysis of soils of Kowloon (Hong Kong) eaten by hybrid rhesus macacques. *J. Chem. Ecol.* 24:195–205.

BROUILLARD, M.-Y., and RATEAU, J.-G. 1989. Pouvoir d'asorption de deux argiles, la smectite et le kaolin sur des entérotoxines bactériennes. *Gastroenterol. Clin. Biol.* 13:18–24.

BROWMAN, D. L., and GUNDERSON, J. N. 1993. Altiplano comestible earths: Prehistoric and historic geophagy of highland Peru and Bolivia. *Geoarchaeology* 8:413–425.

COLLINS, D. A., and MCGREW, W. C. 1988. Habitats of three groups of chimpanzees (*Pan troglodytes*) in Western Tanzania compared. *J. Hum. Evol.* 17:553–574.

CURRIE, C. R., SCOTT, J. A., SUMMERBELL, R. C., and MALLOCH, D. 1999. Fungus-growing ants use antibiotic bacteria to control garden parasites. *Nature* 398:701–704.

DAVIES, A. G., and BAILLIE, I. E. 1988. Soil-eating by red leaf monkeys (*Presbytis rubicunda*) in Sabah, northern Borneo. *Biotropica* 20:252–258.

DAY, P. E. 1965. Particle fractionation and particle size analysis, pp. 545–567, *in* C. A. Black (ed.). Methods of Soil Analysis. American Society of Agronomy, Madison, Wisconsin.

ETKIN, N. L., and ROSS, P. J. 1983. Malaria, medicine and meals: plant use among the Hausa and its impact on disease, pp. 231–359, *in* L. Romanucci-Ross, D. E. Moerman, and L. R. Tancredi (eds.). The Anthropology of Medicine: From Culture to Method. Praeger Scientific, J. F. Bergin, New York.

FOSSEY, D. 1974. Observations on the home range of one group of mountain gorilla (*Gorilla gorilla beringei*). *Anim. Behav.* 22:568–581.

FOSSEY, D. 1983. Gorillas in the Mist. Houghton Mifflin, Boston.

GARDINER, K. R., ANDERSON, N. H., MCCAIGUE, M. D., ERWIN, P. J., HALLIDAY, M. I., and ROWLANDS, B. J. 1993. Adsorbents as antiendotoxin agents in experimental colitis. *Gut* 34:51–55.

GATES, W. P., JAUNET, A.-M., TESSIER, D., COLE, M. A., WILKINSON, H. T., and STUCKI, J. W. 1998. Swelling and texture of iron-bearing smectites reduced by bacteria. *Clays Clay Miner.* 46(5):487–497.

GEISSLER, P. W., MWANIK, D. L., THIONG'O, F., and FRILS, H. 1997. Geophagy among schoolchildren in western Kenya. *Trop. Med. Int. Health* 2:624–630.

GEISSLER, P. W., MWANIKI, D. L., THIONG'O, F., MICHAELSEN, K. F., and FRILS, H. 1998. Geophagy, iron status and anemia among primary school children in Western Kenya. *Trop. Med. Int. Health* 3:529–534.

GEISSLER, P. W., PRINCE, R. J., LEVENE, M., PODA, C., BECKERLEG, S. E., MUTEMI, W., and SHULMAN, C. E. 1999. Perceptions of soil-eating and anaemia among pregnant women on the Kenyan coast. *Soc. Sci. Med.* 48:1069–1079.

GHIO, A. J., KENNEDY, T. P., WHORTON, A. R., CRUMBLISS, A. L., HATCH, G. E., and HOIDAL, J. R. 1992. Role of surface-complexed iron in oxidant generation and lung inflammation induced by silicates. *Am. J. Physiol.* 263:L511–L518.

GILARDI, J. D., DUFFEY, S. S., MUNN, C. A., and TELL, L. A. 1999. Biochemical functions of geophagy in parrots: Detoxification of dietary toxins and cytoprotective effects. *J. Chem. Ecol.* 25:897–922.

HANCOCK, R. G. V. 1978. Some aspects of the analysis of ancient artifacts by neutron activation. *J. Int. Inst. Conserv. Can. Group* 3:21–27.

HANCOCK, R. G. V. 1984. On the source of clay used for Cologne Roman pottery. *Archaeometry* 26:210–217.

HENRY, M., JOLIVET, J. P., and LIVAGE, J. 1990. Aqueous chemistry of metal cation: Hydrolysis, condensation, and complexation. *Struct. Bonding* 25:1–64.

HEYMANN, E. W., and HARTMANN, G. 1991. Geophagy in moustached tamarins, *Sagiunus mystax* (Platyrrhini: Callitrichidae), at the Rio Blanco, Peruvian Amazonia. *Primates* 32:533–537.

HLADIK, C. M. 1977. A comparative study of the feeding strategies of two sympatric species of leaf monkeys: *Presbytis senex* and *Presbytis entellus*, pp. 481–501, *in* T. H. Clutton-Brock (ed.). Primate Ecology: Studies of Feeding and Ranging Behaviour in Lemurs, Monkeys and Apes. Academic Press, New York.

HLADIK, C. M., and GUEGUEN, L. 1974. Géophagie et nutrition minérale chez les primates sauvages. *C.R. Acad. Sci. Paris Ser. D.* 279:1393–1396.

HUFFMAN, M. A. 1997. Current evidence for self-medication in primates: A multidisciplinary perspective. *Yearb. Phys. Anthropol.* 40:171–200.

HUNTER, J. M. 1973. Geophagy in Africa and in the United States: A culture–nutrition hypothesis. *Geogr. Rev.* 63:170–195.

IZAWA, K. 1993. Soil-eating by *Alouatta* and *Ateles. Int. J. Primatol.* 14:229–242.

JOHNS, T. 1990. With bitter herbs shall they eat it: Chemical ecology and the origin of human diet and medicine. University of Arizona Press, Tucson.

JOHNS, T., and DUQUETTE, M. 1991. Detoxification and mineral supplementation as functions of geophagy. *Am. J. Clin. Nutr.* 53:448–456.

JULLIOT, C., and SABATIER, D. 1993. Diet of the red howler monkey (*Alouatta seniculus*) in French Guiana. *Int. J. Primatol.* 14:527–549.

KETCH, L. A. 1998. Microbiological investigations of geophagy in chimpanzees. MSc. thesis. University of Toronto, Toronto.

KNEZEVICH, M. 1998. Geophagy as a therapeutic mediator of endoparasitism in a free-ranging group of rhesus macaques (*Macaca mulatta*). *Am. J. Primatol.* 44:71–82.

KORTLAND, A. 1984. Habitat richness, foraging range and diet in chimpanzees and some other primates, pp. 119–159, *in* D. J. Chivers, B. A. Wood, and A. Bilsborough, (eds.). Food acquisition and processing in primates. Plenum Press, New York.

LAUFER, B. 1930. Geophagy. Anthropology Series XVIII. Field Museum of Natural History.

MAHANEY, W. C. 1990. Glacially crushed quartz grains in Late Quaternary deposits in the Virunga Mountains, Rwanda–indicators of wind transport from the north. *Boreas* 19:81–89.

MAHANEY, W. C. 1999. Paleoclimate and paleonutrition–paleozoopharmacognosy: A timely connection. *Frankf. Geowiss. Arb. Ser.* 25:123–134.

MAHANEY, W. C., WATTS, D. P., and HANCOCK, R. G. V. 1990. Geophagia by mountain gorillas (*Gorilla gorilla beringei*) in the Virunga Mountains, Rwanda. *Primates* 31:113–120.

MAHANEY, W. C., HANCOCK, R. G. V., and AUFREITER, S. 1995. Mountain gorilla geophagy: A possible strategy for dealing with intestinal problems. *Int. J. Primatol.* 16:475–488.

MAHANEY, W. C., HANCOCK, R. G. V., AUFREITER, S., and HUFFMAN, M. A. 1996. Geochemistry and clay mineralogy of termite mound soil and the role of geophagy in chimpanzees of the Mahale Mountains, Tanzania. *Primates* 37:121–134.

MAHANEY, W. C., MILNER, M. W., SANMUGADAS, K., HANCOCK, R. G. V., AUFREITER, S., WRANGHAM, R., and PIER, H. W. 1997. Analysis of geophagy soils in Kibale Forest, Uganda. *Primates* 38:159–176.

MAHANEY, W. C., ZIPPIN, J., MILNER, M., SANMUGADAS, K., HANCOCK, R. G. V., AUFREITER, S., CAMPBELL, S., HUFFMAN, M. A., WINK, M., MALLOCH, D., and KALM, V. 1999. Chemistry, mineralogy and microbiology of termite mound soil eaten by the chimpanzees of the Mahale Mountains, Western Tanzania. *J. Trop. Ecol.* 15:565–588.

MCCONNELL, R. B. 1950. Outline of the geology of Ufipa and Ubende. *Bull. Geol. Surv. Tanganyika* 19.

MEEROVITCH, E. 1982. The jigsaw puzzle of host-parasite relations in amoebiasis begins to take shape, pp. 263–173, *in* E. Meerovitch (ed.). Aspects of Parasitology, A Festschrift Dedicated to the Fiftieth Anniversary of the Institute of Parasitology of McGill University. Institute of Parasitology, McGill University, Montreal.

MINNICH, V., OKCUOĞLU, A., TARCON, Y., ARCASOV, A., CIN, S., YÖRÜKOĞLU, O., RENDA, F., and DEMIRAĞ, B. 1968. Pica in Turkey II. Effect of clay upon iron absorption. *Am. J. Clin. Nutr.* 21:78–86.

MULLER, K.-H., AHL, C., and HARTMANN, G. 1997. Geophagy in masked titi monkeys (*Callicebus personatus* melanochir) in Brazil. *Primates* 30:69–77.

NISHIDA, T. 1990. A quarter century of research in the Mahale Mountains: An overview, pp. 3–35, *in* T. Nishida (ed.). The Chimpanzees of the Mahale Mountains: Sexual and Life History Strategies. The University of Tokyo, Tokyo.

OATES, J. F. 1977. The guereza and its food, *in* T. H. Clutton-Brock, (ed.). Primate Ecology: Studies of Feeding and Ranging Behaviour in Lemurs, Monkeys and Apes. Academic Press, New York.

OATES, J. F. 1978. Water–plant and soil consumption by guereza monkeys (*Colobus guereza*): A relationship with minerals and toxins in the diet? *Biotropica* 10:241–253.

OYAMA, M., and TAKEHARA, H. 1970. Standard soil color chart. Japan Research Council for Agriculture, Forestry and Fisheries.

POIRIER, F. E. 1970. The nilgiri langur (*Presbytis johnii*) of South India, pp. 251–383, *in* L. A. A. Rosenblum, (ed.). Primate Behaviour: Developments in Field and Laboratory Research, Vol. 1. Academic Press, New York.

POLLITT, E. 1993. Iron deficiency and cognitive function. *Annu. Rev. Nutr.* 13:521–537.

RATEAU, J.-G., MORGANT, G., DROY-PRIOT, M.-T., and PARIER, J.-L. 1982. A histological, enzymatic and water-electrolyte study of the action of smectite, a mucoprotective clay, on experimental infectious diarrhea in the rabbit. *Curr. Med. Res. Opin.* 8:233–241.

REICHENAU, E. 1920. Den Wiederkauer-Infusiorien verwandte Formen aus Gorilla und Schimpansen. *Arch. Protistenkd.* 41:1–33.

SAID, S. A., SHIBL, A. M., and ABDULLAH, M. E. 1980. Influence of various agents on absorption capacity of kaolin for *Pseudomonas aeruginosa* toxin. *J. Pharm. Sci.* 69:1238–1239.

SODHI, R. N. S., GRAD, H. A., and SMITH, D. C. 1992. Examination by X-ray photoelectron spectroscopy of the adsorption of chlorhexidine on hydroxyapatite. *J. Dent. Res.* 71:1493–1497.

STAMBOLIC-ROBB, A. 1997. Geophagy amongst free ranging Sumatran orangutans (*Pongo pygmaeus abelii*) of Gunung Leuser National Park and ex-captive Bornean orangutans (*Pongo pygmaeus pygmaeus*) of Sungai Wain Forest, Indonesia. MSc thesis. York University, Toronto.

TUOMAINEN, T.-P., NYYSSÖNEN, K., PORKKALA-SARATAHO, E., SALONEN, R., BAUMGARTNER, J. A., GEISSER, P., and SSALONEN, J. T. 1999. Oral supplementation with ferrous sulfate but not with non-ionic iron polymaltose complex increases the susceptibility of plasma proteins to oxidation. *Nutr. Res.* 19:1121–1132.

UEHARA, S. 1982. Seasonal changes in the techniques employed by wild chimpanzees in the Mahale Mountains, Tanzania, to feed on termites (*Pseudacanthotermes spiniger*). *Folia Primatol.* 37:44–76.

VORTISCH, W., MAHANEY, W. C., and FECHER, K. 1987. Lithology and weathering in a paleosol sequence on Mount Kenya. *Geol. Paleontol.* 21:245–255.

WATTS, D. P. 1984. Composition and variability of mountain gorilla diets in the central Virungas. *Am. J. Primatol.* 7:323–356.

WEINBERG, E. D. 1974. Iron and susceptibility to infectious disease. *Science* 184:952–956.

WHITTIG, L. D. 1965. X-ray diffraction techniques for numerical identification and mineralogical composition, pp. 671–696, *in* C. A. Black, (ed.). Methods of Soil Analysis. American Society of Agronomy, Madison, Wisconsin.

WILEY, S. A., and KATZ, H. S. 1998. Geophagy in pregnancy: A test of a hypothesis. *Curr. Anthropol.* 39(4):532–545.

WINK, M. 1993a. Allelochemical properties and the raison d'être of alkaloids, 43:1–118, *in* G. Cordell (ed.). The Alkaloids. Academic Press, London.

WINK, M. 1993b. Quinolizidine alkaloids, pp. 197–329, *in* P. Waterman, (ed.). Methods in Plant Biochemistry. Academic Press, London.

WINK, M., HOFER, A., BILFINGER, M., ENGLERT, E., MARTIN, M., and SCHNEIDER, D. 1993. Geese and plant dietary allelochemicals—food palatability and geophagy. *Chemoecology* 4:93–107.

WINK, M., MEISSNER, C., and WITTE, L. 1995. Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*. *Phytochemistry* 38:139–153.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

PHENYLHEPTATRIYNE VARIATION IN *Bidens alba* VAR. *radiata* LEAVES

EMILY G. CANTONWINE and KELSEY R. DOWNUM*

Department of Biological Sciences
Florida International University
Miami, Florida 33199

(Received July 5, 2000; accepted October 13, 2000)

Abstract—Variation of phenylheptatriyne (PHT) in leaves of *Bidens alba* (Linn.) var. *radiata* (Shultz-Bip.) was investigated across its Florida range, throughout the year, and in response to the photoenvironment. A survey of PHT in *B. alba* leaves was done at 13 sites in Florida and three sites throughout the year. PHT concentrations differed among populations ($P < 0.001$), but little of the variation was explained by latitude ($R^2 = 0.024$) or longitude ($R^2 = 0.022$). Leaf concentrations of PHT fluctuated throughout the year ($P < 0.001$); they were highest in October and lowest in January and April. Experimental manipulations of light quality and quantity caused increased PHT accumulation when UV wavelengths were filtered out and decreased accumulation under low R/FR treatments. Low light levels did not significantly influence the concentration of PHT on a dry weight basis, but did decrease leaf biomass and PHT levels on a leaf area basis. The results suggest that PHT in *B. alba* leaves varies in nature and that light quality affects PHT biosynthesis in situ.

Key Words—Acetylenes, Asteraceae, *Bidens*, phenylheptatriyne, phytochemical variation, geographic variation, seasonal variation, photoenvironment, light, R/FR, UV radiation.

INTRODUCTION

Secondary compounds vary within plant species. Some of the variation may be genetic; however, Bryant et al. (1983) has proposed that much of the quantitative phytochemical variation within a species may result from the availability of resources for their production. For example, when carbon availability is

* To whom correspondence should be addressed.

0098-0331/01/0200-0313$19.50/0

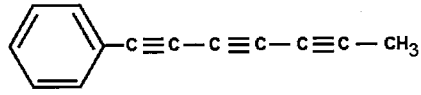
limited by low light, Bryant's hypothesis suggests that carbon would be allocated to growth (i.e., primary processes) rather than biosynthesis of secondary compounds—resulting in decreased production of defensive allelochemicals.

In addition to light quantity, light quality (i.e., wavelengths available) can also influence the levels of secondary compounds by activating enzymes (Tietjen and Materns, 1983) or genes coding for biosynthetic enzymes (Chappel and Hahlbrock, 1984; Lois et al., 1989; Schultze-Lefert et al., 1989). This is true for coumarins and flavonoids, both products of the shikimate pathway with known photomediated mechanisms (Beirer and Oertli, 1983; Hahlbrock and Scheel, 1989; Harborne, 1993).

Acetylenes are plant secondary metabolites derived from common fatty acids. There are about 1000 acetylenic compounds known from 18 plant families and the basidiomycetes (Bohlmann et al., 1973). They are common phytochemical constituents in such flowering plant families as the Asteraceae, Araliaceae, Companulaceae, Santalaceae, and Apiaceae. In many Asteraceae, acetylenes are characterized by conjugated systems of double and triple carbon bonds, often with cyclic or heterocyclic structures. Some acetylenes are toxic toward a variety of organisms that are detrimental to plants; many are phototoxic and require specific wavelengths of UV radiation for expression of their toxicity. Little is known regarding how acetylenes vary in nature or how environmental factors influence their biosynthesis or storage (Ichihara and Noda, 1977; Norton and Towers, 1985; Towers, 1984).

Phenylheptatriyne (1-phenylhepta-1,3,5-triyne, PHT) (Figure 1) is one such phototoxic acetylene that is activated by UV-A radiation (Wat et al., 1979, 1980; Weir et al., 1985). UV-excited PHT has been shown to degrade cellular membranes, resulting in broad-spectrum toxicity against competing organisms, including herbivorous insect larvae (Wat et al., 1981; Arnason et al., 1981), fungi (Bourque et al., 1985; Arnason et al., 1980), bacteria (Geissberger and Sequin, 1991; Towers et al., 1979; Wat et al., 1980; Rabe and Van Studen, 1997; Towers and Hudson, 1987), membrane-bound viruses (Hudson et al., 1986; Towers and Hudson, 1987), and other plants (Campbell et al., 1982).

PHT is found throughout *Bidens alba* var. *radiata* (Asteraceae), although the highest concentrations occur in leaves (Bourque et al., 1985). *B. alba* ranges



Phenylheptatriyne

FIG. 1. Chemical structure of PHT.

throughout Florida, the Caribbean islands, and the Gulf Coast of Mexico (Ballard, 1986) and is part of the *Bidens pilosa* (L.) complex (Ballard, 1986). This complex includes species that are invasive weeds in more than 40 countries (Holm et al., 1977). *B. alba* is used extensively as a food and medicine (e.g., Wat et al., 1980; Ugarte, 1997); much of the research on this complex recognizes PHT as a significant ecological and medicinal factor (Campbell et al., 1982; Meissner et al., 1986; Geissberger and Sequin, 1991; Wat et al., 1980).

This paper investigates the natural variation of PHT in *B. alba*, and explores the influence of photoenvironment on accumulation of this acetylenic compound in *B. alba* leaves.

METHODS AND MATERIALS

Protocol for Geographic and Seasonal Studies. Thirty plants were randomly sampled along a 50-m transect at 13 Florida sites between June 16 and 30, 1998 (Table 1). There was at least 150 km between sites to increase the likelihood of sampling from distinct populations. Plants growing in full sun environments were selected at each sample site. Because *B. alba* grows in disturbed habitats, sites were generally located along roadsides, parking lots, sidewalks, or empty lots. Sampled leaves were restricted to the second leaf from the top of a flowering shoot to minimize variation in PHT levels resulting from leaf position and/or developmental stage.

TABLE 1. PHT IN *B. alba* GROWING IN FLORIDA[a]

Site	Habitat description	Latitude (N)	Longitude (W)	PHT (mean ± SE)	
				nmol/mm^2	μmol/g^{-1}
1	parking lot gully	87°14.05′	30°26.77′	1.00 ± 0.07	21.49 ± 1.69 b
2	roadside	86°08.30′	30°44.22′	0.76 ± 0.04	18.87 ± 1.02 a
3	edge of building	84°34.60′	30°35.59′	0.71 ± 0.05	13.64 ± 0.98 b
4	sidewalk cracks	82°07.20′	30°16.61′	0.86 ± 0.05	15.51 ± 1.04 b
5	open field	83°23.70′	29°40.41′	1.34 ± 0.11	25.38 ± 2.37 b
6	roadside	81°40.27′	28°55.43′	0.55 ± 0.08	11.43 ± 2.05 b
7	roadside	80°50.83′	28°33.36′	0.98 ± 0.07	16.31 ± 1.07 c
8	open field	82°26.92′	28°06.84′	1.07 ± 0.07	21.74 ± 1.19 b
9	parking lot edge	81°29.51′	27°30.70′	1.05 ± 0.10	19.04 ± 1.89 b
10	roadside	80°22.93′	27°22.26′	1.40 ± 0.08	30.40 ± 1.86 a
11	roadside	81°54.13′	26°41.32′	0.91 ± 0.05	19.85 ± 1.16 a
12	roadside	80°28.62′	25°45.69′	1.23 ± 0.07	24.29 ± 1.33 b
13	roadside	81°02.71′	24°43.59′	0.97 ± 0.05	18.74 ± 0.95 b

[a] Site number, habitat description and GPS coordinates for each sample site are listed. DW (±SE) (g): a, 0.004 (0.0002); b, 0.005 (0.0002); c, 0.006 (0.0002).

TABLE 2. PARTIAL FACTORIAL DESIGN OF PHOTOENVIRONMENT EXPERIMENT

	+UV	−UV
High light		
R/FR normal	A, B	C
R/FR low		
Low light		
R/FR normal		D
R/FR low		E

Three leaf discs were removed from leaves on site by using a hole punch. Individual discs measured 6.3 mm in diameter, resulting in a total area of 93.5 $mm^2/3$ discs. PHT was extracted by immersing the three leaf discs into 6 ml of methanol (MeOH) for 14 days. Preliminary studies to determine the stability of extracted PHT in MeOH revealed that PHT concentration remained constant for at least 60 days. Additional leaf discs (three per leaf) were collected and dried for dry weight determination. Seasonal variation in PHT levels was determined by sampling plants at three sites (sites 8, 9, and 12) in October, January, and April. Extracts were analyzed for PHT by high-pressure liquid chromatography (HPLC).

Photoenvironmental Experiments. Seeds were collected in Miami, Florida, surface sterilized with 0.05% sodium hypochlorite (Reddy and Singh, 1992), and germinated. Seedlings were randomly transplanted into 10.1 cm round pots after six days.

The experiment was set up in a partial factorial design with two levels for each of three light factors: quantity of total irradiation [high PPFD (photosynthetic photon flux density) versus low PPFD]; UV radiation (normal UV versus filtered UV), and R/FR (normal R/FR versus low R/FR) (Table 2). There were five treatments and five replications.

Five treatments were created by using energy films or plastics. Treatment A was an open control and had no film or plastic. Treatment B used a transparent plastic (4 mil thickness) that did not significantly alter transmitted light. This treatment served as a control for the plastic and film effects. Treatment C used a clear poly plastic (6 mil thickness) that filtered UV radiation without decreasing PPFD. Treatments D and E used energy films (3M Corp.) that filtered UV radiation and reduced PPFD by about 80%, but altered R/FR differently. Treatment D used a metal sputter-coated film (REAL20) that did not alter normal R/FR. Treatment E used a dye-impregnated film (NEARL20), which reduced R/FR to about 0.23 of normal. A LiCor LI-1800 spectroradiometer was used to quantify light treatments at the start of the experiment (Table 3).

Twenty-five enclosures ($59 \times 75 \times 54$ cm) were made from PVC pipe (2.5 cm) and set up in a Latin-square design. Energy films or plastics were taped

TABLE 3. LEVELS OF UV-A (320–400 nm), PAR (400–700 nm), AND R/FR (656–664/726–734 nm) FOR EACH LIGHT TREATMENT[a]

Light treatment	UV-A (%)	PAR (%)	R/FR
A	100 (45.91 W/m^2)	100 (1700 μmol/m^2/sec)	1.215
B	87.8	95.0	1.200
C	42.8	95.0	1.186
D	4.9	19.9	1.100
E	5.6	20.1	0.276

[a] Values are based on a single measurement (mean of three consecutive scans) made on October 15, 1998.

across the top of each enclosure with duct tape. The sides of the enclosures were left open because preliminary studies revealed that *B. alba* seedlings did not grow well when the enclosure sides were covered. Enclosures were tilted about 5 cm towards the south to allow for rain runoff. Nine pots were placed in 10.1-cm pot trays that rested on two cinder blocks underneath each treatment enclosure. This set-up gave plants approximately 8 hr of treated light each day.

All treatment enclosures had a minimum of eight plants. After two weeks, plants were treated with a soap spray (5 ml Dove liquid detergent in 896 g water) to eliminate aphids. Nearly all plants were infected with leaf-minors by the third week of the experiment. These insects were manually removed from affected leaves.

After four weeks, three leaf discs from the topmost leaf and the second leaf from the bottom (leaf 2) of each plant were extracted by soaking in 5 ml MeOH. Three additional leaf discs were dried to determine dry weight. Extracts were analyzed by HPLC after 14 days.

Quantification of PHT. PHT was extracted from fresh leaf material with HPLC grade MeOH. Extracts were filtered through Whatman No. 1 filter paper, diluted 1 : 1 with DI water, and partitioned against an equal volume of petroleum ether (PE; bp 30°C–60°C). PE was removed by rotary evaporation at 40°C, and the residue was dissolved in HPLC grade MeOH and stored at 4°C. Spectral analysis revealed that PHT was the predominant acetylenic component in *B. alba*, and it was easily identified by its unique UV absorption spectrum (Bohlmann et al., 1973).

PHT concentrations were determined by UV spectroscopy at 250 nm by using dilutions of PHT standard (ϵ = 148,000) (Bohlmann et al., 1973). Calibration curves were created to standardize PHT concentrations with HPLC peak area units. These were updated at the beginning and end of each study to ensure accurate calculations and were based on curves with $R^2 \geq 0.990$.

In all experiments, PHT was quantified by reverse-phase HPLC on a

Hewlett-Packard model 1090M chromatograph equipped with diode array detection. Separations were accomplished on Hypersil ODS microbore columns (Hewlett-Packard, 5 μm, 100 $\times$ 2.1 mm) with a 0.5 ml/min flow rate and a 40°C column temperature. A water–acetonitrile (H_2O/MeCN) solvent system was used to elute compounds from the column. The program was linear, increasing from 0% to 100% MeCN over 6.5 min, and remained at 100% MeCN for 0.5 min. Compound elution was monitored at 250 nm. All extracts were filtered through a 0.25-μm filter before HPLC injection. Injection volumes were 4 μl for geographic extracts and 7 μl for photoexperiment extracts.

Statistical Analysis. One-way analysis of variance (ANOVA) with site as main effect was used to compare leaf dry weight (DW) and two measures of PHT concentration, based on leaf area (PHT per square millimeter) and DW (PHT per gram). Means and standard errors for PHT per square millimeter, PHT per gram and DW were calculated by using descriptive statistics. Sites were distinguished into homogenous subsets with Tukey's post-hoc analysis. Correlation of PHT per square millimeter with DW for all sites combined, and for each site separately, was tested with a one-way ANOVA. Patterns for geographic influence were investigated with linear regression of PHT per gram by site location. Seasonal patterns were tested by using two-way ANOVA with site and month as the main effects.

Photoenvironmental data were separated into three subsets distinguished by light factor and analyzed independently. The influence of light quantity on leaf PHT was examined by using results from treatments C and D; UV and R/FR influence on leaf PHT levels were determined by using treatments B and C, and D and E, respectively. PHT per square millimeter, PHT per gram, and DW differences for light factor levels were tested with a one-way ANOVA, with light factor as main effect. Correlation between PHT per square millimeter and DW for each light factor was tested with one-way ANOVA. Statistical analyses were performed with SPSS base 9.0 (SPSS Software, Chicago, Illinois).

RESULTS

Data Sets. Three data sets are referred to in the results. Two of the data sets reflect different measures of PHT concentration—one based on leaf area (PHT per square millimeter), the other on leaf biomass (PHT per gram). The third data set, DW, refers to the dry weight of three leaf discs (grams per three discs) taken from the same leaf.

Geographic Variation in PHT and Dry Weight. Sites were restricted to open canopy locations, but they varied in geography and disturbed habitat type. Habitat descriptions, means and standard errors of PHT per square millimeter, PHT per gram, and DW for all sites are shown in Table 1.

TABLE 4. TUKEY'S POST-HOC ANALYSIS USING PHT PER GRAM TO DISTINGUISH HOMOGENEOUS SUBSETS OF SITES[a]

	Subset				
	A	B	C	D	E
Site	6				
	3	3			
	4	4	4		
	7	7	7		
		13	13	13	
		2	2	2	
		9	9	9	
		11	11	11	
			1	1	
			8	8	
				12	12
				5	5
					10
P	0.522	0.151	0.147	0.089	0.170

[a] Uses harmonic mean sample size = 30.000; $\alpha = 0.05$.

PHT per gram values differed between sites ($P < 0.001$). Post-hoc analysis distinguished sites into five subsets (Table 4). PHT per square millimeter and DW were also different between sites ($P < 0.001$). Longitude, from north to south, and latitude, from east to west, were not strongly correlated with PHT per gram ($R^2 < 0.024$), PHT per square millimeter ($R^2 < 0.033$), or DW ($R^2 < 0.029$).

Dry weights of three leaf discs ranged from 0.002 to 0.008 g (data not shown). The majority of samples were within 0.003–0.007 g, with sample sizes for these weights between 11 and 141 for all sites combined; there were only two samples for weights 0.002 g and 0.008 g. The correlation coefficient of DW and PHT per square millimeter for all sites combined was small ($R^2 = 0.070$). When sites were analyzed separately, two sites, 6 and 13, showed a significant correlation between PHT per square millimeter and DW ($P < 0.05$) with $R^2 = 0.382$ and 0.386, respectively. When 0.002 g and 0.008 g weights were excluded from analyses their significance was retracted. Other sites had a positive, negative, or no relationship between DW and PHT per square millimeter.

Seasonal Variation in PHT and Dry Weight. There were differences detected throughout the year for PHT per square millimeter ($P < 0.001$), DW ($P < 0.05$), and PHT per gram ($P < 0.001$), but they did not follow the same pattern. Variation across months is shown for PHT per square millimeter, PHT per gram, and DW (Figure 2). Plants sampled in June had higher PHT per square millimeter, PHT per gram, and DW than plants sampled in January and April. Those sampled in January and April were not significantly different from each

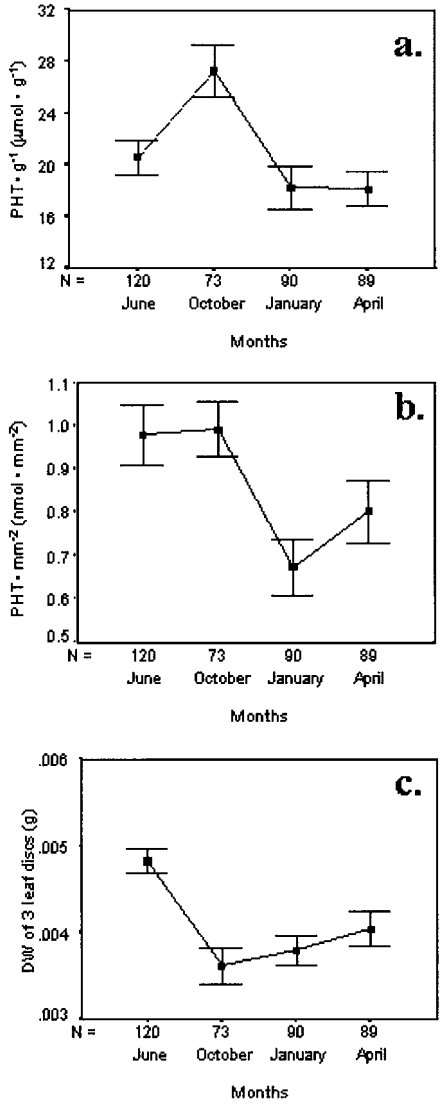


FIG. 2. Mean (± 95% CI) for PHT per gram (a), PHT per square millimeter (b), and DW (c) across months.

other for all analyses. Plants had more PHT per gram in October than plants from other months. DW was positively correlated with PHT per square millimeter ($P < 0.001$; $R^2 = 0.103$) throughout the year.

TABLE 5. INFLUENCE OF PHOTOENVIRONMENT ON PHT AND DRY WEIGHT IN *B. alba* BY LIGHT FACTOR AND LEAF POSITION

Light factor	Factor level	Leaf position	PHT (nmol/mm^2)	PHT (μmol g)	DW (g)
Quantity (intensity)	High	Top	0.81 ± 0.05	23.85 ± 1.46	0.003 ± 0.0002
	Low		0.50 ± 0.03	26.14 ± 2.00	0.002 ± 0.0003
			$P = 0.000^a$	$P = 0.356$	$P = 0.012^a$
	High	Leaf 2	0.26 ± 0.02	8.68 ± 0.59	0.003 ± 0.0002
	Low		0.15 ± 0.01	7.10 ± 0.59	0.002 ± 0.0000
			$P = 0.000^a$	$P = 0.061$	$P = 0.004^a$
UV	High	Top	0.70 ± 0.03	21.92 ± 1.07	0.003 ± 0.0000
	Low		0.81 ± 0.05	23.85 ± 1.46	0.003 ± 0.0002
			$P = 0.038^a$	$P = 0.291$	$P = 0.347$
	High	Leaf 2	0.23 ± 0.02	6.95 ± 0.55	0.003 ± 0.0002
	Low		0.26 ± 0.02	8.68 ± 0.59	0.003 ± 0.0002
			$P = 0.318$	$P = 0.034^a$	$P = 0.195$
R/FR	High	Top	0.50 ± 0.03	26.14 ± 2.00	0.002 ± 0.0003
	Low		0.47 ± 0.03	18.88 ± 1.40	0.002 ± 0.0002
			$P = 0.385$	$P = 0.004^a$	$P = 0.347$
	High	Leaf 2	0.15 ± 0.01	7.99 ± 0.59	0.002 ± 0.0000
	Low		0.15 ± 0.01	7.18 ± 0.56	0.002 ± 0.0000
			$P = 0.923$	$P = 0.923$	

[a] Denotes significance at $P \leq 0.05$.

Effects of Photoenvironment on PHT and Dry Weight. Light quantity affected PHT per square millimeter levels and dry weight. Low light decreased PHT per square millimeter and DW but did not affect PHT per gram. Selective removal of UV radiation resulted in significantly higher PHT per square millimeter for the topmost leaf and PHT per gram for leaf 2. The level of PHT per gram in the topmost leaf was lower under the low R/FR treatment than PPFD equivalent shade treatment (Table 5).

DW influenced PHT per square millimeter for leaves under the light quantity treatments ($P < 0.001$), with $R^2 = 0.224$ for the topmost leaf and $R^2 = 0.204$ for leaf 2, but had an insignificant influence for other light factors. The topmost leaf, the fourth or fifth leaf, had about three times more PHT per square millimeter than leaf 2 for plants included in the analyses, and about 1.5 times more for plants in the open control. Plants from the open control were not used in analyses because they grew differently from plants in the plastic and film treatments. They were significantly shorter and had no lower node branching, unlike plants from the other treatments. PHT levels from plants in the open control were not different from plastic control (UV+) for the top leaf, yet significantly higher for leaf 2.

DISCUSSION

Although there is no evidence that *B. alba* plants from the different sites in this study are reproductively isolated, the distances between sites (ca. 150 km) suggest that this may be enough to restrict gene flow. In addition, *B. alba* flowers do not self-pollinate (Ballard, 1986), increasing the possibility of divergent populations with distance. For these reasons, plants at the different sites were treated as distinct populations.

PHT per gram varied within and among populations throughout Florida. Almost none of the interpopulational variation of PHT per gram could be explained by latitude or longitude. Instead, concentrations were scattered throughout the state in no particular order. Although we restricted populations to open canopy locations, sites varied in soil type, water availability, and level of disturbance. Other environmental factors (i.e., nutrients and water) also influence the biosynthesis of secondary compounds (Gershenzon, 1984), but were not measured.

Foliar levels of PHT per square millimeter and leaf biomass (DW) also varied within and between populations, but PHT per square millimeter was not correlated with DW for most sites. This suggests that intra- and interpopulational variation of PHT per gram in leaves could be due to differences in allocation of resources to PHT biosynthesis over biomass.

Differences in PHT per gram from leaves sampled throughout the year were noted. PHT per gram was high in October followed by June, and low in January and April. Significantly higher PHT per gram in June and October may be a result of greater defense requirements during these months because of stronger herbivore pressure. An alternative hypothesis is that PHT per gram was high in late June and October because there was more carbon available for PHT synthesis (i.e., less was allocated to reproduction). Although *B. alba* flowers year round, there is evidence that closely related *B. pilosa* plants require at least 9 hr of low-light (night) to induce flowering (Kirszenzaft and Felippe, 1978). Day length is longest in late June and may have not been short enough by mid-October to stimulate intensive reproduction. In addition, *B. alba* appears to flower more in South Florida in the winter and spring (personal observation).

PHT per square millimeter and DW differed in leaves over the year. They were correlated in June, January and April, but not in October. October PHT per square millimeter was similar to June levels, but DW decreased. This resulted in the highest PHT per gram levels recorded in the study. The decrease in DW from October leaves without influencing PHT per square millimeter may be a mechanism to increase PHT per gram in response to October pressures by allocating carbon to PHT instead of biomass.

PHT per square millimeter levels were influenced by light quantity, but there was no difference for PHT per gram between light quantity treatments.

This was true in part because PHT per square millimeter and DW were correlated with each other. Because of this relationship, it appears as though PHT per square millimeter and DW were similarly influenced by low PPFD, suggesting that carbon may not have been assimilated into growth significantly more than PHT synthesis, which Bryant's model proposes. The correlation between PHT per square millimeter and DW suggests that PHT per square millimeter production might be linked to leaf tissue biomass rather than restricted by limited light.

Low R/FR decreased PHT per gram for the topmost leaf but did not affect leaf 2. The lack of response from leaf 2 may be due to minimized R/FR differences among treatments since lower leaves were shaded by top leaves and surrounding plants. The response of PHT per gram for the topmost leaf to low R/FR is similar to what has been shown for flavonoids. Flavonoid biosynthesis is induced by R and reversed by FR, resulting in less flavonoid biosynthesis when R/FR is low (Piringer and Heinze, 1954; Siegelman and Hendricks, 1957; Beggs and Wellmann, 1985). Therefore, low R/FR may inhibit PHT biosynthesis by a phytochrome-mediated mechanism similar to flavonoids. More research is needed to answer this question.

Decreased levels of UV radiation significantly increased PHT per square millimeter, for the topmost leaf and PHT per gram for leaf 2. UV apears to influence PHT levels in leaves, most likely affecting cuticular PHT, since epidermal leaf layers absorb most of the UV radiation reaching the leaf (Alenius et al., 1995). There are three possible modes of action that might explain these data. First, more PHT may have been photodegraded by higher levels of UV compared to filtered UV treatments. Photodegradation of PHT occurs when PHT is excited by UV (Marchant, 1987). Second, PHT accumulation may have increased in leaves in response to low UV levels to maintain defensive potential. Downum (1992) suggested that the high concentrations of acetylenes commonly found in roots may be a response to low UV occurrence. Third, low UV might allow plants to accumulate higher levels of PHT with less chance for autotoxicity (i.e., tissues might be able to store higher levels of these phototoxic components in the absence of the activating wavelengths of light). Little is known about polyacetylene autotoxicity, but plant cells are susceptible to UV-activated toxicity by PHT (Campbell et al., 1982). The last two hypotheses could be regulated by UV mechanisms. Additional research is needed to address these complex questions.

Does the photoenvironment explain the natural variation seen in geographic and seasonal studies? The variation detected within and among populations in geographic studies does not appear to be caused by light differences across geographic range. This is not surprising since the sampled populations were not that different in elevation or geography and probably did not vary a great deal in light quantity or quality. Some of the PHT per gram variation may be due to R/FR or UV effects from vegetative shading by neighbor plants.

PHT per square millimeter and DW detected in plants throughout the year

can be explained in part by seasonal fluctuations in light quantity for all months except for October. PPFD is highest in the summer and lowest in the winter (Lee and Downum, 1991). Plants in June, January, and April appear to respond to this variation much like the light quantity experiment—low PHT per square millimeter and DW when PPFD is low, and high PHT per square millimeter and DW when PPFD is high. However, plants sampled in October did not respond like plants from the other months suggesting that an alternative factor (i.e., increased herbivore pressures or flowering stage) may be a more likely explanation.

CONCLUSION

PHT concentrations in *B. alba* leaves are variable. There are significant concentration differences among populations, but the geographic location of sites does not explain much of the observed variation. Geographic variation, due to genetic or environmental differences, was not measured in this study. PHT concentrations in leaves were highest in the fall, and this suggests that the plants may need to defend themselves to the greatest extent during this season. Light quantity did not influence PHT concentrations in leaves, but did affect PHT levels and leaf biomass. Light quality influenced PHT concentrations in leaves and may be a regulatory factor in the biosynthesis of PHT. More research is necessary to demonstrate the effects of light quality factors on PHT biosynthesis.

Acknowledgments—We thank Victor Apanius and Paulette Johnson for statistical guidance, Allan Phipps for laboratory help, Scott Gregor for field support, and University of Florida's Tropical Research and Education Center for land to conduct experiments. This work was funded by a grant from the Tropical Biology Program from the Department of Biological Sciences at Florida International University.

REFERENCES

ALENIUS, C. M., VOGELMANN, T. C., and BORNMAN, J. F. 1995. A three dimensional representation of the relationship between penetration of UV-B radiation and UV-screening pigments in leaves of *Brassica napus. New Phytol.* 131:297–302.

ARNASON, T., WAT, C. K., DOWNUM, K. R., YAMAMOTO, E., GRAHAM, E. A., and TOWERS, G. H. N. 1980. Photosensitization of *Escherichia coli* and *Saccharomyces cervisiae* by phenylheptatriyne from *Bidens pilosa. Can. J. Microbiol.* 26:698–705.

ARNASON, T., SWAIN, T., WAT, C. K., GRAHAM, E. A., PARTINGTON, S., and TOWERS, G. H. N. 1981. Mosquito larvacidal activity of polyacetylenes from species in the Asteraceae. *Biochem. Syst. Ecol.* 9:63–68.

BALLARD, R. 1986. *Bidens pilosa* complex (Asteraceae) in North and Central America. *Am. J. Bot.* 73:1452–1465.

BEGGS, C. J., and WELLMANN, E. 1985. Analysis of light-controlled anthocyanin formation in coleoptiles of *Zea mays* L.: The role of UV-B, blue, red and far-red light. *Photochem. Photobiol.* 41:481–486.

BEIRER, R. C., and OERTLI, E. H. 1983. Psoralen and other linear furanocoumarins as phytoalexins in celery. *Phytochemistry* 22:2595–2597.

BOHLMANN, F., BURKHARDT, T., and ZDERO, C. 1973. Naturally Occurring Acetylenes. Academic Press, London.

BOURQUE, G., ARNASON, J. T., MADHOSINGH, C., and ORR, W. 1985. The photosensitization of the plant pathogen *Fursarium culmorum* by phenylheptatriyne from *Bidens pilosa. Can. J. Bot.* 63:899–902.

BRYANT, J. P., CHAPIN, F. S., III, and KLEIN, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CAMPBELL, G., LAMBERT, J. D. H., ARNASON, T., and TOWERS, G. H. N. 1982. Allelopathic properties of alpha-terthienyl and phenylheptatriyne, naturally occurring compounds from species of Asteraceae. *J. Chem. Ecol.* 8:961–972.

CHAPPEL, J., and HAHLBROCK, K. 1984. Transcription of plant defense genes in response to UV light or fungal elicitor. *Nature* 311:76–78.

DOWNUM, K. R. 1992. Tansley review No. 43 Light-activated plant defense. *New Phytol.* 122:401–420.

GEISSBERGER, P., and SEQUIN, U. 1991. Constituents of *Bidens pilosa* L.: Do the components found so far explain the use of this plant in traditional medicine? *Acta Trop.* 48:251–261.

GERSHENZON, J. 1984. Changes in levels of plant secondary metabolites under water and nutrient stress, pp. 273–320, *in* B. N. Timmermann, C. Steelink and F. A. Loewus (eds.). Recent Advances in Phytochemistry: Phytochemical Adaptations to Stress, Volume 18. Plenum Press, New York, New York.

HAHLBROCK, K., and SCHEEL, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Mol. Biol.* 40:347–369.

HARBORNE, J. B. 1993. The Flavanoids. Advances in Research (1988–1991). Chapman and Hall, London.

HOLM, L. G., PLUCKNETT, D. L., PANCHO, J. V., and HERBERGER, J. P. 1977. The World's Worst Weeds: Distribution and Biology. University of Hawaii Press, Honolulu, pp. 185–190.

HUDSON, J. B., GRAHAM, E. A., and TOWERS, G. H. N. 1986. Investigation of the antiviral action of the photoactive compound phenylheptatriyne. *Photochem. Photobiol.* 43:27–33.

ICHIHARA, K., and NODA, M. 1977. Distribution and metabolism of polyacetylenes in safflower. *Biochem. Biophys. Acta* 487:249–260.

KIRSZENZAFT, S. L., and FELIPPE, G. M. 1978. Effects of photoperiod and growth regulators on flowering of *Bidens pilosa* L. *Ciencia Cult.* 30:357–360.

LEE, D. W., and DOWNUM, K. R. 1991. The spectral distribution of biologically active solar radiation at Miami, Florida, USA. *Int. J. Biometeorol.* 35:48–54.

LOIS, R., DIETRICH, A., HAHLBROCK, K., and SCHULZ, W. 1989. A phenylalanine ammonia-lyase gene from parsley: Structure, regulation and identification of elicitor and light responsive cis-acting elements. *EMBO J.* 8:1641–1648.

MARCHANT, Y. Y. 1987. Photodecomposition of naturally occurring biocides, pp. 168–175, *in* J. R. Heitz and K. R. Downum (eds.). Light-Activated Pesticides. American Chemical Society, Washington, D.C.

MEISSNER, R., NEL, P. C., and BEYERS, E. A. 1986. Allelopathic influence of *Tagetes*- and *Bidens*-infested soils on seedling growth of certain crop species. *S. Afr. J. Plant Soil* 3:176–180.

NORTON, R. A., and TOWERS, G. H. N. 1985. Synthesis of polyacetylenes in tumor callus of *Bidens alba. J. Plant Physiol.* 120:273–283.

PIRINGER, A. A., and HEINZE, P. H. 1954. Effect of light on the formation of a pigment in the tomato fruit cuticle. *Plant Physiol.* 29:467–472.

RABE, T., and VAN STUDEN, J. 1997. Antibacterial activity of South African plants used for medicinal purposes. *J. Ethnopharmacol.* 56:81–87.

REDDY, K. N., and SINGH, M. 1992. Germination and emergence of hairy beggarticks (*Bidens pilosa*). *Weed Sci.* 40:195–199.

SCHULTZE-LEFERT, P., DANGL, J. L., BECKER-ANDRE, M., HAHLBROCK, K., and SCHULTZ, W. 1989. Inducible in vivo DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. *EMBO J.* 8:651–656.

SIEGELMAN, H. W., and HENDRICKS, S. B. 1957. Photocontrol of anthocyanin formation in turnip and red cabbage seedlings. *Plant Physiol.* 32:393–398.

TIETJEN, K. G., and MATERNS, U. 1983. Different response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi. 2. Effects on enzyme activities. *Eur. J. Biochem.* 131:409–413.

TOWERS, G. H. N. 1984. Interactions of light with phytochemicals in some natural and novel systems. *Can. J. Bot.* 62:2900–2911.

TOWERS, G. H. N., and HUDSON, J. B. 1987. Potentially useful antimicrobial and antiviral phototoxins from plants. *Photochem. Photobiol.* 46:61–66.

TOWERS, G. H. N., ARNASON, T., WAT, C. K., GRAHAM, E. A., LAM, J., and MITCHELL, J. C. 1979. Phototoxic polyacetylenes and their thiophene derivatives (effects on human skin). *Contact Dermatitis* 5:140–144.

UGARTE, C. A. 1997. Ethnopharmacology of plants used as both food and medicine in a Totonac community of Mexico. Master's thesis. Florida International University, Miami.

WAT, C. K., BISWAS, R. K., GRAHAM, E. A., BOHM, L., TOWERS, G. H. N., and WAYGOOD, E. R. 1979. Ultraviolet-mediated cytotoxic activity of phenylheptatrilyne from *Bidens pilosa* L. *J. Nat. Prod.* 42:103–111.

WAT, C. K., JOHNS, T., and TOWERS, G. H. N. 1980. Phototoxic and antibiotic activities of plants of the Asteraceae used in folk medicine. *J. Ethnopharmacol.* 2:279–283.

WAT, C. K., PRASAD, S. K., GRAHAM, E. A., PARTINGTON, S., ARNASON, T., and TOWERS, G. H. N. 1981. Photosensitization of invertebrates by natural polyacetylenes. *Biochem. Syst. Ecol.* 9:59–62.

WEIR, D., SCAIANO, J. C., ARNASON, J. T., and EVANS, C. 1985. Photochemistry of the phototoxic polyacetylene phenylheptatriyne. *Photochem. Photobiol.* 42:223–230.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

EFFECTS OF SALINITY ON ENDOGENOUS ABA, IAA, JA, AND SA IN *Iris hexagona*

YONGYIN WANG, SUSAN MOPPER, and KARL H. HASENSTEIN*

Biology Department University of Louisiana at Lafayette
Lafayette, Louisiana 70504-2451

(Received May 25, 2000; accepted October 12, 2000)

Abstract—Phytohormones play critical roles in regulating plant responses to stress. We investigated the effects of salinity on abscisic acid (ABA), indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA) in leaves, stalks, fruits, and seeds of *Iris hexagona*, a native wetland species. Using gas chromatography–mass spectroscopy with selected ion monitoring, our experiments demonstrated significant and different short- and long-term changes in iris phytohormones. ABA and JA generally increased and IAA and SA declined in response to salinity. We conclude that these phytohormones may have separate and interactive effects on how plants respond and adapt to stress in natural environments.

Key Words—Abscisic acid, *Iris hexagona*, jasmonic acid, indole-3-acetic acid, phytohormones, salicylic acid, salinity, stress.

INTRODUCTION

Increasing salinity from human disturbance and climate change is a critical problem worldwide because it has dramatic effects on plant physiology and performance (Levitt, 1980; Abbas et al., 1991; Brugnoli and Bjorkman, 1992; Morgan, 1984; Delesalle and Mazer, 1996; Morales et al., 1998). Salinity changes nutrient and water availability, lowers the quality of arable lands, and alters the structure of ecological communities. Salinity-induced osmotic stress, the physiological equivalent of drought stress, typically reduces growth and photosynthesis in plants (Pasternak, 1987). Stress can also arise from competition of sodium with nutritive ions, toxicity, and injury of cellular structures (Levitt, 1980). Because most studies have been performed with crop plants, we know little about salinity stress in natural plant populations, and we do not yet understand the role of salinity in the evolution of salt tolerance and plant speciation. Here we present

*To whom correspondence should be addressed. e-mail: hasenstein@louisiana.edu

0098-0331/01/0200-0327$19.50/0

our research on plant growth regulators in a noncrop, glycophytic plant (*Iris hexagona*) exposed to salinity stress.

Plants produce proteins in response to abiotic (salinity, drought, temperature) and biotic (pathogens, herbivores) stress. Many of these proteins are induced by phytohormones such as abscisic acid (ABA) (Shinozaki et al., 1998; El-Enany, 2000; Jin et al., 2000), salicylic acid (SA) (Thaler et al., 1999; Hoyos and Zhang, 2000) and jasmonates (Chao et al., 1999; Thaler, 1999). The generic stress hormone ABA is up-regulated by salinity and induces genes involved in salt and osmotic alleviation (Serrano and Gaxiola, 1994). In cucumber leaves, salinity raised ABA levels, which subsequently increased cold and heat resistance of seedlings (Talanova and Titov, 1994). In the halophyte *Suaeda maritima*, ABA concentrations fluctuated in response to salinity (Clipson et al., 1988). ABA delayed the deleterious effect of NaCl and improved tolerance to ionic stress in tobacco callus culture (Eberhardt and Wegman, 1989) and in sorghum (Amzallag et al., 1990).

The observation that ABA accelerated adaptation of cultured tobacco cells to salt, but not to equivalent osmotic concentrations of sorbitol and polyethyleneglycol (LaRosa et al., 1985), suggests that ABA levels change more in response to salt than osmotic stress. Salt-induced ABA mediated the inhibition of leaf expansion and limited the accumulation of Na^+ and Cl^- in leaves, thus protecting bush beans from salt stress (Montero et al., 1997).

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (JAMe), are naturally occurring regulators of plant development (Sembdner and Parthier, 1993). Elevated levels of JA can induce pathogen defense genes in soybean (Farmer et al., 1992; Creelman et al., 1992; Chao et al., 1999), reduce leaf abscission (Curtis, 1984), control the opening of rice florets (Zeng et al., 1999), and modulate salinity (Moons et al., 1997) and osmotic stress (Creelman and Mullet, 1995; Finch-Savage et al., 1996). Herbivory can increase JA production (Agrawal et al., 1999), which induces proteinase inhibitors (Farmer et al., 1992; Thaler et al., 1996; Thaler, 1999) and secondary compounds such as nicotine, a highly effective insecticide (Baldwin, 1994; Thaler et al., 1996; McCloud and Baldwin, 1997).

The induction of JA by wounding or pathogens is attributed to the release of fatty acids, by damaged cell membranes which then metabolize via lipoxygenase to JA (Sembdner and Parthier, 1993). Jasmonic acid and its derivatives also respond to salinity. For example, JAMe levels in rice roots increased significantly in 200 mM NaCl (Moons et al., 1997). Furthermore, pretreatment with JA diminished the inhibitory effect of high salt concentration on growth and photosynthesis of barley (Tsonev et al., 1998). Although JA has been linked with crop plant stress responses (Moons et al., 1997; Creelman and Mullet, 1995; Finch-Savage et al., 1996), there is no information about how salinity affects endogenous JA levels in natural plant populations.

Salicylic acid (SA) belongs to a diverse group of plant phenolics and is

associated with the chemical defense of plants against microbes and herbivores (Raskin, 1992; Thaler et al., 1999). It is widely distributed in monocot and dicot plants (Cleland and Ajami, 1974; Swain et al., 1985; Raskin et al., 1990). Plants are most likely to synthesize SA from cinnamic acid (Chadha and Brown, 1974), via phenylalanine–ammonialyase (PAL) activity. Increased endogenous SA levels coincide with accumulation of pathogen-induced proteins and elevated virus resistance in tobacco (Yalpani et al., 1993). Ethylene and various biotic and abiotic stresses such as salinity, chilling injury, and high temperature are known to increase PAL activity (Enyedi et al., 1992; Martinez-Tellez and Lafuente, 1997; Tomas-Barberan et al., 1997; Dunn et al., 1998), possibly contributing to enhanced SA levels.

SA has antagonistic effects on JA by preventing its accumulation in response to wounding. In flax SA inhibits transcription of allene oxide synthase (AOS), which mediates the conversion of lipoxygenase-derived fatty acid hydroperoxides to unstable allene epoxides and then to JA precursors (Harms et al., 1998). The wound-induced increase of leucine aminopeptidase (LapA) RNAs, which is up-regulated by ABA, JA, salinity, and water deficit, was inhibited by SA (Chao et al., 1999). SA also induces acidic pathogen-related (PR) genes and inhibits basic PR genes, whereas JA does the opposite (Niki et al., 1998). SA also reduced the synthesis of tomato proteinase inhibitors (Doares et al., 1995). Antagonistic interactions between SA and jasmonic acid affect the expression of PR protein genes in tomato (Thaler et al., 1999). Two drought-inducible genes in the drought-tolerant cowpea (*Vigna unguiculata*) were identified after treatment with high salinity and exogenous ABA. One of these genes was also expressed in response to heat stress, methyl jasmonate, and SA (Iuchi et al., 1996), suggesting that SA affects plant resistance to stress.

Indole-3-acetic acid (IAA) plays a major role on regulating plant growth. For example, it controls vascular tissue development, cell elongation, and apical dominance. IAA also responds to salinity in crop plants. There was a significant reduction in IAA levels in rice five days after exposure to NaCl (Nilsen and Orcutt, 1996) and salinity reduced IAA levels 75% in tomato roots (Dunlap and Binzel, 1996).

To our knowledge, this is the first investigation to link ecological responses and phytohormonal induction in natural plant populations under environmental salinity stress. The research is important because it can reveal the endogenous mechanisms that impart stress resistance and provide insight into the potential for native plants to adapt to environmental change.

METHODS AND MATERIALS

Plant Material. Iris hexagona Walter (Iridaceae) is a long-lived perennial that inhabits freshwater wetlands. Plants reproduce clonally via rhizomes and

sexually by fruits that contain up to 60 seeds. Although irises typically occur in fresh-water habitats, our populations are located in the saline soils (4–15 g/liter) of Marsh Island, a 33,000-ha salt marsh located off the coast of Louisiana, USA (92°W 29.5°N).

In March 1997, we collected a total of 300 rhizomes from 10 Marsh Island *I. hexagona* populations and planted them in a common garden in 30 Rubbermaid tubs (220 liters) filled with topsoil. The common garden was located at the Center for Ecology and Environmental Technology at the University of Louisiana at Lafayette. To investigate long-term effects of salinity stress, we randomly assigned tubs to low (LS, 15 mM), medium (MS, 50 mM), and high (HS, 100 mM) salinity treatments, consistent with levels in the natural *I. hexagona* habitat, and maintained the garden for 19 months. In this paper we report on the effects of the low and high salinity treatments.

Salinity Treatment. We established and sustained salinity levels in the common garden with Instant Ocean (Aquarium Systems, Inc., Mentor, Ohio), and monitored tubs weekly with an Orion 125 conductivity meter (Orion Research Inc., Beverly, Massachusetts). ABA, IAA, JA, and SA levels in leaves, seeds, seed pods, and flower stalks were determined at monthly intervals in 1998 and 1999. To investigate short-term responses to salinity we conducted a laboratory experiment and measured iris phytohormones 24 and 48 hr after exposure to 0, 100, 200, and 400 mM NaCl.

Extraction, Purification and Methylation of ABA, IAA, JA and SA. We collected samples on ice and either stored them in a −70°C freezer or extracted them immediately. About 1–2 g of tissue was weighed and ground into powder under liquid N_2. Then 200 ng of internal standards (D_2-ABA, [^{13}C] IAA, D_2-JA) and radioactive tracers—50,000 cpm of [^{3}H] ABA (53 Ci/mmol, Amersham Life Science), [^{3}H] IAA (27 Ci/mmol, Amersham Phamacia Biotech), [^{3}H] JA, 50 Ci/mmol, American Radiolabeled Chemicals Inc.) and 25,000 cpm of [^{14}C] SA (11.7 mCi/mmol, Sigma #S8644, carboxyl group labeled SA) were added and the samples extracted in 10 ml isopropanol overnight at 4°C. The supernatant was centrifuged (2500*g* for 5 min, 4°C), and collected in a 25-ml flask. Three additional extractions with 2 ml of 100% isopropanol were combined and reduced to the aqueous phase in a rotary evaporator and then diluted to ca. 20 ml with water and 0.5 ml of 0.1 M imidazole buffer (pH 8.0). The four components were prepurified using NH_2 columns (Alltech #211150). After priming the columns with methanol, 5% acetic acid, and water, the samples were applied, washed with hexane and methanol, and eluted with 75% methanol containing 0.3 N HCl, dried in a rotary evaporator, and taken up in 3×45 μl 36% MeOH. Then 100 μl of each sample was purified by HPLC (Dionex 500 equipped with a 50×4.6 mm ODS column, Phenomenex 00B-0351-E0) and eluted with 1% acetic acid in 36% MeOH (1 ml/min) for 19 min. The MeOH concentration was raised linearly to 50% within 7 min, and the column was isocratically eluted for

another 9 min. Under these conditions, the retention times for IAA, SA, ABA, and JA were 11, 16, 24, and 30 min, respectively. The two or three 1-ml fractions with the highest radioactivity of each compound were combined, dried in vacuo, methylated with 0.5 ml ethereal diazomethane, dried, and taken up in 50 μl of ethyl acetate. Since no deuterated standard was available for SA, 100 μl of ethyl acetate was used, and losses were determined by measuring the radioactivity of half of that amount. The cumulative losses during the purification resulted in efficiencies of 52–58% for ABA, 59–65% for IAA, 50–52% for JA, and 70–76% for SA.

Determination of ABA, IAA, JA and SA. Aliquots (1–2 μl) of the methylated ABA, IAA, JA, or SA samples were analyzed with an HP 6890 GC equipped with a HP 5973 mass selective detector using selected ion monitoring. Chromatography was carried out on a capillary column (15 m $\times$ 0.25 mm $\times$ 0.25 μm, J&W DB-1701) with helium as carrier gas (1.5 ml/min). For ABA and IAA the oven temperature started at 160°C and after 1 min was ramped to 280°C at 20°C/min. For JA analysis the initial temperature was 120°C, and it was raised at 20°C/min after 1 min. SA analysis started at 60°C for 1 min with a ramp of 20°C/min to 130°C and then 40°C/min to 280°C. Under these conditions, the retention times were 5.25, 4.0, 4.5, and 4.3 min for ABA, IAA, JA, and SA, respectively. The relative abundance of representative fragments for ABA (m/z 190/193), IAA (m/z 130/136), JA (m/z 151/153), and SA (m/z 120) was used for quantification. All analyses were performed in triplicate. The data were calculated as mean $\pm$ SE in micrograms per gram of dry weight (DW).

Statistical Analysis. We used full-factorial and repeated-measure analysis of variance to test the effects of salinity, time, and tissue type on ABA, IAA, JA, and SA concentrations [SAS PROC GLM Version 6 (SAS) Institute, 1994). Data were transformed when necessary to meet assumptions of normality and homogeneity of variance. To determine differences between individual groups, we conducted paired comparisons using Tukey-adjusted least-square means.

RESULTS

Short-Term Effects of Salinity on ABA, JA, IAA, and SA

In laboratory experiments, salinity had short-term effects on all four phytohormones examined (Figure 1). After about eight days, the leaves of plants exposed to 400 mM salt wilted and eventually died but lower salinities were tolerated. ABA content in leaves varied from 0.2 to 1.2 μg/g DW and increased in response to salinity concentration ($F_{3,16} = 24.41$, $P = 0.0001$) and duration of exposure ($F_{1,16} = 4.69$, $P = 0.046$). In paired comparisons, ABA significantly increased after 48 hr of exposure to 400 mM NaCl ($P = 0.0143$). Although 100–200 mM NaCl had a weaker short-term effect on ABA levels, there were

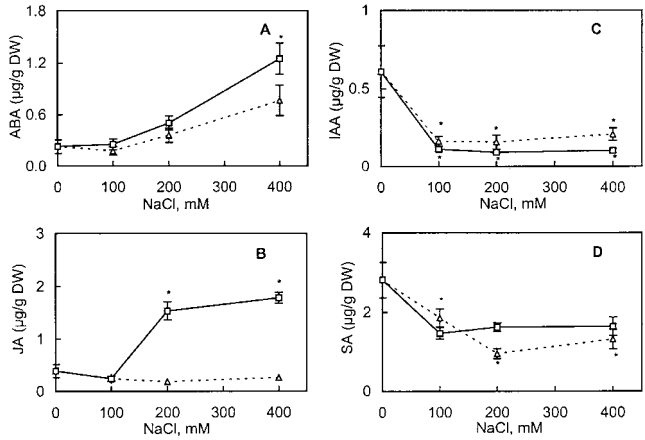


FIG. 1. Effect of 100, 200, and 400 mM NaCl on levels of ABA (A), JA (B), IAA (C) and SA (D) of *Iris hexagona* immature leaves after exposure for 24 (△) and 48 (□) hours. The bars represent standard errors. $^{*}P < 0.05$ as compared with controls (no added NaCl).

significant effects after long-term exposure in the field experiment.

In young leaves, JA content increased in response to salinity concentration ($F_{3,16} = 14.0$, $P = 0.0001$) and duration of exposure ($F_{1,16} = 77.6$ $P = 0.0001$) and ranged from 0.2 to almost 2 μg/g DW (Figure 1B). NaCl at 200 ($P = 0.0026$) and 400 ($P = 0.0008$) mM caused significant increases in JA content within 48 hr.

In contrast to ABA and JA, IAA content rapidly declined in young leaves exposed to salinity (Figure 1C) and was the most sensitive of all four phytohormones to the NaCl treatment ($F_{3,16} = 28.5$, $P = 0.0001$). Within 24 h of exposure to 100 mM NaCl, IAA levels fell from about 0.6 to <0.2 μg/g DW ($P = 0.0001$). The 24-hr IAA reductions were statistically similar to IAA levels after 48 hr of exposure.

Similar to IAA, SA levels in young leaves declined significantly in response to salinity ($F_{3,16} = 17.9$, $P = 0.0001$, Figure 1D). Within 24 hr after treatment with 200 mM NaCl, SA dropped from 2.8 to <1.7 μg/g DW ($P = 0.0001$). The SA reduction was strongest after 24 hr of exposure to 200 ($P = 0.0001$) and 400 mM NaCl ($P = 0.0002$).

Long-Term Effects of Salinity on Phytohormones

Abscisic Acid. In the common garden experiments, leaf ABA levels were greater in high salinity (HS, 100 mM) than low salinity (LS, 15 mM) plants over a six-month time period ($F_{1,4} = 37.7$, $P = 0.004$, Figure 2A). In both salinity

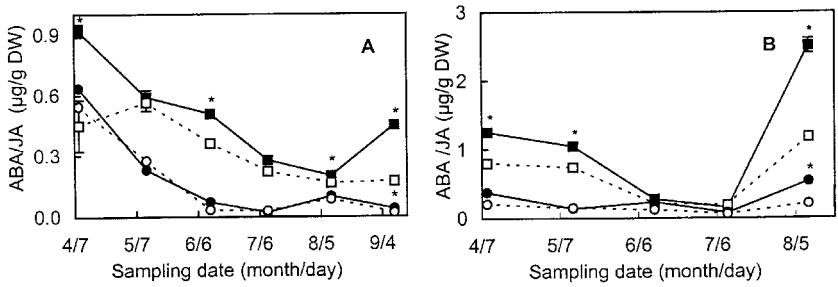


FIG. 2. Temporal change of ABA (squares) and JA (circles) levels in *Iris hexagona* leaves (A) and apex of flower stalks (B) exposed to 100 mM (HS, solid symbols) and ~15 mM (LS, open symbols) salinity. $^{*}P < 0.05$ in pairwise comparison between salinity treatment at the same time.

treatments, ABA levels decreased significantly from May through August, ($F_{1,5} = 37.1$, $P = 0.0001$), then rose significantly in the HS group in September ($P = 0.013$).

After anthesis (early April), the apical parts of HS stalks (Figure 2B) maintained significantly higher ABA levels than LS stalks in every month except June and July ($F_{1,4} = 209.5$, $P = 0.0001$). Towards the end of the growth season (August) ABA levels increased significantly in both LS and HS flower stalks, but the increase was much stronger in HS plants ($P = 0.004$), possibly due to impending abscission.

The ABA concentrations in developing immature flower stalks ranged from <1 to almost 2 μg/g DW (Figure 3A) and were significantly greater under high salinity ($F_{1,36} = 8.22$, $P = 0.0069$). The site of ABA production shifted during stalk development from the basal to the apical region ($F_{4,36} = 2.79$, $P = 0.041$), with the highest concentrations appearing in the central portion of 60-cm stalks ($P = 0.027$) and the apical portion of 90-cm stalks ($P = 0.037$).

The concentration of ABA was higher in developing seeds than pods (Figure 4A), regardless of salinity treatment ($F_{1,4} = 350.6$, $P = 0.0001$). ABA peaked between 20 and 40 days after anthesis ($F_{1,8} = 63.4$, $P = 0.002$) with concentrations of 10–17 μg/g DW. High-salinity seeds had higher ABA levels than low-salinity seeds ($F_{1,8} = 11.3$, $P = 0.01$). By day 60, seeds were fully developed and ABA concentrations had returned to their initial levels in both salinity treatments. The ABA in pods was low throughout seed development (<2 μg/g DW) and was not affected by salinity.

Jasmonic Acid. Salinity had no significant long-term effect on JA levels in the leaves of HS and LS plants (Figure 2A). Similar to the ABA profile, the JA levels decreased significantly early in the growth season from about 0.6 μg/g DW, to <0.1 μg/g DW ($F_{5,15} = 25.0$, $P = 0.001$) and then remained low. After

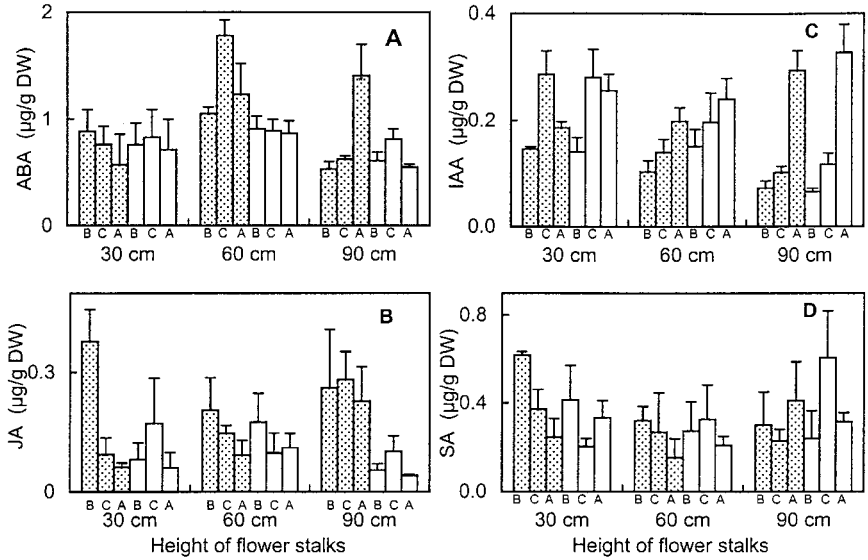


FIG. 3. Distribution of ABA (A), JA (B), IAA (C), and SA (D) in the flower stalks of *Iris hexagona* at different growth stages in the presence of 100 mM (HS, stippled) and ~15 mM (LS, open) salinity. The data are given for the base (B), center (C) and Apex (A) of young (30 cm), medium (60 cm) and mature (90 cm) stalks. $^{*}P < 0.05$ in pairwise comparison between salinity treatment at the same time.

anthesis (Figure 2B), salinity significantly increased JA content in the apical parts of flower stalks ($F_{1,4} = 10.8$, $P = 0.02$). Similar to ABA, JA concentrations rose significantly in HS stalks in August ($P = 0.004$).

Salinity had a significant positive effect on JA in developing stalks (Figure 3B). A higher JA content was measured in HS flower stalks than in LS stalks ($F_{1,36} = 9.43$, $P = 0.004$). In contrast, the JA levels did not vary significantly among the stages of development. On average, slightly more JA was produced in the basal stalk regions ($P = 0.048$), but 90-cm-long stalks showed a higher JA concentration in HS stalks.

There were significant differences between seeds and pods in JA production ($F_{1,8} = 11.1$, $P = 0.01$, Figure 4B) and the pattern of production reversed over time. For example, seeds produced more JA than seed pods in the earliest time period ($P = 0.002$), but significantly less than seed pods in all but the final stage ($P = 0.0001$, 0.0004, 0.02, and 0.76, respectively). In further contrast with ABA, JA levels were highest on average in LS than HS tissue ($F_{1,8} = 7.3$, $P = 0.027$, Figure 4B). Time also affected JA production ($F_{5,4} = 58.2$, $P = 0.0008$). After 40 days, JA concentrations decreased from >1.0 to <0.15 μg/g DW in both salinity

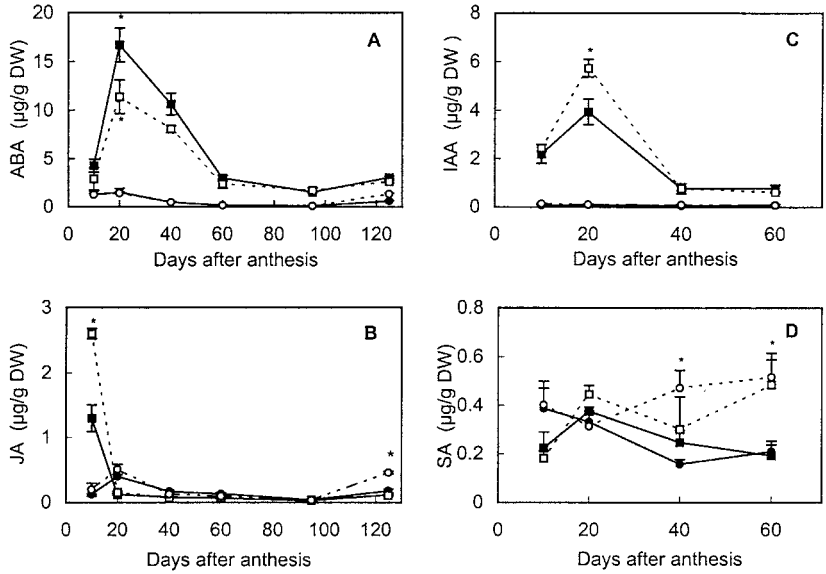


FIG. 4. Temporal change in ABA (A), JA (B), IAA (C), and SA (D) levels of seeds (squares) and pods (circles) of *Iris hexagona* growing in high (HS, 100 mM, solid symbols) and low (LS, ~15 mM, open symbols) salinity following anthesis. $^{*}P < 0.05$ in pairwise comparison between salinity treatment at the same time.

treatments and remained flat for the rest of the growth period, except for a small but significant late-season rise in LS pods ($F_{1,4} = 33.4$, $P = 0.0004$).

Indole-3-acetic Acid. Salinity caused a slight reduction of IAA in developing flower stalks ($F_{1,44} = 3.61$, $P = 0.064$), but tissue type ($F_{2,44} = 30.4$, $P = 0.0001$) and stalk height ($F_{2,44} = 5.31$, $P = 0.009$) had much stronger effects (Figure 3C). The IAA content of growing stalks ranged from <0.1 to 0.35 μg/g DW, and there was an increasing basipetal gradient in the 60-cm and 90-cm stalks of both LS and HS treatments.

Seeds produced significantly more IAA than pods ($F_{1,8} = 163.0$, $P = 0.0001$) and initial seed development was characterized by an increase in IAA ($F_{1,8} = 94.4$, $P = 0.0001$, Figure 4C). HS seeds produced significantly less IAA than LS seeds at 20 days after anthesis ($P = 0.018$) but did not differ thereafter. The IAA content of pods was low throughout seed development and unaffected by salinity.

The weak effect of salinity on IAA levels (Figure 3C) corresponds to the similarity in growth rates of HS and LS flower stalks (Figure 5). Growth rates varied from 3 to 6 cm/day and were most strongly affected by time ($F_{23,272} = 17.4$, $P = 0.0001$) and temperature ($R^2 = 0.4$, $P = 0.0001$).

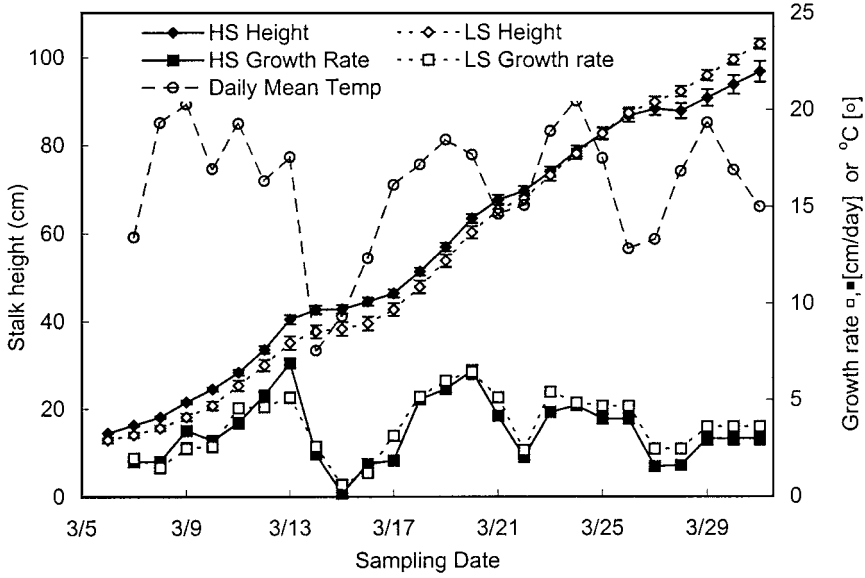


FIG. 5. The effect of salinity on the length and daily growth rate of *Iris hexagona* flower stalks prior to anthesis. Iris were grown in low (~15 mM) and high (100 mM) salinity. For the sake of comparison, the daily mean temperature during this time period is shown as well.

Salicylic Acid. SA ranged from 0.2 to 0.6 μg/g DW (Figure 3D) and salinity had no effect on the production of SA in developing flower stalks. There was a significant effect of stalk height on SA, with 30-cm and 90-cm stalks producing more SA on average than the 60-cm stalks ($F_{2,35} = 6.3$, $P = 0.005$). The SA levels did not develop into a consistent gradient within the stalks.

Salinity had an overall negative effect on SA production in seeds and pods ($F_{1,8} = 8.9$, $P = 0.02$, Figure 4D), and it was most pronounced 60 days after anthesis ($P = 0.006$). Similar to JA, there was a significant interaction between tissue type and time ($F_{3,8} = 7.5$, $P = 0.02$). The SA concentration in LS pods increased over time while that of the HS pods decreased. This interaction was caused primarily by a reversal in the pattern of SA production by seeds and pods 20 days after anthesis ($F_{1,8} = 14.9$, $P = 0.005$).

DISCUSSION

The response of plants to stress involves complex physiological and biochemical responses, including changes in the concentration and ratios of endoge-

nous phytohormones. This is the first study that examines the spatial and temporal fluctuations of four growth regulators in a noncrop plant in response to salinity. The data indicate that salinity has significant and differential effects on the four growth regulators: ABA and JA increase and IAA and SA decline in response to plant salinity stress.

ABA is a generic stress hormone that has multiple functions, including the induction of genes involved in osmotic stress protection (Serrano and Gaxiola, 1994). Our results showed that salinity increased ABA contents in both young and mature *I. hexagona* leaves (Figures 1 and 2). This is consistent with other plant species that increase ABA production in response to salinity (Talanova and Titov, 1994; Clipson et al., 1988; Moons et al., 1997; Montero et al., 1997). ABA concentrations fluctuated among different segments of the flower stalks and during different developmental stages (Figures 2–4), therefore no specific tissue or ontogenetic state can be identified as most responsive to salinity-elevated ABA levels. Generally, the central and apical portions of mature flower stalks contained the highest ABA levels (Figure 3), establishing a basipetal gradient. Therefore, these areas are likely sites of ABA biosynthesis.

The ABA content of seeds is especially important because it influences seed dormancy patterns (Baskin and Baskin, 1998; Skriver and Mundy, 1990). Our determination that the highest ABA concentrations occurred in immature seeds and declined with seed maturation (Figure 4) supports evidence that ABA inhibits germination (Skriver and Mundy, 1990; Baskin and Baskin, 1998). Therefore, the higher level of ABA in HS plants may result in delayed seed germination relative to LS plants.

There are several mechanisms by which elevated ABA could increase plant resistance to stress. ABA can improve water permeability (Glinka and Reinhold, 1971), and it induces late-embryogenesis abundant proteins (LEA), osmoprotectants, and osmolyte biosynthesis genes (Thomas and Bohnert, 1993; Zhu et al., 1997). An ABA-inducible group of proteins (e.g., dehydrins, LEA) are thought to protect cells from water deficit and freezing by stabilizing other proteins and membranes (Thomashow, 1999).

Salinity-induced ABA may affect the reproductive success of iris by influencing embryonic development and the duration of seed dormancy. This notion is supported by the observation that in iris, seed number and germination rate increased in seeds derived from salinity-stressed plants (unpublished data). In contrast to seeds, ABA levels in iris seed pods did not change in response to salinity (Figure 4), indicating that seeds are a major target and possibly site of ABA synthesis.

JA is a fundamental signaling molecule in all plants and has been identified in over 160 plant families (Sembdner and Parthier, 1993). The level of JA in plant tissues varies as a function of tissue type, development, and external stimuli. The highest levels of JA are typically found in flowers and reproduc-

tive tissues (Creelman and Mullet, 1995). JA is known to increase in response to mechanical wounding and water deficit (Creelman and Mullet, 1997), herbivory (Thaler et al., 1996, McCloud and Baldwin, 1997; Thaler et al., 1999), microbial cell wall elicitors (Blechert et al., 1995), the plant signaling peptide systemin (Bergey et al., 1996), and salinity (Tsonev et al., 1998).

Our analysis revealed significant effects of salinity on JA levels. The response is stronger in young leaves and flower stalks than in later stages of development (Figures 1 and 3). Because JA and ABA levels increase in vegetative structures (Figure 2A and B), these hormones may act synergistically in raising salinity tolerance. However, JA levels decline during seed development and were generally reduced in HS plants (Figure 4B). Alternatively, salinity-enhanced ABA levels may inhibit JA production in seeds by reducing the amount of linolenic acid, a precursor of JA biosynthesis (Creelman and Mullet, 1997).

Exogenous JA inhibits seed germination in *Brassica napus* and flax (Staswick and Lehman, 1999). Although we have no evidence that JA inhibits germination of iris seeds, immature LS seeds contain higher concentrations of JA (Figure 4B), and LS seeds germinate at a slower rate than seeds from HS plants (unpublished data). This hypothesis could be tested by external application of JA to seeds.

Salinity significantly reduced IAA levels in leaves and seeds (Figures 1C and 4C), consistent with observations in rice (Nilsen and Orcutt, 1996) and in tomato (Dunlap and Binzel, 1996). Reduced IAA levels may contribute to the reduced above-ground biomass observed in HS-grown iris (unpublished data). Lower IAA contents could also explain the greater number of aborted seeds in HS plants that was noticed during sample prparation. However, this may be compensated for by the increase in the total number of seeds in the HS plants (data not shown).

Significantly higher levels of IAA and zeatin riboside occurred in response to stress in *Rosmarinus officinalis L.*, a Mediterranean shrub that does not accumulate ABA when subjected to water stress (Lopez-Carbonell et al., 1996). Iris accumulated ABA and therefore the combined effect of elevated ABA and reduced IAA levels are likely to diminish growth. The similarity in growth rate and final length of Iris flower stalks from both salinity regimes (Figure 5), and the identical IAA content in developing stalks (Figure 3C) suggests that the transition to the generative growth phase (formation and development of flower stalks) is the most carefully controlled stage of iris ontogeny. The fluctuations of ABA and IAA in the generative tissue (Figure 4A and C) may be an important process for seed development in salinity-stressed plants. The salinity-reduced IAA levels could cause delayed acquisition of resources necessary for generative growth and seed development.

SA and its methyl acetate can inhibit the biosynthesis and activity of JA (Pena-Cortes et al., 1993; Harms et al., 1998), which is supported by the oppo-

site responses of Sa and JA in iris leaves, seeds, and pods (Figures 1B and D, 4B and D). A similar inverse relationship between SA and JA has been reported in *Arabidopsis* (Gupta et al., 2000), suggesting that the ratio of SA and JA may also indicate salinity stress. In our experiments, salinity caused a significant reduction of SA in young iris leaves, seeds, and pods (Figures 1D and 4D). However, there was no difference in SA levels in the LS and HS flower stalks (Figure 3D). Low-salinity seeds and pods had higher SA levels than HS reproductive tissues, and the difference increased with time (Figure 4D). The significant decrease in SA content in young iris leaves (Figure 1) and changed content in seeds and pods (Figure 4D) could have implications for the sensitivity to pathogens. Pathogens increase SA levels (Yalpani et al., 1993; Thaler et al., 1999), and a combined effect of salinity and pathogens would lead to a stronger defense response, ostensibly leading to greater resistance against pathogen attack of stressed plants. The same synergistic effects between salinity and SA possibly account for the induction of drought-inducible genes in cowpea (Iuchi et al., 1996).

We have demonstrated that salinity at levels experienced by natural populations profoundly affects four important iris growth regulators. The reduction of SA and IAA and the increase of ABA and JA in salinity-exposed plants suggest interactive phytohormone effects on plant growth and reproduction. Although iris vegetative biomass is substantially reduced by salinity, the seed number and germination success increased significantly (unpublished data). Our experiments indicate that phytohormones play critical roles in plant responses to salinity. Our data are consistent with the concept that hormonal responses in plants control the allocation to generative and vegetative performance, thereby increasing survival in and adaptation to stressful environments.

Acknowledgments—We are indebted to Ed Mouton and the Louisiana Department of Wildlife and Fisheries for invaluable biological and logistical support. We thank Peter Van Zandt and Mark Tobler for outstanding field and laboratory assistance. This research was supported by National Science Foundation grant DEB-96-32302 to SM and KHH, and by Sigma-Xi and University of Louisiana Graduate Student Organization grants to Y.W.

REFERENCES

ABBAS, M. A., YOUNIS, M. E., and SHUKRY, W. M. 1991. Plant growth, metabolism and adaptation in relation to stress conditions. 14. Effect of salinity on the internal solute concentrations in *Phaseolus vulgaris. J. Plant Physiol.* 138:722–727.

AGRAWAL, A. A., STRAUS, S. Y., and SOUT, M. J. 1999. Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53:1093–1104.

AMZALLAG, G. N., LERNER, H. R., and POLJAKOFF-MAYBER, A. 1990. Exogenous ABA as a modulator of the response of sorghum to high salinity. *J. Exp. Bot.* 41:1529–1394.

BALDWIN, I. T. 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* spegazzi and comes. *J. Chem. Ecol.* 20:2139–2157.

BASKIN, C. C., and BASKIN, J. M. 1998. Seeds: Ecology, Biogeography, and Evolution of dormancy and germination. Academic Press, New York pp. 41–43.

BERGEY, D. R., HOWE, G. A., and RYAN, C. A. 1996. Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proc. Natl. Acad. Sci. U.S.A.* 93:12053–12058.

BLECHERT, S., BRODSCHELM, W., HOLDER, S., KAMMERER, L., KUTCHAN, T. M., MUELLER, M. J., XIA, Z. H., and ZENK, M. H. 1995. The octadecanoic pathway: Signal molecules for the regulation of secondary pathways. *Proc. Natl. Acad. Sci. U.S.A.* 92:4099–4105.

BRUGNOLI, E., and BJORKMAN, O. 1992. Growth of cotton under continuous salinity stress—influence on allocation pattern, stomatal, and nonstomatal components of photosynthesis and dissipation of excess light energy. *Planta* 187:335–347.

CHADHA, K. C., and BROWN, S. A. 1974. Biosynthesis of phenolic acids in tomato infected with *Agrobacterium tumefaciens. Can. J. Bot.* 52:2041–2046.

CHAO, W. S., GU, Y. Q., PAUTOT, V., BRAY, E. A., and WALLING, L. L. 1999. Leucine aminopeptidase RNAs, proteins, and activities increase in response to water deficit, salinity, and the wound signals systemin, methyl jasmonate, and abscisic acid. *Plant Physiol.* 120:979–992.

CLELAND, F. C., and AJAMI, A. 1974. Identification of the flower inducing factor isolated from aphid honeydew as being salicylic acid. *Plant Physiol.* 54:904–906.

CLIPSON, N. J. W., LACHNO, D. R., and FLOWERS, T. J. 1988. Salt tolerance in the halophyte *Suaeda maritima* L. Dum: abscisic acid concentrations in response to constant and altered salinity. *J. Exp. Bot.* 39:1381–1388.

CREELMAN, R. A., and MULLET, J. E. 1995. Jasmonic acid distribution and action in plants: Regulation during development and in response to biotic and abiotic stress. *Proc. Natl. Acad. Sci. U.S.A.* 92:4114–4119.

CREELMAN, R. A., and MULLET, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:355–381.

CREELMAN, R. A., TIERNEY, M. L., and MULLET, J. E. 1992. Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 89:4938–4941.

CURTIS, R. W. 1984. Abscision-inducing properties of methyl jasmonates and, ABA, and ABA-methyl ester and their interactions with ethephone and $AgNO_3$. *J. Plant Growth Regul.* 3:157–163.

DELESALLE, V. A., and MAZER, S. J. 1996. Nutrient levels and salinity affect gender and floral traits in the autogamous *Spergularia marian. J. Plant Sci.* 157:621–631.

DOARES, S. H., NARVAEZ-VASQUEZ, J., CONCONI, A., and RYAN, C. A. 1995. Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 108:1741–1746.

DUNLAP, J. R., and BINZEL, M. L. 1996. NaCl reduces indole-3-acetic acid levels in the roots of tomato plants independent of stress-induced abscisic acid. *Plant Physiol.* 112:379–384.

DUNN, D. C., DUNCAN, L. W., and ROMEO, J. T. 1998. Changes in arginine, PAL activity, and nematode behavior in salinity-stressed citrus. *Phytochemistry* 49:413–417.

EBERHARDT, H. J., and WEGMAN, K. 1989. Effects of abscisic acid and proline on adaptation of tobacco callus culture to salinity and osmotic shock. *Physiol. Plant.* 76:283–288.

EL-ENANY, A. E. 2000. Abscisic acid-responsive proteins induce salinity tolerance in wheat seedlings. *Acta Physiol. Plant.* 22:53–59.

ENYEDI, A. J., YALPNI, N., SILVERMAN, P., and RASKIN, I. 1992. Signal molecules in systemic plant resistance to pathogens and pests. *Cell* 70:879–886.

FARMER, E. E., JOHNSON, R. R., and RYAN, C. A. 1992. Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol.* 98:995–1002.

FINCH-SAVAGE, W. E., BLAKE, P. S., and CLAY, H. A. 1996. Desiccation stress in recalcitrant *Quercus*

robur L. seeds results in lipid peroxidation and increased synthesis of jasmonates and abscisic acid. *J. Exp. Bot.* 47:661–667.

GLINKA, Z., and REINHOLD, L. 1971. Abscisic acid raises the permeability of plant cells to water. *Plant Physiol.* 48:103–105.

GUPTA, V., WILLITS, M. G., and GLAZEBROOK, J. 2000. *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: Evidence for inhibition of jasmonic acid signaling by SA. *Mol. Plant–Microbe Interact.* 13:503–511.

HARMS, K., RAMIREZ, I., and PENACORTES, H. 1998. Inhibition of wound-induced accumulation of allene oxide synthase transcripts in flax leaves by aspirin and salicylic acid. *Plant Physiol.* 118:1057–1065.

HOYOS, M. E., and ZHANG, S. Q. 2000. Calcium-independent activation of salicylic acid-induced protein kinase and a 40-kilodalton protein kinase by hyperosmotic stress. *Plant Physiol.* 122:1355–1363.

IUCHI, S., YAMAGUCHI-SHINOZAKI, K., URAO, T., and SHINOZAKI, K. 1996. Characterization of two cDNAs for novel drought-inducible genes in the highly drought-tolerant cowpea. *J. Plant Res.* 109:415–424.

JIN, S., CHEN, C. C. S., and PLANT, A. L. 2000. Regulation by ABA of osmotic-stress-induced changes in protein synthesis in tomato roots. *Plant Cell Environ.* 23:51–60.

LAROSA, P. C., HANNDA, A. K., HASGAWA, P. M., and BRESSAN, R. A. 1985. Abscisic acid accelerates adaptation of cultured tobacco cells to salt. *Plant Physiol.* 79:138–142.

LEVITT, J., 1980. Responses of Plants to Environmental Stresses, Vol. II. Academic Press, New York.

LOPEZ-CARBONELL, M., ALEGRE, L., PASTOR, A., PRINSEN, E., and VAN ONCKELEN, H. 1996. Variations in abscisic acid, indole-3-acetic acid and zeatin riboside concentrations in two Mediterranean shrubs subjected to water stress. *Plant Growth Regul.* 20:271–277.

MARTINEZ-TELLEZ, M. A., and LAFUENTE, M. T. 1997. Effect of high temperature conditioning on ethylene, phenylalanine ammonialyase, peroxidase and polyphenol oxidase activities in flavedo of chilled "Fortune" mandarin fruit. *J. Plant Physiol.* 150:674–678.

MCCLOUD, E. S., and BALDWIN, I. T. 1997. Herbivory and caterpillar regurgitant amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. *Planta* 203:430–435.

MONTERO, E., CABOT, C., BARCELO, J., and POSCHENRIEDER, C. 1997. Endogenous abscisic acid levels are linked to decreased growth of bush bean plants treated with NaCl. *Physiol. Plant.* 101:17–22.

MOONS, A., PRISEN, E., BAUW, G., and MONTAGU, M. V. 1997. Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *Plant Cell* 92:243–259.

MORALES, M. A., SANCHEZ-BLANCO, M. J., OLMOS, E., TORRECILLAS, A., and ALARCON, J. J. 1998. Changes in the growth, leaf water relations and cell ultrastructure in *Argyranthemum coronopifolium* plants under saline conditions. *J. Plant Physiol.* 153:174–180.

MORGAN, J. M. 1984. Osmoregulation and water-stress in higher-plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 36:299–319.

NIKI, T., MITSUHARA, I., SEO, S., OHTSUBO, N., and OHASHI, Y. S. 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol.* 39:500–507.

NILSEN, E. T., and ORCUTT, D. M. 1996. The physiology of Plants Under Stress—Abiotic Factors. John Wiley & Sons, New York, pp. 118–130.

PASTERNAK, D. 1987. Salt tolerance and crop production—a comprehensive approach. *Annu. Rev. Phytopathol.* 25:271–291.

PENA-CORTES, H., ALBRECHT, T., PRAT, S., WEILER, E. W., and WILLMITZER, L. 1993. Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* 191:123–128.

RASKIN, I. 1992. Roles of salicylic acid in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:439–463.

RASKIN, I., SKUBATZ, H., TANG, W., and MEEUSE, B. J. D. 1990. Salicylic acid levels in thermogenic and non-thermogenic plants. *Ann. Bot.* 66:369–373.

SAS Institute. 1994. SAS/STATS USERS GUIDE. Vol. 1. 4th ed. SAS Institute Inc. Cary, North Carolina.

SEMBDNER, G., and PARTHIER, B. 1993. The biochemistry and physiology and molecular actions of jasmonates. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:569–586.

SERRANO, R., and GAXIOLA, R. 1994. Microbial models and salt stress tolerance in plants. *Crit. Rev. Plant Sci.* 13:121–138.

SHINOZAKI, K., YAMAGUCHI-SHINOZAKI, K., MIZOGUCHI, T., URAO, T., KATAGIRI, T., NAKASHIMA, K., ABE, H., ICHIMURA, K., LIU, Q. A., NANJYO, T., UNO, Y., IUCHI, S., SEKI, M., ITO, T., HIRAYAMA, T., and MIKAMI, K. 1998. Molecular responses to water stress in *Arabidopsis thaliana*. *J. Plant Res.* 111:345–351.

SKRIVER, K., and MUNDY, J. 1990. Gene expression and response to abscisic acid and osmotic stress. *Plant Cell* 2:503–512.

STASWICK, P. E., and LEHMAN, C. C. 1999. Jasmonic acid-signaled responses in plants, pp. 117–150, *in* A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture. APS Press, St. Paul, Minnesota.

SWAIN, A. R., DUTTON, S. P., and TRUSWELL, A. S. 1985. Salicylates in foods. *J. Am. Diet. Assoc.* 85:950–960.

TALANOVA, V. V., and TITOV, A. F. 1994. Endogenous abscisic acid content in cucumber leaves under the influence of unfavorable temperature and salinity. *J. Exp. Bot.* 45:1031–1033.

THALER, J. S. 1999. Jasmonate-inducible plant defenses cause increased parasitism of herbivores. *Nature* 399:686–688.

THALER, J. S., STOUT, M. J., KARBAN, R., and DUFFEY, S. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* 22:1767–1781.

THALER, J. S., FIDANTSEF, A. L., DUFFEY, S. S. and BOSTOCK, R. M. 1999. Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *J. Chem. Ecol.* 25:1597–1609.

THOMAS, J. C., and BOHNERT, H. J. 1993. Salt stress perception and plant growth regulators in the halophyte *Mesembryanthemum crystallinum. Plant Physiol.* 103:1299–1304.

THOMASHOW, M. F. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50:571–599.

TOMAS-BARBERAN, F. A., LOAIZAVELARDE, J., BONFANTI, A., and SALTVEIT, M. E. 1997. Early wound and ethylene-induced changes in phenylpropanoid metabolism in harvested lettuce. *J. Am. Soc. Hortic. Sci.* 122:399–404.

TSONEV, T. D., LAZOVA, G. N., STOINOVA, Z. G., and POPOVA, L. P. 1998. A possible role for jasmonic acid in adaptation of barley seedlings to salinity stress. *J. Plant Growth Regul.* 17:153–159.

YALPANI, N., SHULAEV, V., and RASKIN, I. 1993. Endogenous salicylic acid levels correlate with accumulation of pathogenesis-related proteins and virus resistance in tobacco. *Phytopathology* 83:702–708.

ZENG, X. C., ZHOU, X., ZHANG, W., MUROFUSHI, N., KITAHARA, T., and KAMURO, Y. 1999. Opening of rice floret in rapid response to methyl jasmonate. *J. Plant Growth Regul.* 18:153–158.

ZHU, J. K., HASEGAWA, P. M., and BRESSAN, R. A. 1997. Molecular aspects of osmotic stress in plants. *Crit. Rev. Plant Sci.* 16:253–277.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

PHENOTYPIC PLASTICITY OF *Senecio vulgaris* FROM CONTRASTING HABITAT TYPES: GROWTH AND PYRROLIZIDINE ALKALOID FORMATION

PETER M. FRISCHKNECHT,[1,*] KATHARINA SCHUHMACHER,[1] HEINZ MÜLLER-SCHÄRER,[2] and THOMAS W. BAUMANN[3]

[1]*Department of Environmental Sciences*
Federal Institute of Technology, ETH-Zentrum/HCS
CH-8092 Zürich, Switzerland

[2]*Department of Biology/Ecology*
University of Fribourg, Pérolles
CH-1700 Fribourg, Switzerland

[3]*Institute of Plant Biology, University of Zürich*
Zollikerstrasse 107
CH-8008 Zürich, Switzerland

(Received May 17, 2000; accepted July 26, 2000)

Abstract—The growth–differentiation balance hypothesis (GDB), which postulates a physiological trade-off between growth and differentiation (morphological and chemical), has been tested almost exclusively for carbon-based secondary metabolites. Little attention has been paid to N-based compounds. In this study we aimed to test the predictions of the GDB hypothesis under field conditions for growth and pyrrolizidine alkaloid (PA) formation in *Senecio vulgaris*. We conducted a reciprocal transplant experiment at two sites differing widely in their nutrient supply. These included a conventionally managed vineyard (V) and a strip of local wild flowers between crop fields, which was established to promote species diversity in agroecosystems (C). No fertilizer or pesticides are allowed in such ecological compensation areas. In C, we expected lower growth but higher PA formation than in V. Due to differentiated selection regimes in the two habitat types with regard to nutrient (nitrogen) availability in the soil, we also expected different N-allocation patterns for the genotypes of the two collection sites. Plants of V produced more biomass and were taller than the plants of C. The relatively poor nitrogen conditions in C favored an earlier differentiation towards generative organs. In plants of C, higher concentrations of PAs were found than in plants of V. There exists a close negative correlation between growth and PA formation, indicating a trade-off. The origin of the plant

* To whom correspondence should be addressed. e-mail: frischknecht@umnw.ethz.ch

0098-0331/01/0200-0343$19.50/0

material had only a little effect on PA formation. The observed phenotypic reaction of PA formation in *S. vulgaris* in the two habitats fits quite well the predictions of GDB theory. It is shown that this general response is overlaid by physiological factors leading to a pattern of PA accumulation, which is not readily predictable by nonmechanistic theories.

Key Words—Environment, growth, growth–differentiation balance hypothesis, phenotypic plasticity, pyrrolizidine alkaloids, secondary metabolism, *Senecio vulgaris*.

INTRODUCTION

It is widely accepted that phytochemicals play an important role in ecological interactions by, for example, protecting plants against herbivores and pathogens, providing specialized herbivores with ecological niches, or by modulating litter decomposition (for reviews see Rosenthal and Berenbaum, 1992; Harborne, 1993). Inversely, plant secondary metabolism is known to be influenced by environmental parameters. Since these are increasingly changed by human activities, it is important to understand the related phenotypic reactions or adjustments of secondary metabolism. Specifically, the increase of both CO_2 and nitrogen forms available to plants is a new and relevant issue to be investigated in this context (Heyworth et al., 1998).

Two basic concepts dealing with the phenotypic response of secondary metabolism to the above-mentioned parameters have been proposed. The carbon–nutrient balance hypothesis (CNB) (Bryant et al., 1983) postulates limited growth and reduced N-based phytochemicals, but favored accumulation of carbon-based secondary metabolites such as phenolics, terpenoids, and tannins when carbons dominate over nutrients (primarily nitrogen). An excess of nitrogen (over carbon) results in preferential allocation of nitrogen-based compounds such as alkaloids or cyanogens. Due to such imbalances, growth may remain suboptimal.

The growth–differentiation balance hypothesis (GDB) (Loomis, 1953; Herms and Mattson, 1992) centers on a physiological trade-off between growth and differentiation. Plant tissue grows either by division or expansion of cells, or differentiates chemically and/or morphologically. As both growth and differentiation are dependent on photosynthetic products, they are, in terms of metabolism, negatively correlated with each other, whereby differentiation is thought to include secondary metabolism. The hypothesis states that: when resource availability is low, both growth and differentiation are reduced; at intermediate resource levels differentiation is favored; and at high resource levels growth prevails.

Unfortunately the CNB and GDB hypotheses have been tested almost exclu-

sively for carbon-based secondary metabolites and little attention has been paid to N-based compounds. Nevertheless, the formation pattern of phenolics appears to be in accordance with both hypotheses. Elevated CO_2 led to an increase in the concentration of phenolic compounds, and their concentration was found to decrease after fertilization (e.g., Muzika, 1993; Gebauer et al., 1998; Peñuelas and Estiarte, 1998).

The majority of studies do not support either theory with regard to nitrogen-based secondary metabolites. For instance, *Nicotiana attenuata* is homeostatic in its patterns of nitrogen allocation to nicotine and to growth. Under laboratory conditions, an eightfold nitrogen supply had no effect on either the constitutive or methyljasmonate-induced nicotine accumulation when allometrically related to growth (Lynds and Baldwin, 1998). In *N. sylvestris*, nicotine accumulation continues during nitrogen stress (Baldwin et al., 1993). It was concluded that nitrogen allocation to nicotine is not dependent on nitrogen in excess of growth requirements (Baldwin et al., 1993; Ohnmeiss and Baldwin, 1994). For pyrrolizidine alkaloids (PAs) in *Senecio jacobaeae*, no negative relationship between growth and differentiation could be found (Vrieling and van Wijk, 1994). Deficiencies of mineral nutrients including N did not significantly alter PA concentrations in flower heads of *Senecio vulgaris* under growth chamber conditions (Brown and Molyneux, 1996). GDB theory is supported by the work of Höft et al. (1996) who found that nitrogen supply resulted in enhanced alkaloid accumulation in *Tabernaemontana pachysiphon* under greenhouse conditions. Moderate fertilization was more effective than abundant nitrogen supply.

The above-mentioned studies testing CNB and GDB hypotheses in view of nitrogen-based secondary metabolites were carried out under greenhouse or laboratory conditions and were not conclusive. It is essential to investigate these models under field conditions so that the plants are exposed to the entire array of physical factors. Clarifying the validity of these hypotheses also is important from a nature conservation view. The fulfilment of the GDB predictions in heavily fertilized soil, for instance, suggests a decrease of the allelochemical concentration in the plant body. This will influence not only plant survival but also the fitness of associated specialist insect herbivores (for reviews see Rosenthal and Berenbaum, 1992; Harborne, 1993).

In an agroecosystem of northern Switzerland, we examined GDB in *Senecio vulgaris* by choosing two habitats differing widely in their nutrient supply. These were a conventionally managed vineyard and a strip of local wild flowers between crop fields, which was established to promote species diversity in agroecosystems. No fertilizer or pesticides are allowed in such ecological compensation areas. The Swiss government financially subsidizes farmers for setting aside arable land for ecological purposes.

PA formation in the predominantly inbreeding *S. vulgaris* provides an excellent model to study phenotypic plasticity of PA formation and possible

effects on specialized herbivores. PAs are feeding deterrents for most herbivores (Hartmann, 1999) and are hepatotoxic to vertebrate (Mattocks, 1986) and mutagenic to insects (Frei et al., 1992). The use of PA by specialist insects is well documented (Boppré, 1995; Hartmann, 1999). Specialized insect herbivores such as *Arctia caja* and *Thyria jacobaea* (Lepidoptera: Arctiidae) (Hartmann and Witte, 1995), which were, among other species, associated with *S. vulgaris*, were favored. Both species are recorded in the study area and are on the red list of endangered species (Bundesamt für Naturschutz, 1998).

Sites of PA biosynthesis are the roots, where the key compound senecionine *N*-oxide is produced (Sander and Hartmann, 1989) and its formation is quantitatively controlled. Senecionine *N*-oxide is translocated to the above-ground plant parts (Hartmann et al., 1989) and subsequently subjected to chemical diversification specific to species and populations (von Borstel et al., 1989; Witte et al., 1992). The total amount of alkaloids is not changed by this diversification. Senecionine *N*-oxide and the transformation products are spatially mobile but do not underlie degradation (Hartmann and Dierich, 1998).

A reciprocal transplant experiment was carried out with seed material originating from a vineyard and an ecological compensation area, which were closely located and ecologically similar to the experimental sites. The first aim was to test the predictions of the GDB hypothesis under field conditions. In the compensation area we expected lower growth but higher PA formation than in the vineyard. Secondly, due to differential selection regimes in the two habitat types with regard to nitrogen availability in the soil, we also expected different N-allocation patterns among the genotypes of the two collection sites. Thirdly, we aimed to clarify whether plants growing in compensation areas with low N input may allocate higher levels of PAs and benefit specialized insect herbivores.

METHODS AND MATERIALS

Plant Material and Design of Field Trials. Achenes of *S. vulgaris* were randomly sampled in 1997 in the Klettgau region of northern Switzerland (Canton of Schaffhausen) in both a vineyard and an ecological compensation area ca. 5 km apart. Achenes from 10 individual plants per site were grown for one generation in the greenhouse at the University of Fribourg to reduce possible maternal effects. In April 1998, achenes from five individuals (maternal seed families) from each site were sown in seed trays containing nutrient-poor soil in the greenhouse. After 19 days, seedlings were planted into two field plots (1×3.5 m) located in an ecological compensation area (C) and in a vineyard (V), respectively. All vegetation was removed in these plots before planting the seedlings. Field plots were in close (200 m) to each other and located near where the original material had been collected. In each habitat, 10 individuals from each

seed family and from both origins (C and V) were randomly assigned to plant positions in a regular grid of 15 × 15 cm, resulting in 100 plants per site. In the vineyard trial it was necessary to replant, since all seedlings suffered from herbivory (most probably slugs) during the first night. Replanting resulted in a delay of 14 days for the whole experiment.

Harvesting and Determination of Growth Parameters. Above-ground biomass from each of three randomly selected plants from each family was harvested around midday at 18 and 37 days after planting, i.e., 37 days and 56 days after sowing. After determination of plant height, leaf numbers, and fresh weight, the material was dried at 70°C to measure the dry weight. At the second harvest, plants were dissected into vegetative (stem and leaves) and generative parts (flower heads).

Soil Nitrogen Analysis. At the second harvest, five soil samples were randomly collected from the upper 30 cm of soil and pooled to determine total mineralized nitrogen (N_{min}, NO_3, and NH_4). The analysis was carried out photometrically by Schweizer AG, (Laboratory of Soil Analytics and Environmental Technologies, Thun, Switzerland), according to the N_{min} method of Scharf (1977).

Extraction of PA. The method of Hartmann and Toppel (1987) was slightly modified: the dried and finely powdered plant material was extracted with 0.05 M H_2SO_4 (ca. 25–50 mg/ml) at room temperature for 1 hr by sonication. An aliquot of the extract was transferred into a 2-ml Eppendorf tube and centrifuged. Then, 500 μl of the supernatant was mixed with 300 mg freshly reground (in a mortar) zinc dust and vigorously shaken for 5 hr in order to reduce the PA N-oxides. After centrifugation, 400 μl of the extract was removed and the pH adjusted with concentrated ammonia to ca. 8.0, as estimated by dotting on an indicator stripe. The extract was applied onto a Kieselgur column (Extrelut, Merck, Darmstadt, Germany) in a Pasteur pipet, and after 5 min, elution was carried out with 6 ml CH_2Cl_2. After passive evaporation of the solvent at room temperature, the residue was dissolved in 400 μl methanol. In a few cases, where the plant sample weight was below the standard extraction measure, the volume parameters were adjusted. The purification procedure resulted in a loss of 16, 31, and 27% of retrorsine, seneciphylline, and senecionine, respectively, as determined by reference compounds. The presented values were adjusted accordingly.

HPLC. Separation of PA was performed on a Nucleosil 100-5 ODS (Stagroma) column (5 μm; 4.6 × 250 mm; precolumn 4 × 20 mm) with 50 mM KPi buffer (with 1% THF), pH 2.5 (buffer A) and MeOH (with 1% THF) (buffer B) at a total flow rate of 1 ml/min and by the following gradient (% B over A): 0 min (5), 0–14 min (10), 14–25 min (50), 25–26 min (5); and 26–35 min (5). Parameters were controlled by a Hewlett Packard liquid chromatography (model HP 1090) equipped with a diode array detector set at 216 nm. Peak identification was achieved by comparing UV spectrum and R_t of authentic standards (library

established under separating conditions). Quantification was made by standard curves (external standards). The R_ts (minutes) were as follows: retrorsine (20.5); seneciphylline (23.6); senecionine (26.6). The latter includes the *E* isomer integerrimine.

Statistical Analysis. A nested ANOVA design was adopted as a model with origin (site where mother plants had been collected), genotype (original seed family, individual plant) and environment (experimental site) as main factors. The effect of origin was tested against the genotype within origin, the origin × environment interaction against the genotype × environment interaction, and all other factors and interactions against the residual term.

RESULTS

Genetic Effects. Nested analysis of variance showed no significant effect of origin and origin × environment on both the plant growth and PA formation (Tables 1 and 2). Significant genotype effects on growth were detected for plant fresh and dry weight, plant height, and fruit fresh weight (Table 1). With regard to PA formation, the effects were low and, with the exception of PA concentration related to dry weight, not significant (Table 2). Genotype × environment effects were only found for plant height and for dry weight of flowers at the second harvest (Table 1) but not for PA formation (Table 2).

Environment Effects on Growth Parameters. After 37 days of development, no environmental effect could be observed on plant height, but dry weight differed significantly (Table 1). Plants of C had 51% higher dry weight than plants of V. At this developmental stage no reproductive organs were visible.

After 56 days of development, plants in V were significantly taller than plants in C and the fresh weight of plants of V was 70% higher than those of C (Figure 1). Generally, differences in dry weight were more prominent at the first harvest and differences in fresh weight at the second harvest (Table 1).

Fresh weight and dry weight of flower heads were significantly different (Table 1). Flowers of C had 60% more fresh weight (Figure 1) and 135% more dry weight than the flowers of V due to an advanced stage of differentiation at the same age in C. More than twice as many flower heads had developed in plants of C compared to plants of V (results not shown). The fresh and dry weights of an individual flower head, however, was higher in plants of V.

Plants of V were more succulent at both sampling dates. The dry–fresh weight ratios were higher in plants of C than of V.

Environment Effects on Alkaloid Formation. A highly significant environmenal effect on PA concentration was found when referred to the fresh weight (Table 2). At both harvest times, PA concentrations were about twice as high in plants of C than in plants of V (Figure 2). On a dry weight basis the PA

TABLE 1. RESULTS FOR NESTED ANALYSIS OF VARIANCE: GROWTH AND DEVELOPMENTAL PARAMETERS[a]

Source of variation	First harvest time (37 days)				Second harvest time (56 days)			
	df	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*
Fresh weight								
Origin	1	0.11	0.28	NS	1	0.32	0.01	NS
Genotype within origin	8	3.09	2.37	<0.05	8	347.60	2.60	<0.05
Environment	1	0.40	2.48	NS	1	344.87	20.60	<0.001
Origin × environment	1	0.26	1.00	NS	1	1.28	0.05	NS
Genotype × environment	6	1.58	1.62	NS	8	190.31	1.42	NS
Residual	36	5.87			40	669.72		
Dry weight								
Origin	1	0.00	0.00	NS	1	0.07	0.10	NS
Genotype within origin	8	0.07	3.66	<0.01	8	5.57	2.93	<0.05
Environment	1	0.03	15.03	<0.001	1	0.11	0.46	NS
Origin × environment	1	0.00	0.00	NS	1	0.20	0.07	NS
Genotype × environment	6	0.03	2.27	NS	8	2.54	1.34	NS
Residual	36	0.08			40	9.50		
Plant height								
Origin	1	1.68	0.21	NS	1	101.40	1.36	NS
Genotype within origin	8	63.27	3.05	<0.05	8	597.41	6.48	<0.001
Environment	1	1.30	0.50	NS	1	1026.72	89.06	<0.001
Origin × environment	1	0.95	0.21	NS	1	1.35	0.02	NS
Genotype × environment	6	27.34	1.75	NS	8	602.80	6.54	<0.001
Residual	36	93.49			40	461.15		

[a] NS = not significant.

TABLE 1. CONTINUED

Source of variation	First harvest time (37 days)				Second harvest time (56 days)			
	df	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*
Dry wt/fr. wt ratio								
Origin	1	0.00	1.06	NS	1	0.00	1.18	NS
Genotype within origin	8	0.01	2.01	NS	8	0.00	2.05	NS
Environment	1	0.05	169.18	<0.001	1	0.07	238.67	<0.001
Origin × environment	1	0.00	0.10	NS	1	0.00	0.02	NS
Genotype × environment	6	0.00	0.32	NS	8	0.00	0.77	NS
Residual	36	0.01			40	0.01		
Fr. wt flowers								
Origin					1	0.43	0.18	NS
Genotype within origin					8	19.41	2.41	<0.05
Environment					1	5.40	5.36	<0.05
Origin × environment					1	0.12	0.08	NS
Genotype × environment					8	12.88	1.60	NS
Residual					40	40.28		
Dry wt flowers								
Origin					1	0.00	0.01	NS
Genotype within origin					8	0.39	2.01	NS
Environment					1	0.83	33.79	<0.001
Origin × environment					1	0.00	0.00	NS
Genotype × environment					8	0.58	2.97	<0.05
Residual					40	0.98		

TABLE 2. RESULTS FOR NESTED ANALYSIS OF VARIANCE: PA FORMATION[a]

Source of variation	First harvest time (37 days)				Second harvest time (56 days)			
	df	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*
PA concentration (% dry wt)								
Origin	1	8.52	3.90	NS	1	2.91	3.87	NS
Genotype within origin	8	17.5	1.53	NS	8	6.03	2.28	<0.05
Environment	1	4.62	3.24	NS	1	3.96	11.97	<0.01
Origin × environment	1	1.75	0.90	NS	1	0.64	1.63	NS
Genotype × environment	6	11.72	1.37	NS	8	3.14	1.19	NS
Residual	36	51.42			40	13.23		
PA concentration (% fr. wt)								
Origin	1	0.02	0.44	NS	1	0.05	2.28	NS
Genotype within origin	8	0.39	2.01	NS	8	0.18	1.93	NS
Environment	1	2.53	104.28	<0.001	1	0.82	68.72	<0.001
Origin × environment	1	0.03	0.61	NS	1	0.03	2.26	NS
Genotype × environment	6	0.31	2.10	NS	8	0.09	0.95	NS
Residual	36	0.87			40	0.48		
PA total								
Origin	1	1.22	2.12	NS	1	0.13	0.07	NS
Genotype within origin	8	4.61	1.26	NS	8	15.01	1.71	NS
Environment	1	2.18	4.79	<0.05	1	1.46	1.33	NS
Origin × environment	1	0.6	0.44	NS	1	0.10	0.13	NS
Genotype × environment	6	0.84	0.31	NS	8	6.22	0.71	NS
Residual	36	16.4			40	43.78		

[a] NS = not significant.

TABLE 2. CONTINUED

Source of variation	First harvest time (37 days)				Second harvest time (56 days)			
	df	*SS*	*F*	*P*	*df*	*SS*	*F*	*P*
PA concentration (% fr. wt)								
flowers								
Origin					1	0.26	1.60	NS
Genotype within origin					8	1.29	1.52	NS
Environment					1	0.02	0.22	NS
Origin × environment					1	0.04	0.48	NS
Genotype × environment					8	0.68	0.80	NS
Residual					39	4.14		
PA concentration (% dry wt)								
flowers								
Origin					1	41.79	1.65	NS
Genotype within origin					8	202.67	2.03	NS
Environment					1	1.59	0.13	NS
Origin × environment					1	11.39	0.59	NS
Genotype × environment					8	155.05	1.55	NS
Residual					39	486.62		

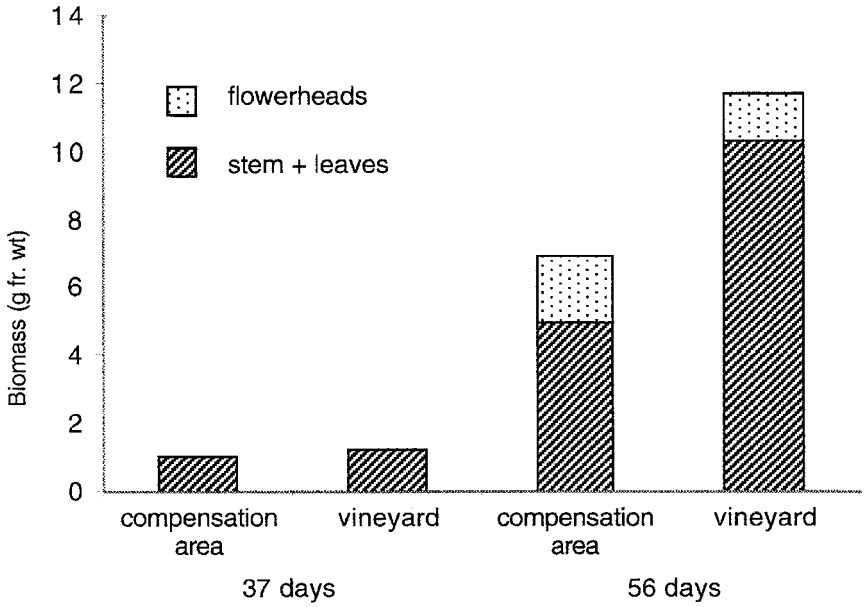


FIG. 1. Biomass (g fr. wt) of *Senecio vulgaris* at 37 days and 56 days of development.

concentrations were 15% and 24% (first and second harvest times, respectively) higher in plants of C than in plants of V. The effect was significant only after 56 days. Total PA content was higher in plants of C than in plants of V (about 70% after 37 days and about 12% after 56 days). The effect was significant only at the first harvest time.

At both sites, PA concentration decreased from the first to the second harvest by a factor of almost 2 (from 0.90 to 0.48 mg/g fresh wt in C and from 0.44 to 0.24 mg/g fresh wt. in V; Figure 2). No environmental effect on PA formation could be found in fruits (Table 2).

The main alkaloid compounds were seneciphylline and senecionine (including the *E* isomer integerrimine) in varying proportions. The portion of retrorsine was below 10%. Correlating with the habitat, no significant alkaloid pattern was found.

Soil Nitrogen. Mineral nitrogen of V was twice as high as of C (33 kg N_{min}/ha and 16 kg/N_{min}/ha, respectively). Compared to sites in the neighborhood, the soil nitrogen content of V was relatively low, because no fertilization was carried out during the experiment. The nitrogen content of C is within the range of sites nearby (Serena Rigamonti, personal communication).

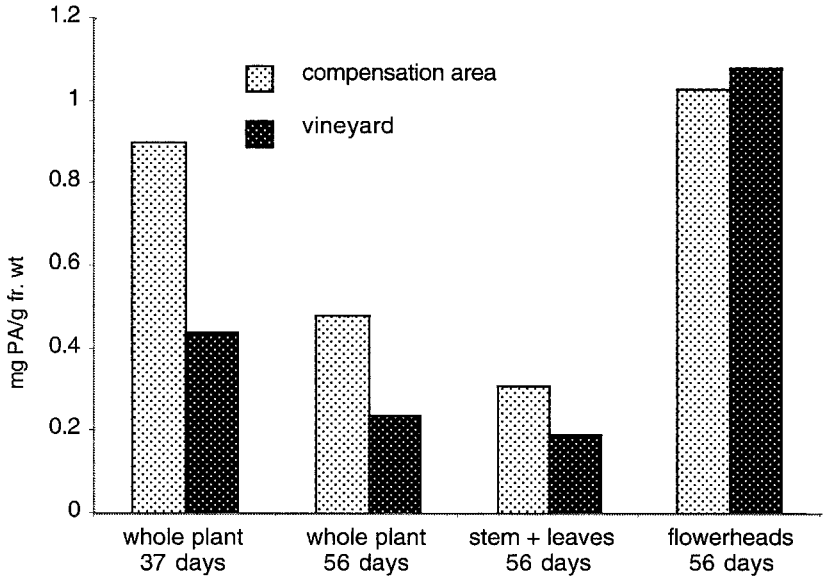


FIG. 2. Alkaloid concentration (mg PA/g fr. wt) in *Senecio vulgaris* at 37 days and 56 days of development.

DISCUSSION

In general, the observed results fit the GDB hypothesis (Loomis, 1953; Herms and Mattson, 1992) quite well. Plants of V with a larger soil nitrogen supply were oriented to growth whereas plants of C were oriented to differentiation. After 56 days, plants of V had a higher fresh weight, were taller, and had more leaves (result not shown) than plants of C. The number of flower heads, however, was twice as high in plants of C than in those of V. The relatively poor nitrogen conditions favored an earlier differentiation towards generative organs. After 37 days, the measured growth parameters (fresh and dry weight, height) did not yet demonstrate higher growth of V and dry weight was even lower in V.

PA formation also followed the GDB hypothesis: higher concentrations were found in plants of C than of V (Figure 2). In accordance with growth development, the difference is more pronounced after 56 days than after 37 days. There exists a significant negative correlation between log biomass and milligrams of PA per gram of biomass ($R^2 = 0.52$, $P < 0.001$ for fresh weight; $R^2 = 0.31$, $P < 0.001$ for dry weight) indicating a trade-off between growth and differentiation (Figure 3). Such a significant negative correlation was also found within each site (for fresh weight: V: $R^2 = 0.43$, $P < 0.001$; C: $R^2 = 0.23$, $P = 0.007$).

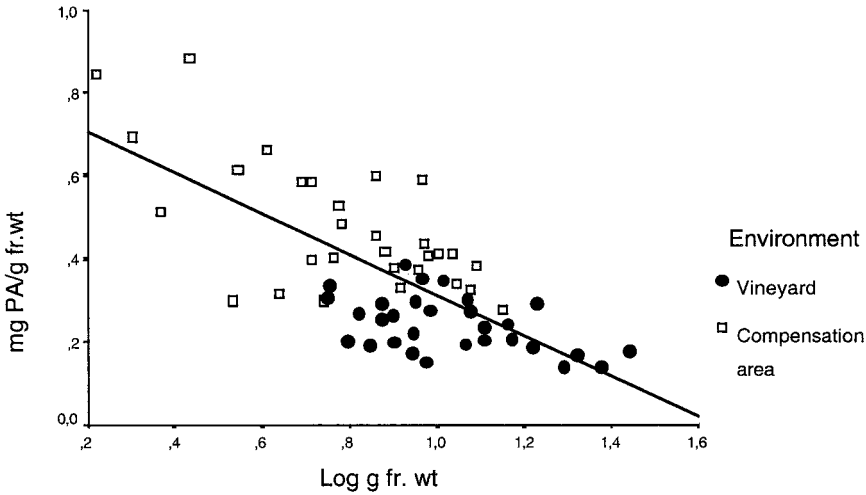


FIG. 3. Correlation between of log fresh weight and alkaloid formation at 56 days of development ($R^2 = 0.52$, $P < 0.001$).

The CNB hypothesis (Bryant et al., 1983) was not supported. Higher availability of nitrogen did not lead to an increased accumulation of PAs.

The fact that seed origin had no significant effect on the various growth and PA parameters assessed is not surprising. A recent plant molecular study carried out on these *S. vulgaris* populations suggests that ecological compensation areas were only recently colonized from adjacent corn fields, with a nitrogen status similar to the vineyard sites (Müller-Schärer and Fischer 2000). The significant genotype effects observed for some of the growth and PA variates indicate, however, genetic variation for these traits and, therefore, the potential for local adaptation.

PAs are synthesized in the roots. In organ cultures their formation is strictly linked to root growth and ceases when root growth stops (Hartmann et al., 1988; Sander and Hartmann, 1989). The shoot–root ratio in *S. vulgaris* correlates positively with the availability of soil nitrogen (Van der Kooij et al., 1998); therefore, plants of C might have a relatively better developed root system than plants of V. If the postulated link between root growth and PA formation exists under field conditions, increased PA formation in C could be an indirect consequence of a morphophysiological adaptation to low mineral nutrient conditions and not a reaction in the sense of the GDB hypothesis. A significant negative correlation between shoot–root ratio and PA concentration could be detected in experiments with seedlings of *S. vulgaris*. A similar trend, although not significant, was found in seedlings of *S. jacobaea*. In both species, a significant positive cor-

relation was found between root biomass and total PAs per seedling (Schaffner et al., unpublished manuscript). The relationship between root growth and PA formation should be further studied under field conditions.

Physiological and biochemical (Hartmann, 1999; Hartmann and Dierich, 1998; Hartmann and Zimmer, 1986) as well as genetic studies (Vrieling et al., 1993) indicate that two different sections of PA formation exist. The root, where biosynthesis of the key compound, senecionine N-oxide, occurs under highly constrained conditions, and the above-ground parts, where a large number of subsequent biosynthetic transformations and tissue- and development-specific accumulations take place. This study supports the model of Hartmann and Dierich (1998) also for an ecological factor. Nitrogen supply is a high constraint for the production of the total amount of PAs (relative to biomass) in the roots. The flexible translocation and allocation of PAs in the above-ground plant parts is independent of the production in the roots. Although alkaloid production relative to biomass was lower in V than in C, alkaloid content in the generative plant parts is equal in V and in C. In an ecological sense, it is reasonable that alkaloid accumulation in vulnerable and valuable plant organs as flowers and fruits are, within a certain range, independent of the supply. Therefore, although the phenotypic reaction of PA formation in *S. vulgaris* in the two sites fits quite well the predictions of GDB theory, the response in detail is overlaid by physiological factors leading to a pattern of PA accumulation, which in its details is hardly predictable by nonmechanistic theories.

The hypothesis that ecological compensation areas with low soil nitrogen may be beneficial to endangered insect specialists that sequester PAs has to be tested further. The results clearly show that PA concentration is higher in plants of C than V, which is in agreement with this hypothesis. The internal allocation pattern may compensate for this reduced production as the level of PA concentration in flowers was found to be independent of the soil nitrogen conditions. Therefore, only specialized insects consuming vegetative plant parts would be able to benefit from increased PA concentrations of ecological compensation sites.

REFERENCES

BALDWIN, I. T., OESCH, R. C., MERHIGE, P. M., and HAYES, K. 1993. Damage-induced root nitrogen metabolism in *Nicotiana sylvestris*: Testing C/N predictions for alkaloid production. *J. Chem. Ecol.* 19:3029–3043.

BOPPRÉ, M. 1995. Pharmakophagie: Drogen, Sex and Schmetterlinge. *Biol. Unserer Zeit* 1:8–17.

BROWN, M. S., and MOLYNEUX, R. J. 1996. Effects of water and mineral nutrient deficiencies on pyrrolizidine alkaloid content of *Senecio vulgaris* flowers. *J. Sci. Food Agric.* 70:209–211.

BRYANT, J. P., CHAPIN, F. S., III, and KLEIN, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

BUNDESAMT für NATURSCHUTZ. 1998. Rote Liste gefährdeter Tiere Deutschlands. *Schriftenr. Landschaftspflege Naturschutz* 55:101–102.

FREI, H., LÜTHY, J., BRAUCHLI, J., ZWEIFEL, U., WÜRGLER, F. E., and SCHLATTER, C. 1992. Structure/activity relationships of the genotoxic potencies of sixteen pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster. Chem. Biol. Interact.* 831:1–22.

GEBAUER, R. L. E., STRAIN, B. R., and REYNOLDS, J. F. 1998. The effect of elevated CO_2 and N availability on tissue concentrations and whole plant pools of carbon-based secondary compounds in loblolly pine (*Pinus taeda*). *Oecologia* 113:29–36.

HARBORNE, J. B. 1993. Introduction to Ecological Biochemistry, 4th ed. Academic Press, London.

HARTMANN, T. 1999. Chemical ecology of pyrrolizidine alkaloids. *Planta* 207:483–495.

HARTMANN, T., and DIERICH, B. 1998. Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: biological need or coincidence? *Planta* 206:443–451.

HARTMANN, T., and TOPPEL, G. 1987. Senecionine N-Oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris. Phytochemistry* 26:1639–1643.

HARTMANN, T., and WITTE, L. 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids, pp. 155–233, *in* S. W. Pelletier (ed.). Alkaloids: Chemical and Biological Perspectives. Elsevier Science, Amsterdam.

HARTMANN, T., and ZIMMER, M. 1986. Organ-specific distribution and accumulation of pyrrolizidine alkaloids during the life history of two annual *Senecio* species. *J. Plant Physiol.* 122:67–80.

HARTMANN, T., SANDER, H., ADOLF, R., and TOPPEL, G. 1988. Metabolic links between the biosynthesis of pyrrolizidine alkaloids and polyamines in root cultures of *Senecio vulgaris. Planta* 177:98–107.

HARTMANN, T., EHMKE, A., EILERT, U., VON BORSTEL, K., and THEURING, C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid N-oxides in *Senecio vulgaris* L. *Planta* 177:98–107.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: To grow or to defend. *Q. Rev. Biol.* 67:283–335.

HEYWORTH, C. J., IASON, G. R., TEMPERTON, V., JARVIS, P. G., and DUNCAN, A. J. 1998. The effect of elevated CO_2 concentration and nutrient supply on carbon-based plant secondary metabolism in *Pinus sylvestris* L. *Oecologia* 115:344–350.

HÖFT, M., VERPOORTE, R., and BECK, E. 1996. Growth and alkaloid contents in leaves of *Tabernaemontana pachysiphon* Stapf (Apocynaceae) as influenced by light intensity, water and nutrient supply. *Oecologia* 107:160–169.

LOOMIS, W. E. 1953. Growth and differentiation—an introduction and summary, pp. 1–17, *in* W. E. Loomis (ed.). Growth and Differentiation in Plants. Iowa State College Press, Ames.

LYNDS, G. Y., and BALDWIN, I. T. 1998. Fire, nitrogen, and defensive plasticity in *Nicotiana attenuata. Oecologia* 115:531–540.

MATTOCKS, A. R. 1986. Chemistry and Toxicology of Pyrrolizidine Alkaloids. Academic Press, London.

MUZIKA, R. M. 1993. Terpenes and phenolics in response to nitrogen fertilisation: A test of the carbon/nutrient balance hypothesis. *Chemoecology* 4:3–7.

MÜLLER-SCHÄRER, H., and FISCHER, M. 2000. Genetic (RAPD-) structure of the annual weed *Senecio vulgaris* in relation to habitat type and population size. *Mol. Ecol.* (submitted).

OHNMEISS, T. E., and BALDWIN, I. T. 1994. The allometry of nitrogen allocation to growth and an inducible defense under nitrogen-limited growth. *Ecology* 75:995–1002.

PEÑUELAS, J., and ESTIARTE, M. 1998. Can elevated CO_2 affect secondary metabolism and ecosystem function? *Trends Evol. Ecol.* 13:20–24.

ROSENTHAL, G. A., and BERENBAUM, M. R. (eds.). 1992. Herbivores: Their Interactions with Secondary Plant Metabolites, vols. I and II, 2nd ed. Academic Press, San Diego.

SANDER, H., and HARTMANN, T. 1998. Site of synthesis, metabolism and translocation of senecionine *N*-oxide in cultured roots of *Senecio erucifolius. Plant Cell Tissue Organ Cult.* 18:19–31.

SCHARF, H. P. 1977. Der Mineralstoffgehalt des Bodens als Massstab für den Stickstoffdüngerbedarf. PhD thesis. Techn. Universität Hannover.

VAN DER KOOIJ, T. A. W., MEIJER, J., and DE KOK, L. J. 1998. The impact of nitrogen nutrition and SO_2 exposure on *Senecio vulgaris* L., *in* pp. 484–487, L. J. De Kok and I. Stulen (eds.). Responses of Plant Metabolism to Air Pollution and Global Change. Backhuys Publishers, Leiden.

VON BORSTEL, K., WITTE, L., and HARTMANN, T. 1989. Pyrrolizidine alkaloid patterns in populations of *Senecio vulgaris*, *S. vernalis* and their hybrids. *Phytochemistry* 28:1635–638.

VRIELING, K., and VAN WIJK, C. A. M. 1994. Estimating costs and benefits of the pyrrolizidine alkaloids of *Senecio jacobaea* under natural conditions. *Oikos* 70:449–454.

VRIELING, K., DE VOS, H., and VAN WIJK, C. A. M. 1993. Genetic analysis of the concentration of pyrrolizidine alkaloids in *Senecio jacobaea. Phytochemistry* 32:1141–1144.

WITTE, L., ERNST, L., ADAM, H., and HARTMANN, T. 1992. Chemotypes of two pyrrolizidine alkaloid-containing *Senecio* species. *Phytochemistry* 31:559–565.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

EFFECTS OF 1,4-BENZOXAZIN-3-ONE DERIVATIVES FROM MAIZE ON SURVIVAL AND FECUNDITY OF *Metopolophium dirhodum* (WALKER) ON ARTIFICIAL DIET

VINCENT CAMBIER,[1,2,*] THIERRY HANCE,[1] and EDMOND DE HOFFMANN[2]

[1] *Unité d'Ecologie et de Biogéographie*
Place Croix du Sud, 5, 1348, Louvain-la-Neuve, Belgium
[2] *Unité de Spectrométrie de masse*
Place Pasteur, 1, 1348, Louvain-la-Neuve, Belgium

(Received December 26, 1999; accepted October 12, 2000)

Abstract—Effects of 2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc) and DIMBOA-Glc *N-O*-methylated (HDMBOA-Glc), two compounds present in high concentration in maize, were tested on the aphid *Metopolophium dirhodum* reared on artificial diet. HDMBOA-Glc and DIMBOA-Glc decrease survival of adults with an LD_{50} of 1 mM and 5.6 mM, respectively, after 72 hr of feeding. These compounds also decrease the fecundity of the aphids at concentrations of 2 mM and 1 mM, respectively. At concentrations of 2 mM HDMBOA-Glc and 8 mM DIMBOA-Glc in the diet, the average lifetime fecundity of 10 females is near zero. Offspring mortality on diet with 2 mM DIMBOA-Glc is significantly higher than with the control diet. In contrast, HDMBOA-Glc has no effect on the survival of offspring. The possibility that these compounds protect Poaceae against aphids is discussed.

Key Words—Poaceae, maize, 1,4-benzoxazin-3-ones, hydroxamic acids, DIMBOA, aphids, *Metopolophium dirhodum*, artificial diet, plant resistance.

INTRODUCTION

Poaceae such as maize contain 1,4-benzoxazin-3-one derivatives in high quantity (0.2% fresh weight). These compounds belong to three classes—hydroxamates, lactams, and *N-O*-methylated derivatives—and are present in noninjured plants

*To whom correspondence should be addressed.

0098-0331/01/0200-0359$19.50/0

only as β-glucosides (Cambier et al., 1999). When plants are crushed, β-glucosidases transform glucosides into corresponding aglycones (Wahlroos and Virtanen, 1959).

Aglycone hydroxamic acids and mainly DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) have been associated with resistance to insects such as *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) (Houseman et al., 1992) and *Diabrotica virgifera virgifera* (Leconte) (Coleoptera: Chrysomelidae) (Xie et al., 1990) and to several aphid species: *Rhopalosiphum maidis* (Fitch) (Long et al., 1977), *Metopolophium dirhodum* (Walker) (Argandona et al., 1980), *Schizaphis graminum* (Rondoni) (Corcuera et al., 1985), *Sitobion avenae* (Fabricius) (Thackray et al., 1990), *Rhopalosiphum padi* (L.) (Thackray et al., 1990), and *Diuraphis noxia* (Kurdjumov) (Mayoral et al., 1996). Argandona et al. (1980, 1981) found negative correlations between hydroxamic acid concentration in the plant and aphid infestation and distribution. The decrease of growth rate and intrinsic rate of increase (r_m) of the aphid populations with the increase of hydroxamic acid concentration also has been shown (Argandona et al., 1980; Thackray et al., 1990). Experiments with artificial diet have shown that DIMBOA is toxic and deterrent to aphids (Argandona et al., 1983). Givovich and Niemeyer (1995) have confirmed the feeding deterrent effect of this compound by electronic monitoring of aphid feeding.

A compound present in the phloem sap, the main nutrition site of aphids, can be both antixenotic (deterrent) and antibiotic. A compound present in the path followed by the aphid stylet during penetration of the plant can act only as a feeding deterrent.

DIMBOA is not detected in the phloem sap (Molyneux et al., 1990; Givovich et al., 1992, 1994; Caillaud and Niemeyer, 1996). Thus, this compound can not act as an antibiotic. Furthermore, we recently found that DIMBOA is not present in maize seedlings during an aphid attack (unpublished data). Indeed, DIMBOA-Glc and DIMBOA β-glucosidase are stored in different compartments (extravacuolar and vacuolar spaces, respectively) (Massardo et al., 1994). So, it is possible that during aphid nutrition, the β-glucosidase is not released from the vacuolar space (Dreyer and Campbell, 1987), and consequently, DIMBOA-Glc is not transformed into DIMBOA. However, this transformation undoubtedly occurs when a plant is crushed by insects such as *Ostrinia nubilalis* or *Diabrotica virgifera virgifera*. Therefore, we believe that DIMBOA is not the main resistance factor to aphids in maize seedlings.

On the contrary, the glucoside of DIMBOA and/or other compounds related to DIMBOA could potentially protect maize plants against aphids. Indeed, we have detected in the honeydew produced by *Metopolophium dirhodum* several glucosides of 1,4-benzoxazin-3-one derivatives, such as DIMBOA-Glc and HDMBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one). These results suggest that DIMBOA-Glc and

HDMBOA-Glc are present in the phloem sap and are ingested by aphids. Furthermore, we have previously shown that these compounds are present in high concentrations in plants (Cambier et al., 2000). Finally, we have observed that plant resistance to *M. dirhodum* decreases when DIMBOA-Glc and HDMBOA-Glc concentrations decrease in aerial parts.

Some authors previously have reported the potential effects of DIMBOA-Glc on aphids reared on artificial diets. According to Corcuera et al. (1985), this compound at concentrations as low as 0.25 mM decreases the reproduction rate of *Schizaphis graminum*, and at a concentration of 4 mM causes the death of 50% of the nymphs after only 24 hr. Givovich and Niemeyer (1995) monitored the feeding behavior of five cereal aphids (*Rhopalosiphum maidis*, *Rhopalosiphum padi*, *Metopolophium dirhodum*, *Schizaphis graminum*, and *Sitobion avenae*) on artificial diet via electrical penetration graphs (EPG) and showed that all species were equally sensitive to the feeding deterrent effect of DIMBOA-Glc. Concerning HDMBOA-Glc, its effects on aphids reared on artificial diet have never been reported.

The purpose of our work is to determine effects of DIMBOA-Glc and HDMBOA-Glc on survival and fecundity of aphids *Metopolophium dirhodum* by using an artificial diet. The results will give us information on the potential roles of these compounds in the plant resistance to aphids.

MATERIALS AND METHODS

Isolation of Compounds. HDMBOA-Glc and DIMBOA-Glc were isolated from maize (Magda, Sesnord, and Aztec varieties) aged 3–5 days. Aerial parts of 500 plants were cut and placed immediately in 500 ml of boiling methanol for 30 min. After filtration, the green extract was evaporated under vacuum at 35°C to a volume of 50 ml. The extract was placed in a refrigerator for several days, filtered, and evaporated to dryness under vacuum at 35°C. The dried residue was dissolved in 15 ml water. Each sample was prepurified on a Sep-Pack C18 cartridge (Waters, Milford, Massachusetts).

The purification was accomplished by preparative HPLC. Samples were chromatographed with a Spectra System AS 3000 on a 10-mm × 25-cm microbore reverse phase C18 preparative column (10 μm, econosphere). A two-solvent system was used to generate the mobile phase: solvent A was 100% water, and solvent B was 100% methanol. Solvents were degassed and filtered. The flow rate was 4.7 ml/min. The mobile phase at the initiation of each run was an 8 : 2 ratio (A to B). After injection of 500 μl of sample, a 4-min linear gradient from 8 : 2 to 5 : 5 A/B was applied and held for another 16 min. Elution was monitored at 280 nm with a Spectra System UV 1000 wavelength detector. The mobile phase flow was connected to a fraction collector.

DIMBOA-Glc and HDMBOA-Glc were identified and quantified from their UV spectra. Purity was verified by mass spectrometry.

Feeding and Diet Composition. Aphids were fed from artificial diet placed between two layers of Parafilm M stretched over Petri dishes. The diet was a mixture of sucrose, amino acids, vitamins, and mineral salts, derived from that described by Auclair (1965).

Effects of DIMBOA-Glc and HDMBOA-Glc on Aphids. Individuals of *Metopolophium dirhodum* (Walker) were collected from naturally infested maize and wheat in Belgium and were reared on wheat seedlings grown in the laboratory at 22 ± 2°C with a 16L : 8D regime. Adults of unknown age were placed on the diets. One day later, nymphs 0–24 hr old were collected and placed on *Triticum aestivum* (Torfirda variety). After eight days in these conditions, nymphs had reached the adult stage. These apterous adults aged 0 ± 1 day were used in the experiments.

Four concentrations of DIMBOA-Glc (1, 2, 4, and 8 mM) and four concentrations of HDMBOA-Glc (0.25, 0.5, 1, and 2 mM) were separately added to the diet to give a range of concentrations similar to those found in aerial parts of maize seedlings. Control diets did not contain DIMBOA-Glc and HDMBOA-Glc. Ten Petri dishes were used with each concentration, and ten apterous adults of *M. dirhodum* aged 0 ± 1 day were placed in each dish. Every day, during a six-day period, survival and fecundity were noted, diet was renewed, and the offspring were discarded, except on the second day. On the second day, nymphs were collected in the order to determine effects of DIMBOA-Glc and HDMBOA-Glc on the offspring originating from these adults.

Impacts of DIMBOA-Glc and HDMBOA-Glc were determined as follows. One hundred nymphs aged 0 to 24 hr laid by adults in 10 Petri dishes containing 0, 1, and 2 mM of DIMBOA-Glc and 0, 0.25, 0.5, and 1 mM of HDMBOA-Glc were placed in other dishes and fed the same diet as the mother. Every day, for four days of experiments with HDMBOA-Glc and for five days with DIMBOA-Glc, survival of nymphs was determined and diet was renewed. Artificial diets containing 4 and 8 mM of DIMBOA-Glc and 2 mM of HDMBOA-Glc were not tested on the offspring because fecundity of adults was too low at these concentrations.

Statistical Analysis. On the third day of the experiment, concentration–mortality regressions were estimated by Probit analysis (Finney, 1971). Data from all bioassays were corrected for control mortality with Abbott's (1925) formula.

On the sixth day of the experiment, one-way analyses of variance (ANOVA) were performed on all aphid survival data (10 per concentration × 5 concentrations). Then, a Scheffé test allowed us to rank the means of the different concentrations and control. These analyses were performed on SAS software (SAS, 1985) for each compound.

Cumulative fecundity curves for each HDMBOA-Glc or DIMBOA-Glc concentration were established. Because of its logistical shape, a fitting by the DUD method (Doesn't Use Derivatives) (SAS, 1985) was performed according to the following equation:

$$y = c/1 + e^{z}$$

where $z = -(x-a)/b$, y represents the value of the cumulative fecundity at age x, a is the inflexion point, b is the inverse of the slope, and c is the plateau value. The equation represents the total number of offspring produced in one generation by an adult female. The DUD method allowed the calculation of a confidence interval for each parameter. We assumed that values were significantly different when confidence intervals did not overlap (Van Impe and Hance, 1993; Kennedy and Hance, 1995).

The number of nymphs laid by the 10 adult females present in each dish during one generation was also determined by this method. Nevertheless, in this case, the number depends on fecundity and survival of the females.

RESULTS

Effects of HDMBOA-Glc on Aphids. With diets containing HDMBOA-Glc, adult aphid survival was always lower ($\alpha = 0.05$) than with the control diet (Figure 1). Survival decreased with the increase in concentration. After six days, 97% of aphids fed on the diet containing 2 mM of HDMBOA-Glc were dead compared to only 18% on the control diet. Mortality seemed particularly high during the second and third days of the experiment and is similar to that observed when aphids are not fed.

LD_{50} and LD_{95} of HDMBOA-Glc on aphids after three days of feeding were 1 mM (limits: 0.8 to 1.1) and 3.8 mM, (limits: 3 to 5.5), respectively.

Concerning the cumulative fecundity per female (Figure 2), no significant difference was observed between the control and concentrations of 0.25, 0.5, and 1 mM of HDMBOA-Glc. A sharp decrease in fecundity was, nevertheless, observed for the 2 mM concentration. However, if we consider the global fecundity per replication (combining survival of the adult females and fecundity), and if we plot the plateau value in relation to HDMBOA-Glc concentration (Figure 3), we observe a strong linear relationship, $y = -52.7x + 114.1$ ($r^2 = -0.99$), stressing the importance of the combined effect (adult survival + fecundity) on the development of the next generation.

Surprisingly, nymphs laid by adult females reared on the artificial diet containing concentrations of HDMBOA-Glc at 0.25, 0.5, and 1 mM did not show any difference in mortality over the 4 days of their rearing.

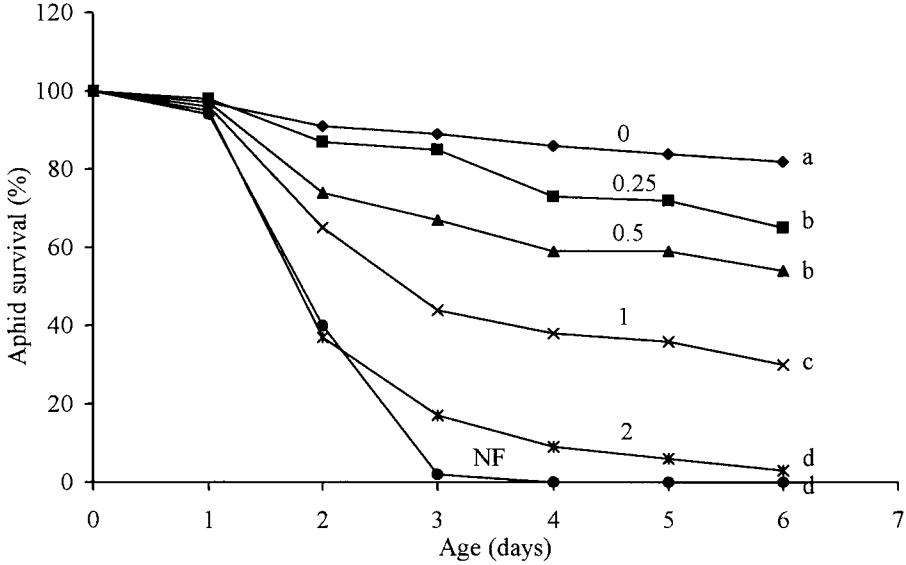


FIG. 1. Survival of *M. dirhodum* adults at different concentrations of HDMBOA-Glc. Numbers represent concentration (mM) in the diet. NF = not fed. On the sixth day of the experiment, significant differences of adult survival are indicated with different letters (Scheffé test; $\alpha = 0.05$).

Effects of DIMBOA-Glc on Aphids. On the sixth day, adult survival (Figure 4) on diets containing 0, 1, and 2 mM of DIMBOA-Glc is not significantly different from controls. Incorporation of higher concentrations of DIMBOA-Glc in the diet, 4 and 8 mM, decreased survival significantly ($\alpha = 0.05$). The LD_{50} and LD_{95} of DIMBOA-Glc on aphids after three days of feeding are 5.6 mM (limits: 5 to 6.5) and 18.6 mM, (limits 14 to 28.5), respectively.

Maxima of logistical curves representing the cumulative fecundity per female (Figure 5) are always significantly different ($\alpha = 0.05$) from control diets. However, no difference appears at concentrations of 1, 2, and 4 mM. At 8 mM concentration, fecundity is near zero. As with HDMBOA-Glc, we observed a linear relationship between the maximum of the logistical equation, c, and DIMBOA-Glc concentration (Figure 6). The equation is $y = -8.1x + 69.8$ ($r^2 = -0.98$).

Mortality of the offspring reared for five days on artificial diet with increasing concentrations of DIMBOA-Glc at 0, 1, and 2 mM was 3, 12, and 24%, respectively. Only the last concentration gave significantly higher mortality ($\alpha = 0.05$).

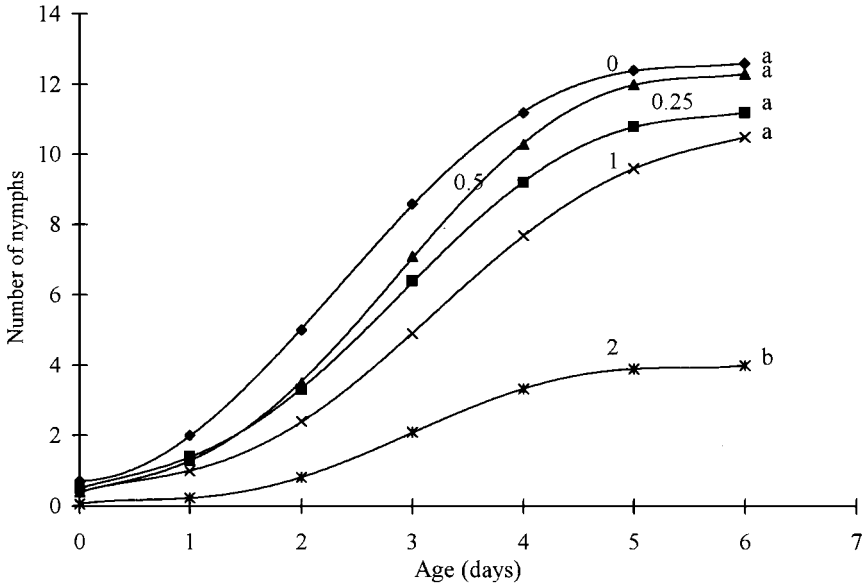


FIG. 2. Curves of cumulative fecundity per *M. dirhodum* female feeding on diets containing different concentrations of HDMBOA-Glc. Experimental points are adjusted to a logistical curve by the DUD method (see Materials and Methods). Number on the curve represents concentration (mM) in the diet. Different letters mean that plateau values, *c*, are significantly different.

DISCUSSION

Our results show that DIMBOA-Glc and HDMBOA-Glc have an important negative effect on survival and fecundity of *M. dirhodum* adults fed on artificial diet at concentrations similar to those found in maize aerial parts (Cambier et al., 2000). Global fecundity is low when DIMBOA-Glc and HDMBOA-Glc concentration in the diet is 8 mM and 2 mM, respectively. HDMBOA-Glc is more active than DIMBOA-Glc on aphid survival. Indeed, after three days of feeding, the LD_{50} and LD_{95} are, respectively, 5.6 and 4.9 times lower that these of DIMBOA-Glc.

Recently, Escobar et al. (1999) tested antifeeding and antibiotic effects of 25 compounds, including benzoxazinones, benzoxazolinones, and N-glyoxylamide derivatives towards the aphid *Sitobion avenae* in diet bioassays. Their results showed that these compounds increased the mortality at a concentration of 2 mM in the diet. Nevertheless, no glucosylated derivatives were tested. In this paper, we show that glucosides present in maize plants, such as HDMBOA-Glc, have

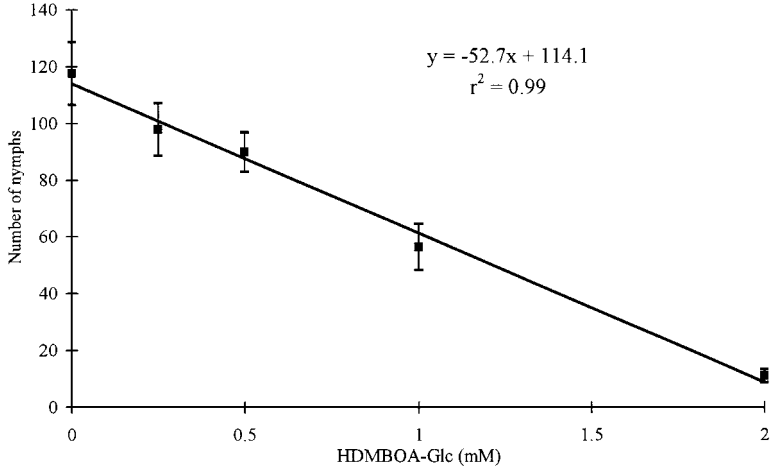


FIG. 3. Average fecundity of 10 *M. dirhodum* females during their life (maximum of the cumulative fecundity curve) that fed on diets containing different concentrations of HDMBOA-Glc. Vertical lines at data points show standard errors.

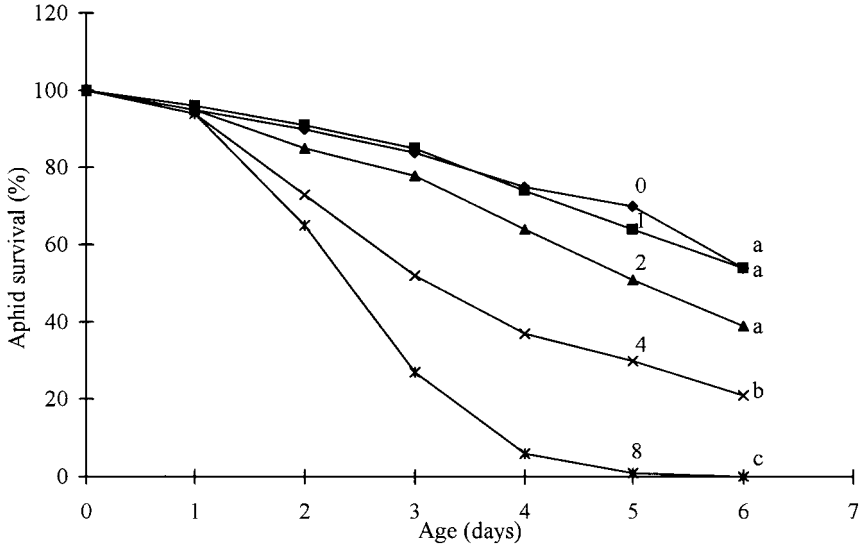


FIG. 4. Survival of *M. dirhodum* adults in the presence of different concentrations of DIMBOA-Glc. Numbers on the line represent concentration (mM) in the diet. On the sixth day of the experiment, significant differences of adult survival are indicated with different letters (Scheffé test; $\alpha = 0.05$).

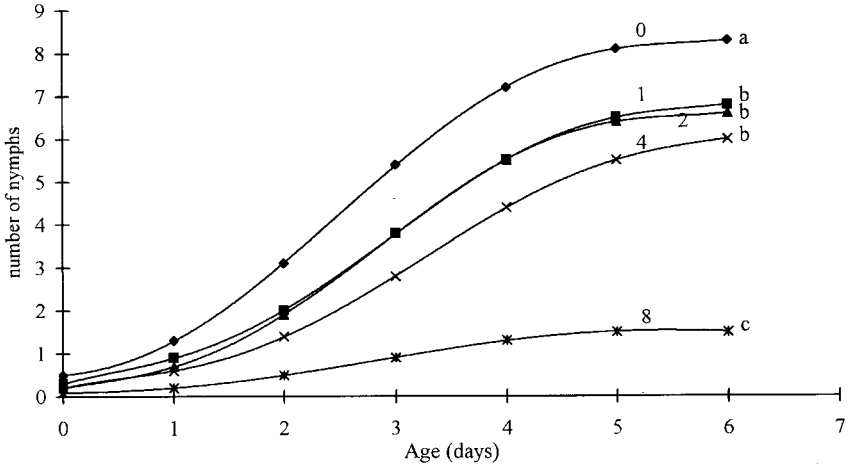


FIG. 5. Curves of cumulative fecundity per *M. dirhodum* female feeding on diets with different concentrations of DIMBOA-Glc. Experimental points are adjusted to a logistical curve by the DUD method (see Materials and Methods). Numbers on the curve represents concentration (mM) in the diet. Different letters mean that plateau values, *c*, are significantly different.

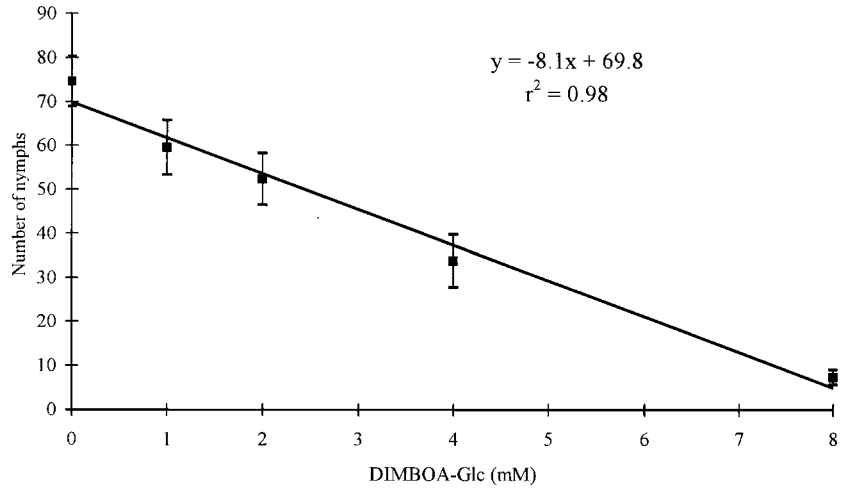


FIG. 6. Average fecundity of 10 *M. dirhodum* females during their life (maximum of the cumulative fecundity curve) that fed on diets containing different concentrations of DIMBOA-Glc. Vertical lines at data points show standard errors.

negative effects on aphids fed on artificial diets. Thus, in future investigations, it seems essential to study their roles in the plant resistance to aphids.

DIMBOA-Glc and HDMBOA-Glc appeared to act as strong feeding deterrents. Indeed, during the first three days, when aphids are fed on a diet containing these compounds, adult survival decreases so that it follows the same pattern as that observed when they are not fed. Furthermore, when concentrations of these compounds are high in the diet, fecundity of the adults is near zero, the quantity of honeydew released is low, and the number of wandering aphids is increased.

The survival of offspring laid by females fed on artificial diets containing HDMBOA-Glc seems not to be affected. The major reason is probably that the HDMBOA-Glc concentrations (0.25, 0.5, and 1 mM) were too low. A DIMBOA-Glc concentration of 2 mM weakly but significantly decreased survival of the offspring laid.

We have never been able to establish a *M. dirhodum* population on maize plants less than 15 days old. On these young plants, aphids wandered and starved after several days. Thus, maize seems to have developed an important defense mechanism against aphids at the more sensitive stage.

Concentrations of DIMBOA-Glc and HDMBOA-Glc are high in aerial parts of maize one day after seed germination. These concentrations are 13 mM and 1.5 mM, respectively (Cambier et al., 2000). Fourteen days later, they drop to 1.5 mM and 0.25 mM, respectively. Thus, concentrations decrease rapidly with plant age, and, we observe that populations of aphids begin to develop. Finally, as DIMBOA-Glc and HDMBOA-Glc are present in the phloem sap and probably in the vascular and mesophyll tissues (Argandona and Corcuera, 1985; Massardo et al., 1994), we suggest that the defense mechanism of the maize seedlings is due to the deterrent effect of these compounds.

Acknowledgments—The authors are grateful to Professor G. Van Impe for Probit analyses. V. Cambier has been supported by a PhD fellowship from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A). T. Hance is a Research Associate of the National Fund for Scientific Research (Belgium). The authors are indebted to the Belgium National Fund for Scientific Research (F.N.R.S).

REFERENCES

ABBOTT, W. S. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265–267.

ARGANDONA, V. H., and CORCUERA, L. J. 1985. Distribution of hydroxamic acids in *Zea mays* tissues. *Phytochemistry* 24:1–15.

ARGANDONA, V. H., LUZA, J. G., NIEMEYER, H. M., and CORCUERA, L. J. 1980. Role of hydroxamic acids in the resistance of cereals to aphids. *Phytochemistry* 19:1665–1668.

ARGANDONA, V. H., NIEMEYER, H. M., and CORCUERA, L. J. 1981. Effect of content and distribution of hydroxamic acids in wheat on infestation by the aphid *Schizaphis graminum. Phytochemistry* 20(4):673–676.

ARGANDONA, V. H., CORCUERA, L. J., NIEMEYER, H. M., and CAMPBELL, B. C. 1983. Toxicity and feeding deterrency of hydroxamic acids from gramineae in synthetic diets against the greenbug, *Schizaphis graminum. Entomol. Exp. Appl.* 34:134–138.

AUCLAIR, J. L. 1965. Feeding and nutrition of the pea aphid, *Acyrthosiphon pisum* (Homoptera: Aphidae), on chemically defined diets of various pH and nutrients levels. *Ann Entomol. Soc. Am.* 58(6):855–875.

CAILLAUD, C. M., and NIEMEYER, H. M. 1996. Possible involvement of the phloem sealing system in the acceptance of a plant as host by an aphid. Experientia 52:927–931.

CAMBIER, V., HANCE, T., and DE HOFFMANN, E. 1999. Noninjured maize contain several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal.* 10:119–126.

CAMBIER, V., HANCE, T., and DE HOFFMANN, E. 2000. Variation of DIMBOA and related compounds content in relation to the age and plant organ in maize. *Phytochemistry* 53:223–229.

CORCUERA, L. J., QUEIROLO, C. B., and ARGANDONA, V. H. 1985. Effects of 2-β-D-glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one on *Schizaphis graminum* (Rondani) (Insecta, Aphididae) feeding on artificial diets. *Experientia* 41:514–516.

DREYER, D. L., and CAMPBELL, B. C. 1987. Chemical basis of host-plant resistance to aphids. *Plant, Cell Environ.* 10:353–361.

ESCOBAR, C. A., SICKER, D., and NIEMEYER, H. M. 1999. Evaluation of DIMBOA analogs as antifeedants and antibiotics towards the aphid *Sitobion avenae* in artificial diet. *J. Chem. Ecol.* 25(7):1543–1554.

FINNEY, D. J. 1971. Probit Analysis. Cambridge University Press.

GIVOVICH, A., and NIEMEYER, H. M. 1995. Comparison of the effect of hydroxamic acids from wheat on five species of cereal aphids. *Entomol. Exp. Appl.* 74:115–119.

GIVOVICH, A., MORSE, S., CERDA, H., NIEMEYER, H. M., WRATTEN, S. D., and EDWARDS, P. J. 1992. Hydroxamic acid glucosides in honeydew of aphids feeding on wheat. *J. Chem. Ecol.* 18(6):841–846.

GIVOVICH, A., SANDSTRÖM, J., NIEMEYER, H. M., and PETTERSON, J. 1994. Presence of a hydroxamic acid glucoside in wheat phloem sap, and its consequences for performance of *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). *J. Chem. Ecol.* 20(8):1923–1930.

HOUSEMAN, J. G., CAMPOS, F., THIE, N. M. R., PHILOGÈNE, B. J. R., ATKINSON, J., MORAND, P., and ARNASON, J. T. 1992. Effect of the maize-derived compounds DIMBOA and MBOA on growth and digestive processes of european corn borer (Lepidoptera: Pyralidae). *J. Econ. Entomol.* 85(3):669–674.

KENNEDY, J., and HANCE, T. 1995. Varietal screening based on demographic parameters: resistance of tea to *Brevipalpus phoenicis* (Acari: Tenuipalpidae). *Environ. Entomol.* 24(6):1481–1486.

LONG, B. J., DUNN, G. M., BOWMAN, J. S., and ROUTLEY, D. G. 1977. Relationship of hydroxamic acid content in corn and resistance to the corn leaf aphid. *Crop Sci.* 17.

MASSARDO, F., ZUNIGA, G. E., PEREZ, F. J., and CORCUERA, L. J. 1994. Effects of hydroxamic acids on electron transport and their cellular location in corn. *Phytochemistry* 35:873–876.

MAYORAL, A. M., TJALLINGII, W. F., and CASTANERA, P. 1996. Probing behaviour of *Diuraphis noxia* on five cereal species with different hydroxamic acid levels. *Entomol. Exp. Appl.* 78:341–348.

MOLYNEUX, R. J., CAMPBELL, B. C., and DREYER, D. L. 1990. Honeydew analysis for detecting phloem transport of plant natural products. *J. Chem. Ecol.* 16(6):1899–1909.

SAS. 1985. SAS user's Guide: Statistics, Version 5 ed. Cary, North Carolina.

THACKRAY, D. J., WRATTEN, S. D., EDWARDS, P. J., and NIEMEYER, H. M. 1990. Resistance to the aphids *Sitobion avenae* and *Rhopalosiphum padi* in Gramineae in relation to hydroxamic acid levels. *Ann. Appl. Biol.* 116:573–582.

VAN IMPE, G., and HANCE, T. 1993. Une technique d'évaluation de la sensibilité variétale au

Tétranyque tisserand, *Tetranychus urticae* Koch (Acari: Tetranychidae). Application au haricot, au concombre, à la tomate et au fraisier. Agronomie 13:739–749.

WAHLROOS, O., and VIRTANEN, I. 1959. The precursors of 6-methoxy-benzoxazolinone in maize and wheat plants, their isolation and some of their properties. *Acta Chem. Scand.* 13(9):1906–1908.

XIE, Y. S., ARNASON, J. T., PHILOGÈNE, B. J. R., and LAMBERT, J. D. H. 1990. Role of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in the resistance of maize to western corn rootworm, *Diabrotica virgifera virgifera* (Leconte) (Coleoptera: Chrysomelidae). *Can. Entomol.* 122:1177–1186.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

ISOLATION AND STRUCTURAL IDENTIFICATION OF A GERMINATION INHIBITOR IN FIRE-RECRUITERS FROM THE CALIFORNIA CHAPARRAL

LOUISE M. EGERTON-WARBURTON,[1,*] and EMILIO L. GHISALBERTI[2]

[1]*Center for Conservation Biology and Department of Botany and Plant Sciences*
The University of California
Riverside, California 92521

[2]*Department of Chemistry*
The University of Western Australia
Nedlands 6907, Western Australia

(Received March 16, 2000; accepted September 17, 2000)

Abstract—A role for inhibitors as regulators of seed dormancy in fire-dependent annuals and their nondormant (fire-independent) congeners was examined in the family Hydrophyllaceae. From seeds of the fire-dependent species, *Emmenanthe penduliflora*, *Phacelia minor*, *P. brachyloba*, and *P. grandiflora*, extracts were obtained that were found to be largely self-inhibitory and potent inhibitors of seed germination in nondormant congeners. Lower activity was detected in the fire-independent species, *P. tanacetifolia* and *P. campanularia*. The inhibitory activity was associated with the new sucrose ester, 6-*O*-linoleyl-α-D-glucopyranosyl-β-D-fructofuranoside.

Key Words—Chaparral, fire, germination inhibition, sucrose esters, Hydrophyllaceae, *Emmenanthe penduliflora*, *Phacelia brachyloba*, *Phacelia campanularia*, *Phacelia grandiflora*, *Phacelia minor*, *Phacelia tanacetifolia*.

INTRODUCTION

Fire is a dominant force in the ecology and evolution of plant communities in the California chaparral (Axelrod, 1958). The chaparral postfire environment is notable for the diversity of adaptive flora (Sweeney, 1956; Christensen and Muller, 1975) and in particular the rich complex of annual and short-lived woody species that are cued for germination by charred wood or smoke (e.g., Wicklow,

*To whom correspondence should be addressed.

0098-0331/01/0200-0371$19.50/0

1977; Keeley and Keeley, 1987). The life history of these "fire-recruiters" is one characterized by prolonged, deep seed dormancy in a soil seed bank, reliance on smoke for germination, and establishment and seed set in the first spring after a fire. In turn, these seeds recharge the persistent soil seed bank until the next fire and ensure the maintenance of the population over time.

Despite our understanding of the effects of fire on seed ecology and controls on germination behavior (Wicklow, 1977; Keeley et al., 1985; Keeley and Keeley, 1987; Keeley, 1992), considerably less is known about the mechanisms that maintain or enforce the prolonged, deep seed dormancy. Sweeney (1956) suggested that physical mechanisms controlled dormancy and germination in fire-recruiters. Structures adjacent to the embryo (*Phacelia*, *Allophyllum*, *Eucrypta*) or seed coat (*Camissonia*), or the selective permeability of the seed coat to water and solutes (*Emmenanthe*, *Antirrhinum*, *Chaenactis*) and the changes therein following exposure to smoke confirm a role for (morpho-) physiological dormancy (Quick, 1943, 1947; Egerton-Warburton, 1998, and unpublished data). However, physiological dormancy is often linked to the presence of germination inhibitors (Baskin and Baskin, 1998), and embryo coverings can either contain or prevent the leaching of inhibitors (Bewley and Black, 1982). To date, a role for endogenous inhibitors in maintaining seed dormancy in fire-recruiters is unknown. In this paper, we report for the first time the nature and chemical identity of a biologically active compound, the sucrose ester 6-*O*-linoleyl-α-D-glucopyranosyl-β-D-fructofuranoside, in seeds of the fire-recruiters, *Emmenanthe penduliflora*, *Phacelia minor*, and *P. brachyloba* (family Hydrophyllaceae), and its potential role in the regulation of dormancy and germination.

METHODS AND MATERIALS

Seed Materials. We assayed for inhibitors in seed of the fire-dependent species, *Emmenanthe penduliflora* Benth., *Phacelia minor* (Harvey) Thell., *P. brachyloba* (Benth.) A. Gray, and *P. grandiflora* (Benth.) A. Gray. All these species display a requirement for smoke-stimulated germination: dormant seed demonstrates 0–2% germination in comparison to >75% germination following exposure to smoke. Comparisons of inhibitory activity were made with seed of nondormant (fire-independent) congeners, *P. tanacetifolia* Benth. and *P. campanularia* A. Gray. Seeds of these species germinated readily in deionized water (mean germination >95%). Seeds of each species were collected from recently burned sites in southern California (fresh), obtained from the Rancho Santa Ana Botanic Garden (RSABG) Seed Collection (stored), or from commercial sources as follows: *E. penduliflora* (N = 3 accessions from recently burned sites and RSABG accession 19174), *P. minor* (N = 4, RSABG 16375, 16268), *P. brachyloba* (N = 3, RSABG 19141, 15943), and *P. grandiflora* (N = 2, RSABG 16851),

P. tanacetifolia (S&S Seeds, CA), and *P. campanularia* (Seeds of the Southwest, NM, RSABG 17578).

Extraction of Germination Inhibitors. Aliquots of seed of an individual species, each 0.5 g, were ground under liquid nitrogen to a fine powder and then steeped in 5 ml 90% EtOH for 24 hr at 18°C. The resultant slurry was pulse centrifuged to pellet the solids, after which the supernatant was removed and evaporated to complete dryness under a stream of compressed N_2. The pellet was resuspended in 5 ml of deionized water and steeped for a further 24 hr. The slurry was again centrifuged and then the aqueous portion removed and dried under N_2. Dry extracts were stored at −20°C until required for analysis and bioassays. All species and accessions were extracted in triplicate on at least three different occasions.

Analytical Methods. For TLC, precoated AL SIL G/UV (Whatman) plates were used and the compounds were detected by spraying with 0.5% thymol in 5% H_2SO_4–ethanol. ^1H and ^{13}C NMR spectra were obtained for d_4-MeOH solutions by using a Bruker ARX-500 instrument operating at 500.1 (^1H) and 125.8 MHz (^{13}C). Mass spectrometry was carried out with a VG Autospec Mass Spectrometer (70 eV) in the Fast Atom Bombardment mode with *m*-nitrobenzylalcohol as the matrix.

Seed Germination Bioassays Conditions. Individual extracts were used in bioassays without further purification since ^1H and ^{13}C NMR analyses demonstrated that extracts were fundamentally composed of a single sucrose ester (e.g., *P. brachyloba*), and all efforts to purify the extracts resulted in rapid hydrolysis of them. Dried extracts for testing were dissolved in 200 mM KH_2PO_4 buffer (pH 7.1) and pipetted into 6-cm-diameter Petri dishes containing two Whatman No. 1 filter papers. Filter papers were prerinsed with methanol, then rinsed in deionized water and air dried to remove any substances that could influence germination (Peterson and Harrison, 1991). Each species and sample was tested by using a final concentration of 25 mg dry weight seed per milliliter of buffer. Clean, intact bioassay seed of *P. tanacetifolia* or smoke-treated *E. penduliflora* (described below) were then placed in the prepared dishes and incubated for 48 hr at 18–20°C under a photoperiod of 14L : 10D. Petri dishes containing buffer only were used as controls. Each species and extract was tested at least three times. On each occasion, three replicates containing 50 seeds per dish were used to test the inhibition response. Germination was scored as positive if the length of the radicle was at least as long as the seed. Differences in the number of seeds germinating following incubation in ethanolic or aqueous extracts were analyzed with a *t* test (Zar, 1984). Germination of the bioassay species after incubation was analyzed first by using a χ^2 test among extracts and then between bioassay species (*E. penduliflora*, *P. tanacetifolia*) for each test species extract by using a *t* test. Germination data were also converted to a response index (*RI*) (Williamson and Richardson, 1988), where *RI* was calculated as: if $T > C$, then $RI = 1 - C/T$

and if $T < C$, then $RI = T/C - 1$. The term T is the germination response following incubation in the extract, and C is the percentage germination in the control (buffer). RI ranges from -1 to $+1$, where -1 indicates complete inhibition and $+1$ indicates stimulation of germination.

Concentration Effects. The degree of germination inhibition and ID_{50} (the inhibitory dose for 50% of the seeds tested) were evaluated by using a graded concentration curve with extracts ranging from 0 to 10 mg/ml of the bioactive compound, a sucrose ester. The relationship between the amount of sucrose ester in solution and germination inhibition or radicle length was analyzed with correlation and regression of the line of best fit (Zar, 1984).

Leaching Versus Deactivation of Inhibitors. The potency of endogenous inhibitors during germination and the potential for leaching of compounds during this period was evaluated in *E. penduliflora*. Batches of *E. penduliflora* seed (RSABG 19174) were soaked for 24 hr in Regen 2000 (dilution 1 : 1000 in deionized water), a commercially available smoke extract. Half of the seed batch was dry blotted on KimWipes, placed in Petri dishes containing Whatman No. 1 filter papers moistened with deionized water, and germinated at 18°C and a photoperiod of 14L : 10D. Up to 88% of smoke-water treated *E. penduliflora* germinated within 10 days of commencing imbibition. For that reason, we sampled seeds and germinants every two days for their inhibitor status during this period. The remaining seeds were soaked in deionized water (1 : 2, seeds–water), and subsamples of leachate were removed for bioassay every second day to coincide with the germination profile. Data were analyzed with a two-way analysis of variance for time (days) and extract source (leachate, germinant).

RESULTS

Aqueous and ethanolic extracts of seeds from all fire-dependent species were equally efficacious inhibitors of germination. We detected no significant difference ($P > 0.05$) in germination of *P. tanacetifolia* seed incubated in either extract; both sources consistently resulted in no germination. For that reason, the data reported here represent the results of combined germination responses to both extractions.

Seeds of nondormant (fire-independent) species, *P. tanacetifolia* and *P. campanularia*, exhibited little inhibitory activity (Table 1). In contrast, extracts from seeds of the fire-dependent species, *E. penduliflora*, *P. minor*, *P. brachyloba*, and *P. grandiflora*, were potent inhibitors of germination on both fire-dependent (*E. penduliflora*) and -independent bioassay species (*P. tanacetifolia*) (χ^2, $P < 0.05$). In both test species, extracts from all *Phacelia* species resulted in total inhibition of germination. The strongly negative RI values also reflect inhibition of germination. Extracts of *E. penduliflora* also inhibited the germination of *P. tanaceti-*

TABLE 1. PERCENTAGE GERMINATION AND RESPONSE INDEX (RI) OF BIOASSAY SPECIES, *Phacelia tanacetifolia* AND *Emmenanthe penduliflora*, FOLLOWING INCUBATION IN EXTRACTS OF FIRE-DEPENDENT AND -INDEPENDENT SPECIES

Source of extract	Bioassay species: *Phacelia tanacetifolia* (fire-independent)	Bioassay species: *Emmenanthe penduliflora* (fire-dependent)	Significance (*t* test)[a]
Fire-dependent			
Emmenanthe penduliflora			
% germination	0	2.8	ns
RI	−1	−0.96	
Phacelia minor			
% germination	0	0	ns
RI	−1	−1	
Phacelia brachyloba			
% germination	0	0	ns
RI	−1	−1	
Phacelia grandiflora			
% germination	0	0	ns
RI	−1	−1	
Fire-independent			
Phacelia campanularia			
% germination	90	78	ns
RI	−0.1	−0.06	
Phacelia tanacetifolia			
% germination	80	73	ns
RI	−0.2	−0.12	
Buffer only (control)			
% germination	100	83	n/a

[a] n/a, statistical analysis not applicable; ns, not significant ($P > 0.05$).

folia. However, a small proportion of the *E. penduliflora* bioassay seedlot germinated in the presence of the *E. penduliflora* extract. Such findings indicate that bioactive compounds are not totally self-inhibitory and, possibly, that the embryo is less sensitive to its own inhibitors than those of other species. The inhibitory effect was also readily reversible; the transfer of seeds of bioassay species to dishes containing deionized water resulted in 80–100% germination.

The degree of germination inhibition was directly correlated with the amount of bioactive compound in solution (line of best fit, polynomial $r = 0.964$, $P < 0.05$; Figure 1). Sucrose linoleate was identified as a bioactive compound in seed extracts (see below), and the sucrose ester demonstrated an ID_{50} of 3.4 mg/ml solution (Figure 1). An increasing concentration of sucrose ester in solution also resulted in a proportionate reduction in the elongation of the radicle

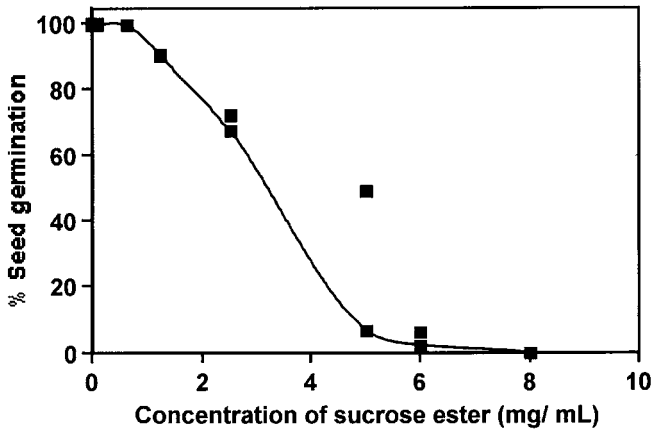


FIG. 1. Percentage germination of *P. tanacetifolia* seeds in response to increasing concentrations of sucrose linoleate extracted from seed of *E. penduliflora*.

(linear $r = 0.979$, $P < 0.05$; Figure 2), brown discoloration and curling of the radicle, and the loss of root hairs.

Inhibitory compounds were not leached from *E. penduliflora* seeds during germination (Figure 3), as bioassay of leachates gave 98–100% germination throughout the germination period. In contrast, inhibitors were detected within *E. penduliflora* seeds until germinants emerged, as indicated by the significant

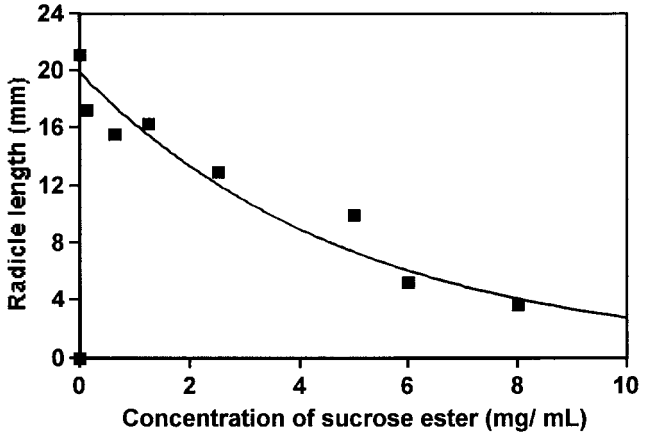


FIG. 2. Mean radicle length of *P. tanacetifolia* germinants following incubation in increasing concentrations of sucrose linoleate from *E. penduliflora* seed.

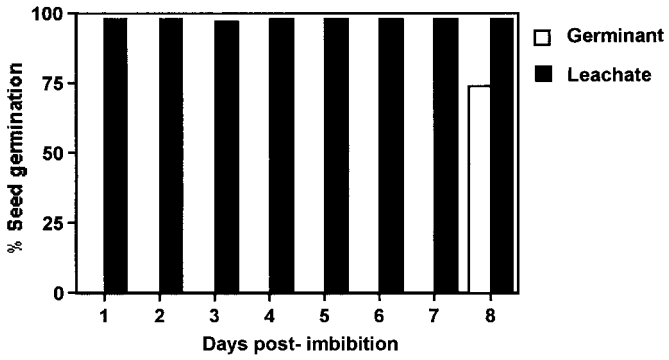


FIG. 3. Percentage germination of *P. tanacetifolia* seed following incubation in leachate or extracts of germinating seed of *E. penduliflora* during the germination period.

increase ($P < 0.05$) in bioassay germination at 10 days postimbibition (to 74%). These data indicate that release from inhibition was associated with deactivation, and not leaching, of the inhibitory compound(s). In addition, the absence of bioactive compounds in leachate reduces the potential for their role in allelopathic activity.

The extract from *P. brachyloba* appeared from thin-layer chromatography (TLC) to contain essentially a single compound as an amorphous solid. The proton and carbon NMR spectra indicated the presence of an unsaturated fatty acid residue that was identified as a linoleyl group by comparison with the spectra of linoleic acid (Pouchert and Behnke, 1976). However, a signal at δ_C 174.7 in the ^{13}C NMR spectrum indicated the presence of an ester, the free acid showed a signal for the carboxylic acid carbon at δ_C 180.1. The other signals in the NMR spectra of the compound suggested that the remainder of the molecule was a disaccharide (signals for 12 carbons in the region δ_C 61–106 (Table 2). Support for this conclusion came from FAB/MS measurements. The mass spectrum showed peaks at m/z 603 ($[M-H]^+$; 13%), 758 ($[M+matrix+H]^+$; 17%) and 263 (linoleyl ion, 18%) (see, for example, FAB mass spectra of sucrose esters) (de Koster et al., 1993). Detailed analysis of the NMR spectra provided evidence for the nature of the disaccharide unit as sucrose. Importantly, signals for three methyleneoxy carbons (δ_C 64.1, 63.4, 62.2, all triplets) and two acetal carbons (δ_C 105.3, 93.6, singlets) were of diagnostic significance. The location of the fatty acid residue at C-6 of the glucose unit was deduced as follows. The chemical shift and multiplicity (δ 4.35, J 3.9, 12.0 Hz; 4.17, J 6.0, 12.0 Hz) of the methyleneoxy protons of the esterifying alcohol indicated that the ester group was at C-6 or C-6′. It has been noted that the presence of an ester group at C-6′ results in shielding of the C-5′ from δ 82–84 to δ 78–79 (Matzusaki et al., 1992; Miyase et al.,

TABLE 2. NMR DATA FOR SUCROSE UNIT OF SUCROSE LINOLEATE (**1**) AND MODEL COMPOUND SIBIRICOSE A_3 (**2**)

	δ_H (J, Hz)		δ_C	
Atom	**1**	**2**	**1**	**2**
1	5.38 d (3.7)	5.42 d (4)	93.6	93.6
2	3.41 dd (9.7, 3.8)	3.46 dd (9.5, 3.5)	73.2	73.2
3	3.70 m	3.76 m	74.7	74.6
4	3.35 dd (9.5, 9.5)	3.45 dd (9.5, 9.5)	71.3	71.7
5	4.10 m	4.14 m	74.4	72.2
	4.17 dd (12.0, 6.0)	4.44 dd (12.0, 4.5)		
6			64.1	64.2
	4.35 dd (12.0, 3.9)	4.55 dd (12.0, 3.0)		
	3.59 d (12.3)	3.61 d (12.0)		
1′			63.4	64.7
	3.63 (12.3)	3.64 d (12.0)		
2′			105.3	105.3
3′	4.09 d (8.3)	4.10 d (8.0)	79.3	79.5
4′	4.10 dd (8.3, 8.3)	4.01 dd (8.0, 8.0)	75.7	76.0
5′	3.70 m	3.67 m	83.8	83.8
	3.75 m	3.74 m		
6′			62.2	63.9
	3.80 m	3.76 m		

1999; Ohya et al., 1994). Since **1** shows C-5′ at δ 83.8, the ester group must be located at C-6. Finally, comparison of the NMR spectra of sucrose linoleate (**1**) with those of the model compound sibiricose A_3 (**2**) (Miyase et al., 1999) shows close correspondence, supporting the structure proposed.

Similar examination of the extracts from *P. minor* and *E. penduliflora* revealed the presence of **1** as the major compound, although traces of other sucrose esters varying in the nature of the fatty acid could be detected.

Given the structure of the compound (**1**; Figure 4) and the observation that inhibition was readily reversible by transferring seed to deionized water, it is tempting to suggest that the most likely mechanism of deactivation is hydrolysis of the ester resulting in the formation of free sucrose and fatty acid in solution.

DISCUSSION

Sucrose esters have been shown to be inhibitors of seed germination in the Solanaceae, (Matsuzaki et al., 1988) and Caryophyllaceae (Peterson et al., 1998). We have now shown that sucrose linoleate constitutes a potent inhibitor to seed germination in *Phacelia* and *Emmenanthe* of the family Hydrophyllaceae. Notably, we detected significant differences in the yield of the sucrose ester

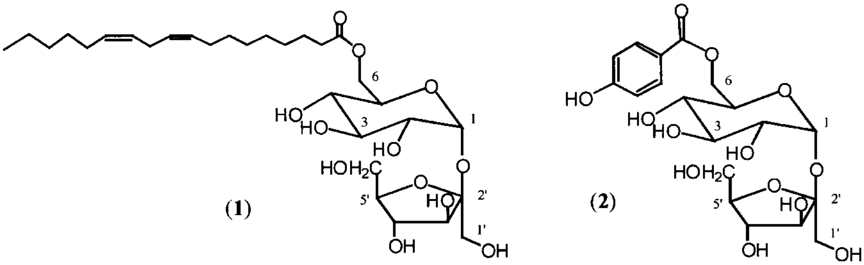


FIG. 4. Structure of sucrose linoleate (**1**) and the model compound, sibiricose A_3 (**2**).

among species based on life history traits (fire-dependent versus -independent). These findings denote, firstly, a familial similarity in the underlying biochemical pathways in these, and possibly other members, of the family Hydrophyllaceae, and secondly, that the sucrose ester concentration was a causal factor in maintaining dormancy or inhibiting germination in fire-recruiters. We infer that the sucrose ester regulates seed germination since other inhibitors, such as abscisic acid, benzoic acid, coumarin, lactones, or cinnamic acids, were not detected in extracts of *Emmenanthe* or *Phacelia* seed (Bewley and Black, 1982; Hillhorst, 1995).

Differences in germination strategies, in this instance sucrose ester yield, among related species may arise either on the basis of genetic differentiation or adaptation to environmental effects. Because sucrose linoleate occurred among phylogenetically related species, it is possible that the biosynthetic cascade for sucrose ester synthesis already exists. Accordingly, the up-scaling of this proximate signal and the production of sucrose esters in fire-recruiters may represent adaptation to the environment, including the maternal environment during development, because they contribute to the regulation of germination (Westoby, 1981).

Apart from the direct inhibition of germination, there are two mechanisms by which sucrose linoleate may contribute to the control of dormancy in Hydrophyllaceae and, in turn, prolong longevity of the soil seed bank. Sucrose esters may exhibit selective insecticidal properties (Puterka and Severson, 1995; Neal et al., 1994; Chortyk et al., 1996, 1997) and antibacterial and antimicrobial properties (Marshall and Bullerman, 1986; Holley et al., 1987; Chortyk et al., 1993). Such properties may limit seed herbivory or the deterioration of seed via the action of soil microbes, respectively.

Sucrose linoleate also may be closely associated with biochemical processes within the seed. Both the hydrophilic (sucrose) and lipophilic (fatty acid) nature of sucrose esters may control seed and cellular moisture; the sucrose moiety retains inherent seed moisture, while the lipophilic group repels the uptake of

water and, in turn, limits imbibition. Sucrose esters also slow cellular respiration and delay ripening, thus prolonging longevity (Bauchot et al., 1995). In addition, sucrose esters have strong emulsifying properties and have been associated with the prevention of thermal decomposition of proteins during either heating or cooling (Jandacek and Webb, 1978; Katsuragi, 1997); these properties are relevant to seed survival during fire, hot summers, and cool winters in the cismontane chaparral. Such mechanistic and functional traits may explain, at least in part, seed dormancy and seed bank persistence in fire-dependent Hydrophyllaceae.

Whether sucrose esters are explicitly linked to dormancy is a key question. Selection rarely acts on specific individual traits in isolation, but instead often on suites of traits in a correlated manner (Roff, 1992). In this context, dormancy in fire-recruiters may be a two-step process that comprises (morpho-) physiological and biochemical mechanisms operating in concert. Firstly, seed coat impermeability may regulate water and gas exchange (Egerton-Warburton, 1998), and secondly, sucrose esters (biochemical) may inhibit germination, reduce respiration, limit total seed moisture, and possibly enhance viability in the dormant phase (this study). Accordingly, we suggest that seed germination involves breaking both (morpho-) physiological and biochemical barriers in a temporal sequence. The exposure of seeds to smoke results in an increase in seed coat permeability and the uptake of water (Egerton-Warburton, 1998); in turn, imbibition may promote hydrolysis of the sucrose ester.

Acknowledgments—We thank David Reznick for assistance with seed collecting, Michael Wall at Rancho Santa Ana Botanic Garden for kindly providing many of the seed accessions used in the study, S&S Seeds for donation of *Phacelia tanacetifolia* seed, Sam Crace of Forest Flavors Inc. for the gift of Regen2000, and William Thomson for generous use of laboratory facilities.

REFERENCES

AXELROD, D. I. 1958. Evolution of the Madro-Tertiary geoflora. *Bot. Rev.* 24:433–509.

BASKIN, C. C., and BASKIN, J. M. 1998. Seeds. Ecology, Biogeography and Evolution of Dormancy and Germination. Academic Press, San Diego.

BAUCHOT, A. D., JOHN, P., SORIA, Y., and RECASENS, I. 1995. Sucrose ester-based coatings formulated with food-compatible antioxidants in the prevention of superficial scald in stored apples. *J. Am. Soc. Hortic. Sci.* 120:491–496.

BEWLEY, J. D., and BLACK, M. 1982. Physiology and Biochemistry of Seeds in Relation to Germination, Vol. 2. Viability, Dormancy and Environmental Control. Springer-Verlag, Berlin.

CHORTYK, O. T., SEVERSON, R. F., CUTLER, H. C., and SISSON, V. A. 1993. Antibiotic activities of sugar esters isolated from selected *Nicotiana* species. *Biosci. Biotech. Biochem.* 57:1355–1356.

CHORTYK, O. T., POMONIS, J. G., and JOHNSON, A. W. 1996. Syntheses and characterizations of insecticidal sucrose estes. *J. Agric. Food Chem.* 44:1551–1557.

CHORTYK, O. T., KAYS, S. J., and TENG, Q. 1997. Characterization of insecticidal sugar esters of *Petunia. J. Agric. Food Chem.* 45:270–275.

CHRISTENSEN, N. L., and MULLER, C. H. 1975. Effects of fire on factors controlling growth in *Adenostoma* chaparral. *Ecol. Monogr.* 45:29–55.

DE KOSTER, C. G., PAJARRON, A. M., HEERMA, W., and HAVERKAMP, J. 1993. Fast atom bombardment mass spectrometry of sucrose monocaprate and sucrose monolaurate. *Biol. Mass Spectron.* 22:277–284.

EGERTON-WARBURTON, L. M. 1998. A smoke-induced alteration of the sub-testa cuticle in seeds of the post-fire recruiter, *Emmenanthe penduliflora* Benth. (Hydrophyllaceae). *J. Exp. Bot.* 49:1317–1327.

HILLHORST, H. W. M. 1995. A critical update on seed dormancy. I. Primary dormancy. *Seed Sci. Res.* 5:61–73.

HOLLEY, J. D., KING, R. R., and SINGH, R. P. 1987. Glandular trichomes and the resistance of *Solanum berthaultii* (PI 473340) to infection from *Phytophthora infestans. Can. J. Plant Pathol.* 9:291–294.

JANDACEK, R. J., and WEBB, M. R. 1978. Physical properties of pure sucrose octaesters. *Chem. Phys. Lipids* 22:163–176.

KATSURAGI, T. 1997. Interaction between surfactants and fats. Annual Meeting of the American Oil Chemists Association, Chicago.

KEELEY, J. E. 1992. Seed germination and life history syndromes in the California chaparral. *Bot. Rev.* 57:81–116.

KEELEY, J. E., and KEELEY, S. C. 1987. Role of fire in the germination of chaparral herbs and suffrutescents. *Madroño* 34:240–249.

KEELEY, J. E., MORTON, B. A., PEDROSA, A., and TROTTER, P. 1985. Role of allelopathy, heat and charred wood in the germination of chaparral herbs and suffructescents. *J. Ecol.* 73:445–458.

MARSHALL, D. L., and DULLERMAN, L. B. 1986. Antimicrobial activity of sucrose fatty acid ester emulsifiers. *J. Food Sci.* 51:468–470.

MATSUZAKI, T., KOSEKI, K., and KOIWAL, A. 1988. Germination and growth inhibition of surface lipids from *Nicotiana* species and identification of sucrose esters. *Agric. Biol. Chem.* 52:1889–1897.

MATZUSAKI, T., SHINOZAKI, Y., HAGIMORI, M., TOBITA, T., SHIGEMATZU, H., and KOIWAI, A. 1992. Novel glycerolipids and glycolipids from the surface lipids of *Nicotiana benthamiana. Biosci. Biotech. Biochem.* 56:1565–156.

MIYASE, T., NOGUCHI, H., and CHEN, X.-M. 1999. Sucrose esters and xanthone C-glycosides from the roots of *Polygala sibirica. J. Nat. Prod.* 62:993–996.

NEAL, J. W., BUTA, J. G., PITTARELLI, G. W., LUSBY, W. R., and BENTZ, J.-A. 1994. Novel sucrose esters from *Nicotiana gossei:* Effective biorationals against selected horticultural insect pests. *J. Econ. Entomol.* 87:1600–1607.

OHYA, I., SINOZAKI, Y., TOBITA, T., TAKAHASHI, H., MATZUSAKI, T., and KOIWAI, A. 1994. Sucrose esters from the surface lipids of *Nicotania cavicola. Phytochemistry* 37:143–145.

PETERSON, J. K., and HARRISON, H. F. 1991. Isolation of substance from sweetpotato (*Ipomoea batatas*) periderm tissue that inhibits seed germination. *J. Chem. Ecol.* 17:943–951.

PETERSON, J. K., SNOOK, M. E., HARRISON, H. F., and MASON, P. F. 1998. Isolation and structural identification of sucrose esters from corn spurrey (*Spergula arvensis*) inhibition of seed germination. *J. Chem. Ecol.* 24:1803–1816.

POUCHERT, C. J., and BEHNKE, J. 1976. The Aldrich Library of ^{13}C and ^{1}H FT NMR spectra. Aldrich Chemical Company, Milwaukee, p. 785.

PUTERKA, G. J., and SEVERSON, R. F. 1995. Activity of sugar esters isolated from leaf trichomes of *Nicotiana gossei* to pear psylla (Homoptera: Psyllidae). *J. Econ. Entomol.* 88:615–619.

QUICK, C. R. 1943. Certain methods for forcing the germination of seeds. *J. Calif. Hortic. Soc.* 4:95–102.

QUICK, C. R. 1947. Germination of *Phacelia* seeds. *Madroño* 9:17–20.

ROFF, D. A. 1992. The Evolution of Life Histories: Theory and Analysis. Chapman and Hall, New York.

SWEENEY, J. R. 1956. Responses of vegetation to fire. A study of the herbaceous vegetation following chaparral fires. *Univ. Calif. Publ. Bot.* 28:143–216.

WESTOBY, M. 1981. How diversified seed germination behavior is selected. *Am. Nat.* 118:882–885.

WICKLOW, D. T. 1977. Germination response of *Emmenanthe penduliflora* (Hydrophyllaceae). *Ecology* 58:201–205.

WILLIAMSON, G. B., and RICHARDSON, D. 1988. Bioassays for allelopathy: measuring treatment response with independent controls. *J. Chem. Ecol.* 14:181–188.

ZAR, J. H. 1984. Biostatistical Analysis, 2nd ed. Prentice-Hall, New Jersey.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

PHYTOTOXICITY OF VULPIA RESIDUES: III. BIOLOGICAL ACTIVITY OF IDENTIFIED ALLELOCHEMICALS FROM *Vulpia myuros*

MIN AN,[1,*] J. E. PRATLEY,[2] and T. HAIG[2]

[1] *Environmental and Analytical Laboratories*
[2] *Farrer Centre for Conservation Farming*
Charles Sturt University
Wagga Wagga, NSW 2678, Australia

(Received October 11, 1999; accepted October 1, 2000)

Abstract—Twenty compounds identified in vulpia (*Vulpia myuros*) residues as allelochemicals were individually and collectively tested for biological activity. Each exhibited characteristic allelochemical behavior toward the test plant, i.e., inhibition at high concentrations and stimulation or no effect at low concentrations, but individual activities varied. Allelopathins present in large quantities, such as syringic, vanillic, and succinic acids, possessed low activity, while those present in small quantities, such as catechol and hydrocinnamic acid, possessed strong inhibitory activity. The concept of a phytotoxic strength index was developed for quantifying the biological properties of each individual allelopathin in a concise, comprehensive, and meaningful format. The individual contribution of each allelopathin, assessed by comparing the phytotoxic strength index to the overall toxicity of vulpia residues, was variable according to structure and was influenced by its relative proportion in the residue. The majority of compounds possessed low or medium biological activity and contributed most of the vulpia phytotoxicity, while compounds with high biological activity were in the minority and only present at low concentration. Artificial mixtures of these pure allelochemicals also produced phytotoxicity. There were additive/synergistic effects evident in the properties of these mixtures. One such mixture, formulated from allelochemicals found in the same proportions as occur in vulpia extract, produced stronger activity than another formulated from the same set of compounds but in equal proportions. These results suggest that the exploration of the relative composition of a cluster of allelopathins may be more important than simply focusing on the identification of one or two compounds with strong biological activity and that synergism is fundamental to the understanding of allelopathy.

*To whom correspondence should be addressed. e-mail address: man@csu.edu.au

0098-0331/01/0200-0383$19.50/0

Key Words—Phytotoxicity, allelopathy, allelopathins, vulpia, bioassay, synergism, biological activity, phenolic compounds.

INTRODUCTION

To identify and quantify compounds contained in plant residues is part of the process of discovering agents of allelopathy. Plants contain thousands of natural products, but not all are implicated as being allelopathic (Bell and Charlwood, 1980; Rice, 1984). Plant allelopathins may be released, either as metabolites or derivatives, into the environment by such processes as volatilization, root exudation, leaching, or residue decomposition and are thereby available to exert their allelopathic effect upon other plants (Putnam and Tang, 1986).

Conclusive establishment of an allelopathic interaction is often a complicated issue, as the biological activities of putative allelopathins are governed by a number of different factors. Such matters as individual chemical structure (Williams and Hoagland, 1982; Einhellig, 1989); antagonistic, additive, sequential, or synergistic effects (Inderjit and Mallik, 1997; Blum et al., 1999); and various biotic factors (Grime, 1979; Blum, 1995; Harborne, 1997) all impact upon observed activity. While various criteria have been proposed for the demonstration of allelopathy (Fuerst and Putnam, 1983; Willis, 1985; Blum et al., 1999), the full implementation of these criteria is in reality extremely difficult to achieve and may perhaps be unrealizable (Blum et al., 1999). Although it is expected that donor-plant allelopathins carried through soil will undergo various abiotic and biotic processes before making contact with the roots of receptor plants, their ultimate effect is to inhibit the growth or functions of that receptor. In this study, we have adopted an achievable demonstration of allelopathy that first identifies the compounds released from *V. myuros* residues, and then follows with bioassay tests of individual compound activity, determination of activity threshold concentrations, and the testing of various compound combinations for additive or synergistic effects (Rice, 1985; Putnam and Tang, 1986).

The weed *Vulpia myuros* is known to have characteristics that make it unattractive to livestock, as well as competitive against crop and pasture plants, so that our investigation was conducted not only to obtain a better understanding of its allelopathic fundamentals, but also to help develop strategies to better manage the vulpia weed problem of southern Australia at a practical ecosystem level. In our second publication on vulpia allelopathy (An et al., 2000), the main compounds released from vulpia residues were identified and quantified. The specific aims of this study were to test the biological activities of the previously isolated compounds (both individually and collectively) and to quantify their individual activities as well as the specific contributions of each to the overall phytotoxicity of their mixture.

METHODS AND MATERIALS

Of the 21 compounds previously identified in residues of vulpia (An et al., 2000), only 20 were used in this study, as hydroferulic acid was not available.

Bioassay Procedure

Preparation of Authentic Compound Solutions. A concentration series of 1, 10, 100, 250, 500, 750, and 1000 ppm was made for each of the 20 identified compounds (Table 1) by using commercially obtained authentic substances. 250

TABLE 1. PHYTOTOXIC STRENGTH OF IDENTIFIED ALLELOCHEMICALS AS MEASURED BY WHEAT (CV. VULCAN) SEEDLING BIOASSAY

	Inhibition index		Threshold concentration (ppm)		I_{50} (ppm)	
Chemical name	Coleoptile	Root	Coleoptile	Root	Coleoptile	Root
Coniferyl alcohol	0.68	2.60	500	500	>1000	775
Protocatechuic acid	1.01	2.95	500	250	>1000	712
Succinic acid	1.08	4.48	500	100	>1000	631
3,4-Dimethoxyphenol	1.86	3.85	250	250	>1000	585
Hydrocaffeic acid	1.93	4.25	250	100	941	576
Syringic acid	1.96	4.25	500	10	932	602
Hydroquinone	1.96	4.52	500	250	924	542
p-Hydroxybenzoic acid	2.60	4.81	250	500	886	463
Vanillic acid	2.68	5.98	500	100	800	373
p-Hydroxy-phenylacetic acid	2.75	6.65	10	1	886	97
3-(4-Hydroxyphenyl) propanoic acid	2.79	5.29	1	1	729	458
Gentisic acid	2.97	5.61	1	100	712	441
Mix 1[a]	3.21	5.69	250	100	669	407
p-Coumaric acid	3.35	6.02	100	250	678	373
Pyrogallol	3.38	6.55	100	100	729	275
Ferulic acid	3.69	6.24	250	1	534	352
Catechol	3.84	6.87	100	1	644	200
Mix 2[b]	3.96	6.66	250	1	492	326
2-Hydroxy-3-phenyl propanoic acid	4.18	6.75	10	250	483	246
Hydrocinnamic acid	6.16	8.43	100	1	288	85
Salicylic acid	6.41	7.90	10	100	273	169
Benzoic acid	6.46	7.61	100	100	309	200

[a] Mix 1: 20 compounds mixed in equal proportions.
[b] Mix 2: 20 compounds mixed in the proportions found in vulpia ether fraction.

ml of 1000 ppm stock solution was first prepared for each compound by dissolving 0.25 g standard in a minimal volume of methanol and adding the remainder as distilled water to 250 ml. Each stock solution was then diluted to the required concentrations in 100-ml volumetric flasks. The same concentration series was also prepared for mixtures of these 20 compounds. Two kinds of 20-compound mixtures were prepared for the bioassay. The first was made by combining each of the compounds in equal proportions (mix 1). The second (mix 2 in Table 1) was made by combining each compound in a proportion equal to that determined to be present in the vulpia ether fraction (An et al., 2000).

Bioassay. The bioassay procedures developed previously (An et al., 1997a) were employed to test the biological activity of identified compounds using wheat as the indicator species. Fifteen pregerminated wheat (*Triticum aestivum* L. cv. Vulcan) seeds were placed in each Petri dish lined with filter paper. Five milliliters of compound solution was added. Control treatments received 5 ml of distilled water instead of compound solution. After 48 hr, the lengths (millimeters) of the longest seminal root and coleoptile were measured. The average length of radicle/coleoptile per Petri dish was used for statistical analysis. The bioassay was arranged in a randomized complete block design with three replicates.

Data Analyses

Seedling Elongation. The average radicle/coleoptile length per Petri dish was used for statistical analyses. The method of whole-range assessment developed by An et al. (1997b) was employed. The approach was to calculate the inhibition area between test plant response of the control (i.e., 100%) over the whole range of compound concentrations on the x axis, and the phytotoxicity curve on the test plant, as generated by these compound concentrations (Figure 1). Thus:

$$\text{inhibition area} = \int_{C_T}^{1000} [100 - f(C)]dC$$

where C is the compound concentration in ppm, and C_T is the threshold concentration for inhibition in the test plant. The computation of the area was done with MicroOrigin software. Biological activity across the whole range of concentrations was then calculated based on "inhibition index," which was defined as the percentage of the maximum area inhibited. Thus:

$$\text{inhibition index} = (\text{inhibition area}/\text{total area}) \times 100,$$

where the total area is defined as $\int_0^{1000} 100\ dC$

I_{50} Value and Threshold Concentrations. In addition to the inhibition index

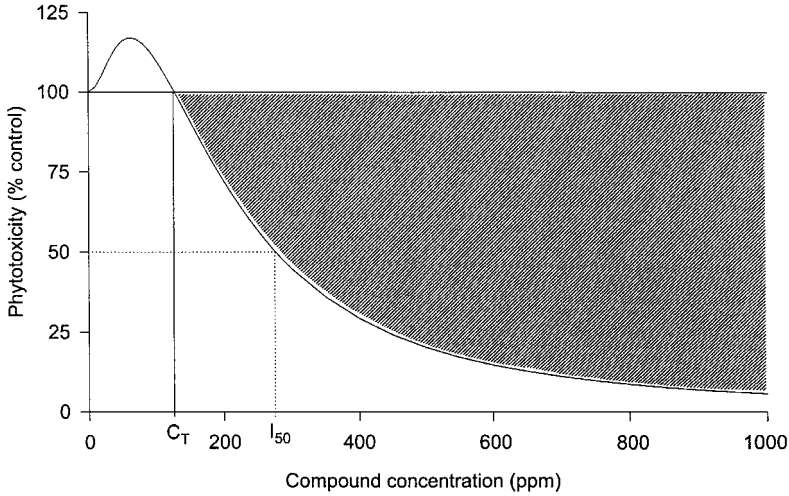


FIG. 1. Diagrammatic representation of seedling response to compound concentrations. The shaded section represents the inhibition area of the test plant by compound concentrations. C_T—the threshold concentration for causing inhibition; I_{50}—the concentration causing 50% of inhibition in the test plant.

values, the I_{50} value and the threshold concentrations at which a compound started to cause inhibition were also considered as important components for comprehensive assessment of a compound's biological activity. The I_{50} values (concentration required to cause 50% inhibition in the test plant) for coleoptile and root inhibition, respectively, were determined for each compound from the equation of best fit, the criterion for best fit being $r > 0.95$, which was significant at $P = 0.01$. Some compounds did not cause 50% inhibition over the range of compound concentrations tested. The I_{50} for those compounds was recorded as >1000 ppm. The inhibition threshold concentrations were those concentrations of the compound treatments at which coleoptile and root inhibition of the test plant were just recordable.

Overall Assessment. All parameters described, including inhibition index values of radicle/coleoptile lengths, threshold concentrations, and I_{50} values, were combined into a concise format to evaluate the overall biological activity of the compounds identified. All data were transformed to reduce the effect of differences in units and values among groups and to provide a homogeneous form for further analysis. Data from the threshold concentrations of inhibition were first transformed by subtraction from 500, while data from I_{50} were transformed by subtraction from 1000. Other group data remained intact. The standard deviation within each group was calculated. All data within each group were

divided by the standard deviation value for that group. All data in the six groups underwent an analysis of variance with MicroStat software, and the outcomes, as an average of six values for each compound, were displayed as a columnar list of integrated compound biological activity values from small to large. This concise-format value was defined as the phytotoxic strength index and gave a relative indication of biological activity for a given compound. A large index value means that a compound has strong biological activity, while a small value implies weak or no activity. Subsequently, all compounds were partitioned into groups based on the LSD value determined, that is, the value of the first compound in one group was significantly different from that of the first one in the group below (Table 2). Compounds in two adjacent groups are not necessarily all significantly different from each other, as overlap may occur.

TABLE 2. PHYTOTOXIC STRENGTH OF IDENTIFIED ALLELOCHEMICALS AS MEASURED BY OVERALL ASSESSMENT

Chemical name	Phytotoxic strength index	Biological activity	Average inhibition (% control)	
			Coleoptile	Root
Coniferyl alcohol	0.54	Weak	86.9	69.3
Protocatechuic acid	0.94			
Succinic acid	1.35			
Hydroquinone	1.41			
3,4-Dimethoxyphenol	1.46			
p-Hydroxybenzoic acid	1.55			
Syringic acid	1.59			
Hydrocaffeic acid	1.72			
Vanillic acid	2.04	Medium	77.7	58.6
Mix 1	2.34			
p-Coumaric acid	2.38			
Gentisic acid	2.47			
3-(4-Hydroxyphenyl)propanoic acid	2.50			
Pyrogallol	2.66			
Ferulic acid	2.70			
p-Hydroxyphenylacetic acid	2.84			
Mix 2	3.04	Strong	67.0	50.3
Catechol	3.05			
2-Hydroxy-3-phenylpropanoic acid	3.14			
Benzoic acid	3.44			
Salicylic acid	3.60			
Hydrocinnamic acid	3.72			

RESULTS

Individual Compounds

All 20 compounds tested exerted significant biological activities over the range of test concentrations (Figure 2). Typical allelochemical behavior was observed on the test plant, i.e., inhibition at high concentrations, stimulation or no effect at low concentrations. In terms of seedling inhibition, all compounds caused greater inhibition on root elongation than on coleoptile length. Threshold concentrations for both root and coleoptile inhibition were between 1 and 500 ppm. I_{50} levels for root inhibition were lower than for coleoptile inhibition. This indicated that the compounds had a greater effect upon plant roots than upon coleoptiles. The biological activities among compounds were not equal. Large differences in biological activity were evident among these 20 compounds, the active extent being dependent on the individual structure rather than on the compound class (Table 2). Generally, measured parameters (Table 1) were consistent with the strength of biological activities, even though some exceptions occurred. By combining all parameters considered, the compounds were partitioned into three groups in terms of the strength of their biological activity: strong, medium, and weak (Table 2). Since the phytotoxic strength for a specific compound is not a constant, but is related to the test plant species, the plant process, and the environmental conditions (Einhellig, 1986), only a relative concept was adopted here.

Most of the compounds tested fell either within the weak (40% of total) or medium (35% of total) classification, with only 25% being considered as strong. The amounts of compounds contained in the vulpia residue were not positively

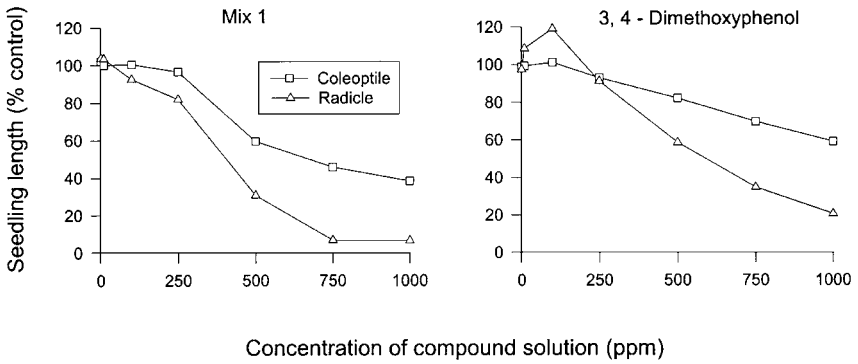


FIG. 2. Representative biological activities of identified allelochemicals as measured by wheat seedling growth.

TABLE 3. RELATIVE CONTRIBUTION OF EACH ALLELOCHEMICAL TO OVERALL ROOT PHYTOTOXICITY AS MEASURED IN MIX 2

Allelochemical	Relative contribution to phytotoxicity[a]	Phytotoxic strength index[b]
Catechol	0.14	3.05
Hydroquinone	0.30	1.41
Coniferyl alcohol	0.31	0.54
Hydrocinnamic acid	0.32	3.72
3,4-Dimethoxyphenol	0.40	1.46
Hydrocaffeic acid	0.86	1.72
p-Coumaric acid	1.45	2.38
Protocatechuic acid	2.01	0.94
Pyrogallol	2.23	2.66
Ferulic acid	2.53	2.70
p-Hydroxyphenylacetic acid	2.63	2.84
Gentisic acid	3.11	2.47
p-Hydroxybenzoic acid	3.21	1.55
Benzoic acid	5.14	3.44
2-Hydroxy-3-phenyl-propanoic acid	6.17	3.14
Syringic acid	11.75	1.59
Salicylic acid	12.65	3.60
Succinic acid	12.77	1.35
3-(4-Hydroxyphenyl) propanoic acid	12.87	2.50
Vanillic acid	19.51	2.04

[a] Calculation = $(C_i / \sum C_i) \times 100$, i = catechol, . . . , vannilic acid; C = phytotoxic strength index of individual compound multiplied by the mass of each compound in mix 2.
[b] As computed for Table 2.

correlated with their phytotoxic strength. Hydrocinnamic acid and catechol were found only in trace levels in the vulpia residue, but both exhibited strong phytotoxic activity. The quantity-dominant compounds such as vanillic, succinic, syringic, and the 3-(4-hydroxyphenyl)propanoic acids (but not salicylic acid) were found in weak and medium strength groups. This later led to the use of the phytotoxic strength index (Table 3) to evaluate the relative contribution of each compound to plant growth inhibition.

Combinations of Compounds

The combination of 20 compounds, whether mixed in equal portions by weight (mix 1) or in proportions reflecting the composition in vulpia residues (mix 2), exhibited appreciable biological activity. The general behavior of the mixtures was similar to that of individual compounds, i.e., inhibition occurred at high concentration and stimulation at low concentration, but there were additive and synergistic effects.

Those mixture concentrations at which each compound had a concentration below its own inhibition threshold still caused significant inhibition (Table 1, Figure 2). Mix 1 concentrations of 669 ppm and 407 ppm suppressed 50% of coleoptile and radicle lengths, respectively. Each compound was represented at a level of 33.5 ppm and 20.4 ppm, respectively, at which none of the compounds caused 50% inhibition on its own. Average I_{50} values for coleoptile inhibition were 960.4 ppm for the weak strength group, 724.0 ppm for the medium group, and 399.4 ppm for the strong group. Average I_{50} values for root inhibition were 610, 338, and 180 ppm for the above three groups, respectively. The threshold concentrations for mix 1 were 250 and 100 ppm for coleoptile and root inhibition, respectively. The concentrations of each compound contained in those two particular formulations of mix 1 were 12.5 ppm and 5.0 ppm, respectively, far below the inhibition threshold concentration for individuals in the majority of cases.

The significant inhibition of both root and coleoptile by the 750 ppm mix 1, containing 37.5 ppm of each compound, was equivalent to the average effects of the single compounds in the weak and medium strength groups at 1000 ppm and equaled the average for the individual compounds of the strong group at the 750 ppm level. None of the individual compounds exhibited significant inhibition at a concentration near 37.5 ppm.

The combination of these 20 allelochemicals in their natural weight proportions (i.e., mix 2) had biological activity similar to mix 1, except that it was stronger. In Table 2, mix 2 was classed within the strong group, while mix 1 was in the medium group. The stronger biological activity of mix 2 than of mix 1 indicated an enhanced effect among the compounds. No attempt was made to separate additive from synergistic effects. Mix 2 reflected a composition of compounds more likely to occur in the field. An attempt, by using the phytotoxic strength index, was made to evaluate the relative contribution of each allelochemical in mix 2 to the root phytotoxicity it caused (Table 3). Like their biological activities and levels present in the vulpia residues, the relative contributions of individual compounds to the overall phytotoxicity varied greatly. Vanillic, salicylic, succinic, syringic, and 3-(4-hydroxyphenyl)propanoic acids contributed most to the vulpia toxicity (more than 60% of total contributions), while hydroquinone, coniferyl alcohol, catechol, 3,4-dimethoxyphenol, and hydrocinnamic acid contributed little (Table 3).

DISCUSSION

All identified compounds exerted biological activity. Their phytotoxic strength was influenced by their concentrations as well as individual structure. The compounds were not equally inhibitory, and an overall assessment of

six significant parameters measured for each divided the 20 compounds into three groups: strong, medium, and weak phytotoxicity. Hydrocinnamic, salicylic, benzoic, 2-hydroxy-3-phenylpropanoic acids, and catechol possessed the greatest phytotoxicity; and coniferyl alcohol, protocatechuic, hydroquinone, 3,4-dimethoxyphenol, *p*-hydroxybenzoic, syringic, hydrocaffeic, and succinic acids the weakest; the rest were of an intermediate level.

Williams and Hoagland (1982) separated 12 phenolic compounds into four groups: very active, active, slightly active, and nonactive, based upon visual observations. Five of these chemicals appear in the list of 21 compounds found in the vulpia residue. The activity order reported for these five is consistent with the data presented here. Salicylic acid was reported as having markedly higher toxicity, while protocatechuic acid exhibited minimal activity (Einhellig, 1989).

The combination of the 20 allelochemicals resulted in an additive or synergistic effect. The inhibition threshold of mixtures was below most individual inhibition thresholds. It has been accepted in the literature that allelopathic interference often results from the combined effects of several different compounds (Rasmussen and Einhellig, 1977; Blum et al., 1985; Einhellig, 1987; Inderjit and Mallik, 1997). In a field situation where the amount of an allelochemical is often found to be well below the concentration for inhibition in a bioassay, such a concurrent interaction of allelochemicals may well be the explanation for the observed allelopathic phenomenon. The inhibition produced by the mixture of 20 allelochemicals combined in the same proportion found in the vulpia residue (mix 2) was compared with the inhibition produced by the crude ether fraction (An et al., 2000) by using as comparator the root inhibition area. The range of concentrations of 0–180 ppm for mix 2 was equivalent to the range of 0–1000 ppm for the crude ether fraction.

The result showed that the inhibition by mix 2 accounted for about 65% of the original inhibition produced by the whole ether fraction. Although the two bioassays were not conducted at the same time, they provide an indication that some compounds other than the chosen 20 allelochemicals identified are involved in the allelopathic interference by the vulpia residue. Indeed, GC-MS methylation analysis (An, 1995) identified about 25 compounds. If considering those additional compounds and the fact that syringic and hydroferulic acids were quantity-dominant compounds in the vulpia residue composition, the further inclusion of hydroferulic acid, with or without those additional compounds, in the mixtures for biological activity tests, would certainly increase the accountability of the allelochemical mixture for overall vulpia toxicity.

The concept of a phytotoxic strength index was developed for characterizing biological properties of allelochemicals and for quantifying their individual behaviors in a concise, comprehensive, and meaningful format. With such a single value index, the biological characteristics of 20 compounds were clearly distinguished. Even though the index was only adopted here for relative pur-

poses, its application went beyond the original context. For example, the index was used for assessing the relative contributions of each individual compound to the overall vulpia phytotoxicity. It was revealed that the relative phytotoxic contribution of an individual compound is largely correlated with its concentration. Even though hydrocinnamic acid and catechol had high phytotoxicity, their effects were greatly diminished by their trace levels in the vulpia residues; whereas the weak or medium toxicities of vanillic, syringic, and succinic acids were compensated for by their higher concentrations. The composition of allelochemicals in vulpia residues was dominated by the compounds that have only a low or medium phytotoxic strength but contribute most to the overall phytotoxicity, while a minority of compounds have high phytotoxic strength but are present only at trace levels. The more that allelochemicals are assessed for their phytotoxic strength index in a collective context, the more useful and meaningful the index will become, and contribute more to the understanding and application of allelopathy.

The combination of allelochemicals in equal proportions (mix 1) yielded less inhibition than the combination of allelochemicals in their natural proportions, even though the contribution to the mix of some compounds with high phytotoxicity had increased. This indicates that the composition of a natural allelochemical mixture is a more complex matter than the simple additive effect of 20 compounds. The synergistic effect is often far more profound than the mere additive effect. The contribution of each individual compound to the whole matrix of allelochemicals would be affected not only by its individual phytotoxic strength and the concentration present, but also most likely by the mixture's natural composition. This suggests that exploration of the composition of a cluster of allelochemicals may be more important than concentrating on the identification of one or two allelochemicals with strong biological activity. The relative composition of allelopathic mixtures in nature is fundamental to our understanding of allelopathy.

Acknowledgments—The financial support of the Grains Research & Development Corporation, Australia, is gratefully acknowledged.

REFERENCES

AN, M. 1995. Allelopathy in vulpia residues. PhD thesis. Charles Sturt University, Wagga, Australia.

AN, M., PRATLEY, J. E., and HAIG, T. 1997a. Phytotoxicity of vulpia residues: I. Investigation of aqueous extracts. *J. Chem. Ecol.* 23:1979–1995.

AN, M., PRATLEY, J. E., and HAIG, T. 1997b. Genotypic variation of plant species to the allelopathic effects of vulpia residues. *Aust. J. Exp. Agric.* 37:647–60.

AN, M., PRATLEY, J. E., and HAIG, T. 2000. Phytotoxicity of vulpia residues: II. Separation, identification and quantitation of allelochemicals from *Vulpia myuros. J. Chem. Ecol.* 26:1465–1476.

BELL, E. A., and CHARLWOOD, B. V. (eds.). 1980. Secondary Plant Products. Encyclopedia of Plant Physiology, New Series, Volume 8. Springer-Verlag, New York, 674 pp.

BLUM, U. 1995. The value of model plant-microbe-soil systems for understanding processes associated with allelopathic interactions: one example, pp. 127–131, *in* Inderjit, K. M. M. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications, ACS Symposium Series 582. American Chemical Society, Washington, D.C.

BLUM, U., DALTON, B. R., and SHANN, J. R. 1985. Effects of various mixtures of ferulic acid and some of its microbial metabolic products on cucumber leaf expansion and dry matter in nutrient culture. *J. Chem. Ecol.* 11:619–641.

BLUM, U., SHAFER, S. R., and LEHMAN, M. E. 1999. Evidence for inhibitory allelopathic interactions involving phenolic acids in field soils: Concepts vs. an experimental model. *Crit. Rev. Plant. Sci.* 18(5):673–693.

EINHELLIG, F. A. 1986. Mechanisms and modes of action of allelochemicals, pp. 171–188, *in* A. R. Putnam and C. S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

EINHELLIG, F. A. 1987. Interactions among allelochemical and other stress factors of the plant environment, pp. 343–357, *in* G. R. Waller (ed.). Allelochemicals: Role in Agriculture and Forestry. ACS Symposium Series 330. Americal Chemical Society, Washington, D.C.

EINHELLIG, F. A. 1989. Interactive effects of allelochemicals and environmental stress, pp. 101–118, *in* C. H. Chou and G. R. Waller (eds.). Phytochemical Ecology: Allelochemicals, Mycotoxins and Insect Pheromones and Allomones. Academia Sinica Monograph Series NO. 9. Taipei, Taiwan.

FUERST, E. P., and PUTNAM, A. R. 1983. Separating the competitive and allelopathic components of interference: Theoretical principles. *J. Chem. Ecol.* 9:937–944.

GRIME, J. P. 1979. Plant Strategies and Vegetation Processes. Wiley, Chichester, U.K.

HARBORNE, J. B. 1997. Role of phenolic secondary metabolites in plants and their degradation in nature, pp. 67–74, *in* G. Cadisch and K. E. Giller (eds.). Driven by Nature, Plant Litter Quality and Decomposition. CAB International, Wallingford.

INDERJIT, and MALLIK, A. U. 1997. Effect of phenolic compounds on selected soil properties. *For. Ecol. Man.* 92:11–18.

PUTNAM, A. R., and TANG, C. S. 1986. The Science of Allelopathy. John Wiley & Sons, New York, 317 pp.

RASMUSSEN, J. A., and EINHELLIG, F. A. 1977. Synergistic inhibitory effects of *p*-coumaric and ferulic acids on germination and growth of grain sorghum. *J. Chem. Ecol.* 3:197–205.

RICE, E. L. 1984. Allelopathy, 2nd ed. Academic Press, New York, 421 pp.

RICE, E. L. 1985. Allelopathy—an overview, pp. 81–105, *in* G. A. Cooper-Drive, T. Swain and E. E. Conn (eds.). Recent Advances in Phytochemistry: Chemically Mediated Interaction Between Plants and Other Organisms. Plenum Press, New York.

WILLIAMS, R. D., and HOAGLAND, R. E. 1982. The effects of naturally occurring phenolic compounds on seed germination. *Weed Sci.* 30:206–212.

WILLIS, R. J. 1985. The historical bases of the concept of allelopathy. *J. Hist. Biol.* 18:71–102.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

PHYTOTOXICITY OF VULPIA RESIDUES: IV. DYNAMICS OF ALLELOCHEMICALS DURING DECOMPOSITION OF VULPIA RESIDUES AND THEIR CORRESPONDING PHYTOTOXICITY

MIN AN,[1,*] J. E. PRATLEY,[2] and T. HAIG[2]

[1] *Environmental and Analytical Laboratories*
[2] *Farrer Centre for Conservation Farming*
Charles Sturt University
Wagga Wagga, NSW 2678, Australia

(Received October 11, 1999; accepted July 30, 2000)

Abstract—The behavior and dynamics of 20 identified allelochemicals in vulpia residues were both collectively and individually monitored, and their kinetic phytotoxicity was assessed. The total content of the identified allelochemicals in decaying vulpia residues increased from 0.31 to 1.24 mg/g dry residue over a 21-day decomposition period, while the total phenolic content increased from 1.86 to 2.16 mg/g dry residue. This corresponded to a phytotoxicity increase from 42% to 82% of radical inhibition. Allelochemicals changed in composition and quantity over the duration of the residue decomposition. Addition of soil to the residues reduced the total allelochemical contents extracted and altered the dynamic pattern. In the same period, the total content of allelochemicals declined from 0.061 to 0.046 mg/g residue + soil, with the total phenolics reduced from 0.20 to 0.11 mg/g residue + soil, corresponding to a radical length increase from 53% to 109% of control. Only 14 of the identified allelochemicals were detected in the mix of soil and residues, in contrast to 20 present in the residues alone. The implications of these findings are discussed.

Key Words—Phytotoxicity, allelopathy, allelochemical(s), vulpia, phenolic compounds, dynamics, plant residues.

INTRODUCTION

The monitoring of allelochemical dynamics in the environment is one necessary step for establishing conclusive proof of allelopathy (Putnam and Tang, 1986;

*To whom correspondence should be addressed. e-mail address: man@csu.edu.au

0098-0331/01/0200-0395$19.50/0

Dalton, 1999). Once allelochemicals are released into the environment, they are immediately subject to various physicochemical and biological processes, and they may be detoxified or toxified by soil organisms and/or may serve as a carbon skeleton for the production of new toxins by organisms in the soil (Blum et al., 1999). The composition and quantity of allelochemicals may vary substantially over time or with changed environmental conditions (Shindo and Kuwatsuka, 1977; Wójcik-Wojtkowiak et al., 1990; Dalton, 1999; Okumura et al., 1999). Phenolic acids, well-known potential allelochemicals, are readily converted from one structure to another with different phytotoxicities (e.g., ferulic acid to vanillic acid) by soil-borne microbes (Blum, 1998). For example, the content of *p*-hydroxybenzoic acid increased simultaneously with a rapid decrease in *p*-coumaric acid during the incubation of rice straw (Shindo and Kuwatsuka, 1975a,b). Phytotoxicity dynamics during residue decomposition has been well documented (Patrick et al., 1963; Chou and Lin, 1976; Mason-Sedun and Jessop, 1988). In general, decaying plant residues exhibit the most severe inhibition at the early stages of decomposition. As decomposition proceeds, the phytotoxicity declines. Only stimulation is evident at the later stages of decomposition (Mason-Sedun and Jessop, 1988; An et al., 1996a). Concentration changes of allelochemicals from decomposing plant residues have been shown to be related to phytotoxicity dynamics (Chou and Patrick, 1976; Tang and Waiss, 1978). An et al. (1996a) also theoretically demonstrated such a correlation from the viewpoint of mathematical modeling. However, little information is available on allelochemical dynamics during residue decomposition, either individually or collectively. Such matters as peak concentration, background concentration levels, shifts in composition, and the duration of peak concentration are largely unknown.

We have shown that the phytotoxicity of an aqueous extract of vulpia residues increased as decomposition proceeded, reached a maximum after 60 days of decomposition, and gradually diminished thereafter (An et al., 1997). Addition of soil to vulpia residues alleviated its allelopathic potential (An et al., 1993). Twenty-one compounds were identified and quantified in those vulpia residues (An et al., 2000), and their biological activity, characteristics, and individual contributions to overall phytotoxicity were subsequently assessed (An et al., 2001).

The objectives of this study were to elucidate the behavior of the identified allelochemicals during the decaying process of vulpia residues under various conditions, to monitor their quantity changes both collectively and individually, to gain insight into the allelochemical kinetics, and to clarify the correlation between phytotoxicity and allelochemical dynamics.

METHODS AND MATERIALS

Vulpia Residues and Soil. Material was obtained from pure mature stands of *V. myuros.* The residue material was cut 1.0 cm above the soil surface by hand,

oven dried at 40°C for 72 hr, and stored in dry conditions until commencement of the analysis.

About 1000 g soil was obtained from the top 10 cm layer of a vulpia-free field from the Charles Sturt University farm in Wagga Wagga, Australia. The soil was dried at 40°C, crushed, and passed through a 2-mm sieve.

Experimental Design and Decomposition Samples. Three treatment series were undertaken: residue alone, soil alone, and a mixture of residue with soil (residue + soil). Six decomposition periods, 1, 3, 7, 10, 14, and 21 days, for each treatment were used. For the residue alone treatment, 25 g dried vulpia residues, ground in a Wiley mill (1-mm mesh), were placed in a 500-ml glass jar with a lid, and 75 ml distilled water were added and mixed well. For the soil alone treatment, 25 g soil was placed in a 500-ml glass jar, and 7.5 ml distilled water was added to reach the field capacity of the soil. All samples were incubated in the dark at 20°C and allowed to decompose for the time period mentioned above. The air in the jar was exchanged through three 2-mm-diameter holes in the lid. The moisture content of the samples was kept constant by daily weighing the jars and supplementing the evaporated water loss.

Preparation of Aqueous Extracts. Aqueous extracts were obtained by adding 340, 290, and 350 ml distilled water to each sample of residue alone, soil alone, and residue + soil treatments, respectively, stirring for 10 min, and allowing them to settle for 5 min. The mixtures were decanted, filtered, squeezed through one layer of cheesecloth, centrifuged for 40 min at 3900 rpm, and then filtered through one layer of Whatman No. 1 filter paper. The volume of aqueous extract recovered from each of the three treatments was similar, about 270 ml. Each extract was partitioned into two lots and subjected to bioassay and chemical analysis, respectively.

Bioassay for Testing Phytotoxicity. The bioassay was carried out in 9-cm plastic Petri dishes lined with one piece of Whatman No. 1 filter paper. Fifteen germinated wheat (*Triticum aestivum* cv. Vulcan) seeds, radical length 3–5 mm, were transplanted in each dish, and 5 ml of sample extract was added. Control treatments received 5 ml of distilled water. The dishes were placed in an incubator, in the dark, at 24°C. After 48 hr, the lengths (millimeters) of the longest seminal root and coleoptile were measured. The average length per Petri dish was used for statistical analysis. The bioassay was arranged in a randomized complete block design with three replicates.

Extraction and Separation of Allelochemicals. Acetone (750 ml) was added to 250 ml of the aqueous extract from each treatment. The mixture was stirred at slow speed overnight at room temperature. Proteins and lipids in the supernatant were precipitated and filtered by vacuum filtration through two layers of Whatman Nos. 4, 1, and 42 filter papers. The precipitate was discarded. The acetone was removed from the extract by rotary evaporation below 40°C. The clear aqueous extract was sequentially partitioned twice with 500 ml hexane,

then five times with 300 ml diethyl ether. The hexane fraction was discarded, and the diethyl ether fraction was dried with Na_2SO_4, concentrated to dryness and weighed.

Determination of Total Phenolics. After evaporation of acetone from the above aqueous extracts, 30 ml sample extract was taken for estimating total phenolic contents by colorimetric procedures that used Folin-Ciocalteau phenol reagent (Sigma-Aldrich Australia) (Lowe, 1993).

For the soil alone treatment, 10 ml of extract were placed in test tubes, and then 3 ml 20% Na_2CO_3 solution (w/v) was added to each tube, followed by 1 ml Folin-Ciocalteau reagent. The solutions were mixed well and allowed to stand for 1 hr at room temperature (20–25°C); the absorbance was read at 750 nm in a spectrophotometer against a distilled water blank. For the residue alone, the sample was replaced by 0.5 ml extract and 9.5 ml distilled water. For residue + soil, the sample comprised 5 ml extract and 5 ml distilled water. The total phenolic substances in aqueous extracts were calculated from the calibration curve prepared from solutions of 1, 2.5, 5, 7.5, and 10 μg/ml vanillic acid and were reported as micrograms vanillic acid equivalents per milliliter of extract.

Qualitative and Quantitative Determination of Allelochemicals. The 21 allelochemicals identified before (An et al., 2000) were targeted in three experimental treatments through the decomposition period. The sample preparation and GC-MS analyses were the same as previously described (An et al., 2000). All samples were first subject to silylation and then analyzed by using a Hewlett-Packard 5890 series II gas chromatograph coupled with a VG Trio-2 mass spectrometer. Target compounds in samples were identified by comparison with GC retention times and MS of reference compounds. Quantitation was done by an internal standard method with multiple-point calibration curves. All samples were run in triplicate.

RESULTS

Phytotoxicity of Decomposing Vulpia Residues

With treatment of residue alone, all aqueous extracts exerted a strong inhibition on the test plant, with 42–82% radical length being inhibited (Figure 1a). The decomposition of residue accelerated the development of phytotoxicity. After one day of decomposition, the residue extract caused 42% inhibition of root length, and 11% of coleoptile length. As decomposition proceeded, phytotoxicity steadily increased and reached its maximum at day 21, and inhibition on root and coleoptile length of wheat increased to 82% and 72%, respectively (Figure 1a and b).

Addition of soil to residue altered the residue phytotoxicity pattern (Figure 1). The aqueous extracts from the mixture of residue + soil only exhibited

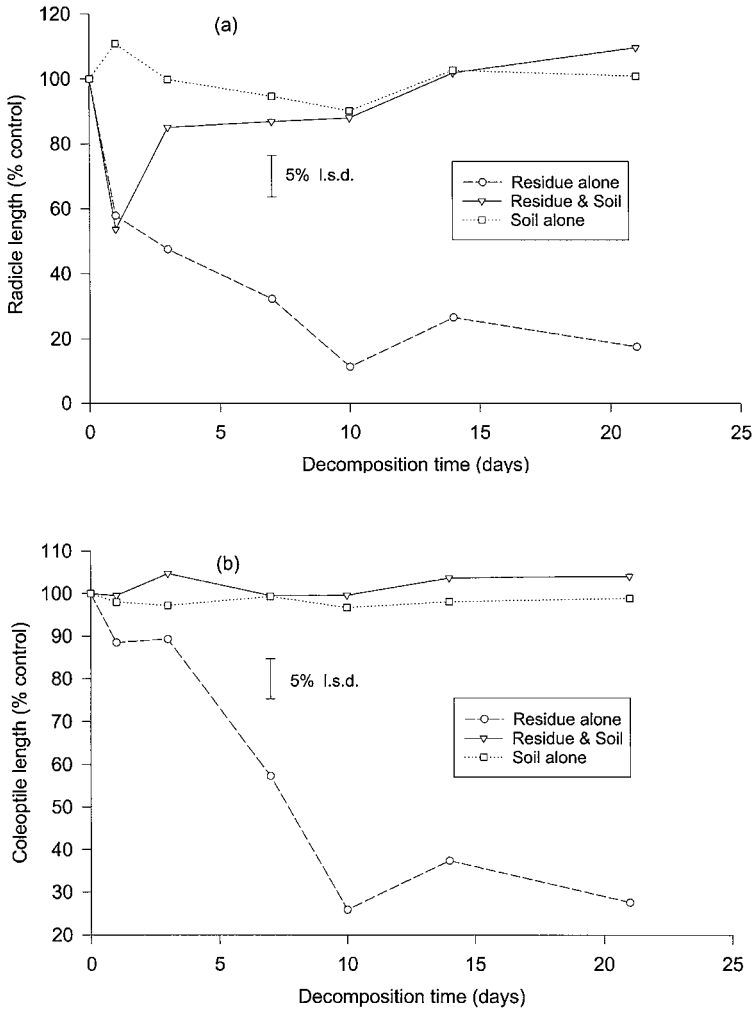


FIG. 1. Phytotoxicity dynamics of three experimental treatments as measured by seedling length of test plants (wheat cv. Vulcan): (a) measured by radicle length; (b) measured by coleoptile length.

severe phytotoxic effects on root length of wheat at an early stage of decomposition, i.e., at day 1 it inhibited up to 45% of radical length. As decomposition time increased, phytotoxicity rapidly decreased. By day 14 of decomposition, the phytotoxicity completely disappeared. At day 21, radical growth was actually stimulated. The coleoptile responses were no different from the control over

the whole decomposition period. The treatment of soil alone showed no effects on wheat growth, and only fluctuated around the control responses.

Total Phenolic Contents and Phytotoxicity

Total phenolic contents in residue alone were the highest among the three treatments, and steadily increased as the decomposition proceeded (Figure 2). Over a 21-day period, the total phenolics in aqueous extracts increased from 137 to 159 ppm, which accounted for 0.19% and 0.22% residue dry weight, respectively. The soil alone had the lowest phenolic contents. Its concentrations fluctuated between 1.2 and 6.1 ppm, which only accounted for 0.001 and 0.007% soil dry weight. The dynamics of total phenolics in residue + soil was different from both the residue-alone and soil-alone treatments. Addition of soil to residue reduced total phenolics, as well as changed their pattern compared to the residue alone. The total phenolic contents of residue + soil ranged from 15.0 to 44.3 ppm, about 10-fold decrease compared to the residue alone.

The phytotoxicity of all treatments was closely related to their corresponding total phenolic contents (Figures 3 and 4). The 42% radical inhibition in residue alone corresponded to a 137 ppm total phenolic content, while 15 ppm total phenolic content in residue + soil corresponded to a radical length increase of 110% control. Total phenolics in soil alone varied between 1.0 and 6.0 ppm, while the corresponding phytotoxicity only fluctuated around the control

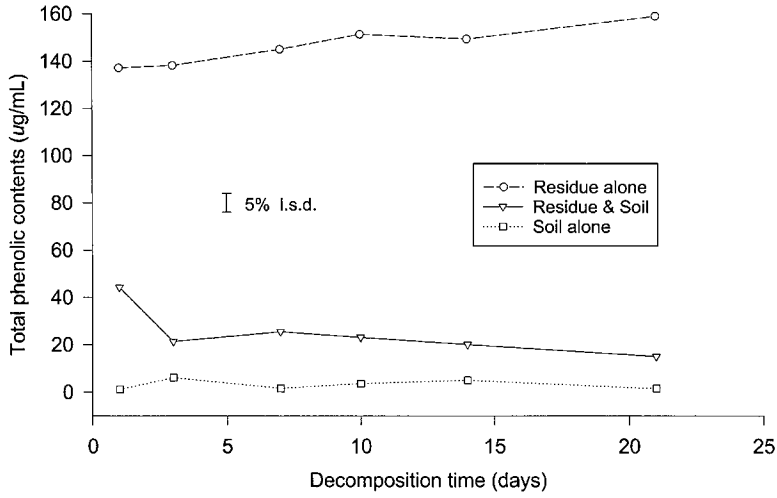


FIG. 2. Dynamics of total phenolic contents in three experimental treatments.

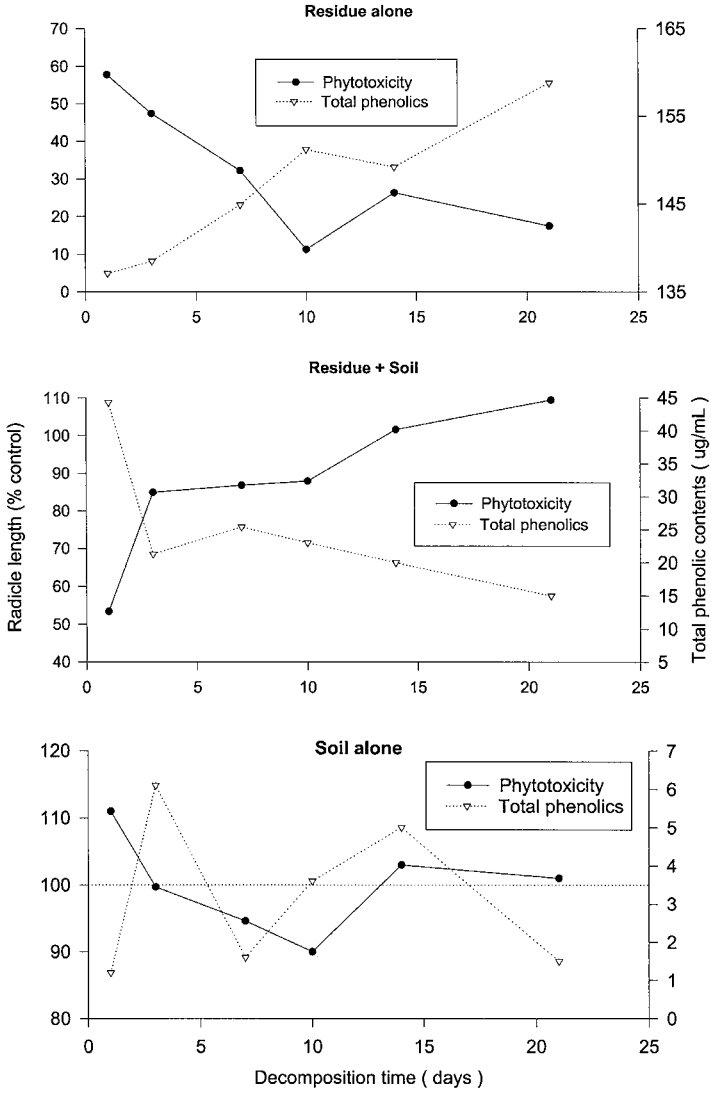


FIG. 3. Phytotoxicity dynamics of three experimental treatments with the corresponding dynamics of their total phenolic contents.

responses. In general, high total phenolic contents in aqueous extracts corresponded to severe inhibition, while low contents were associated with much less inhibition or no inhibition, even stimulation.

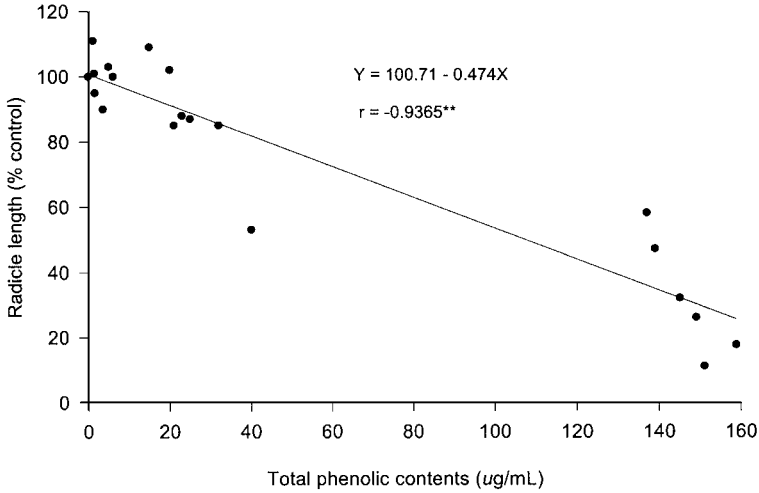


FIG. 4. The correlation between phytotoxicity of aqueous extracts of three treatments and their total phenolic contents.

Dynamics of Composition and Quantity of Allelochemicals

Residue. Among the 21 allelochemicals monitored, all except pyrogallol were detected. However, their presence and quantities were varied considerably, depending on individual compounds as well as the decomposition process (Table 1). Hydrocaffeic and *p*-hydroxybenzenepropanoic acids appeared as the decomposition proceeded, whereas the appearance of hydroquinone, salicylic, and gentisic acids fluctuated over the course of decomposition. The quantities of compounds, such as benzoic acid, succinic acid, catechol, 3,4-dimethoxyphenol, salicylic acid, α-hydroxybenzenepropanoic acid, *p*-hydroxybenzenepropanoic, syringic, and hydroferulic acids, were generally increased in the course of decomposition. With compounds such as *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic acids, their quantities were initially high, but decreased from day 7 to day 14 of decomposition, then increased at day 21. Other compounds, such as hydroquinone, coniferyl alcohol, hydrocinnamic, *p*-hydroxyphenylacetic, gentisic, protocatechuic, and hydrocaffeic acids remained relatively constant over the decomposition period.

The sum of the allelochemicals generally increased over the decomposition period, from 306 to 1236 μg/g residue, which accounted for 0.03% and 0.12% residue weight, respectively, and accounted for 16% and 57% total phenolic contents. The dynamic pattern was similar to that of total phenolics.

Resiue + Soil. Compared to the 20 allelochemicals detected in the residue-alone treatment, only 14 were detected in the residue + soil. The appearance of

TABLE 1. DYNAMICS OF ALLELOCHEMICALS IN COMPOSITION AND QUANTITY IN RESIDUE-ALONE TREATMENT

Chemical name	Quantity dynamics of allelochemicals (μg/g residue)					
	Decomposition time (days)					
	1	3	7	10	14	21
Benzoic acid	7.27	11.22	14.75	21.08	21.83	40.29
Succinic acid	30.08	32.37	29.13	32.17	75.60	158.37
Catechol	0.50	0.55	8.64	7.05	15.09	14.07
Hydroquinone	2.12				2.87	
Hydrocinnamic acid	0.64	0.62	0.86	1.70	1.23	2.55
3,4-Dimethoxyphenol	3.90	3.64	6.26	4.99	6.44	9.82
Salicylic acid	9.86	4.25	2.53			22.34
α-Hydroxybenzenepropanoic acid	13.38	14.08	14.40	16.15	33.76	28.28
p-Hydroxybenzoic acid	28.02	33.71	14.98	19.40	17.21	35.09
p-Hydroxyphenylacetic acid	14.60	15.05	14.54	16.54	19.17	17.81
p-Hydroxybenzenepropanoic acid		14.76	22.77	34.03	27.93	60.03
Vanillic acid	51.30	64.69	30.69	40.60	32.44	85.39
Gentisic acid	15.25	16.13	13.69		17.78	16.55
Protocatechuic acid	19.63	24.65	17.57	17.74	22.33	29.72
Syringic acid + Hydroferulic acid	21.34	25.87	135.68	130.79	286.39	628.69
Coniferyl alcohol	7.72	8.09	7.67	9.00	10.26	8.83
p-Coumaric acid	25.53	26.66	8.94	10.06	10.95	14.30
Hydrocaffeic acid			7.85	8.80	10.09	8.95
Ferulic acid	54.81	54.24	10.83	10.22	18.50	55.01

these 14 allelochemicals varied with decomposition time and with the individual compound (Table 2). Catechol, salicylic, *p*-hydroxybenzenepropanoic, vanillic, syringic, and *p*-coumaric acids were only detectable in some stage of the whole decomposition process. Hydroferulic acid was only present at day 1 of decomposition, thereafter only syringic acid was detectable. The number of allelochemicals in the composition varied with the decomposition time. At day 1, 14 allelochemicals were detected, but the number decreased afterwards. The quantities of compounds such as benzoic acid, succinic, *p*-hydroxybenzoic, vanillic, and *p*-coumaric acids decreased as the decomposition proceeded, while others remained relatively constant.

In general, even though the quantities of total allelochemicals were slightly higher at an early stage of decomposition, they basically remained constant. In contrast with the residue alone, the total quantities of allelochemicals declined, reduction ranging from 83 to 97% over the decomposition period. However, their proportions to total phenolic content increased as the decomposition proceeded, similar to the residue alone treatment.

Soil. The results were the same as for the residue + soil treatment. Only 14

TABLE 2. DYNAMICS OF ALLELOCHEMICALS IN COMPOSITION AND QUANTITY IN RESIDUE + SOIL TREATMENT

	Quantity dynamics of allelochemicals (μg/g residue + soil)					
	Decomposition time (days)					
Chemical name	1	3	7	10	14	21
Benzoic acid	3.94	0.77	2.16	5.92	4.34	2.57
Succinic acid	11.39	8.95	16.53	12.80	12.95	11.76
Catechol	0.18	0.15	0.26	0.21		0.18
Hydrocinnamic acid	0.36	0.19	0.35	0.24	0.24	0.20
Salicylic acid	0.52			0.51	0.58	
α-Hydroxybenzenepropanoic acid	5.36	4.19	7.74	5.87	6.02	5.43
p-Hydroxybenzoic acid	6.25	3.50	6.95	6.09	5.50	4.32
p-Hydroxyphenylacetic acid	5.86	4.54	8.37	6.42	6.49	5.80
p-Hydroxybenzenepropanoic acid	5.67					
Vanillic acid	9.97	6.21		8.57	8.82	7.93
Syringic acid	1.78[*]			1.84	1.87	1.73
Coniferyl alcohol	3.01	2.37	4.29	3.29	3.36	3.06
p-Coumaric acid	4.23					
Ferulic acid	2.80	2.21	3.97	3.03	3.10	2.80

[a] Syringic and Hydroferulic acids.

allelochemicals were detected, but salicylic acid in the residue + soil was substituted by protocatechuic acid. The appearance of all compounds except *p*-hydroxyphenylacetic, ferulic, hydrocinnamic acids, catechol, and succinic acid, fluctuated over the decomposition period (Table 3), as did the total quantities. The decomposition period did not affect allelochemical contents in the soil. The allelochemical levels in the soil were low, about 1.2–10.1 μg/g dry soil, accounting for only 0.0001–0.0010% dry weight, significantly lower than both the residue and the residue + soil treatments.

DISCUSSION

Phytotoxicity dynamics varied with the experimental treatments. Residue alone showed a steady increase in phytotoxicity as the decomposition proceeded. Soil alone did not show any significant stimulation or inhibition over the whole decomposition period, while the addition of soil to residue, i.e., residue + soil, significantly alleviated the phytotoxicity of vulpia residue. This is consistent with previous findings (An et al., 1993, 1997). Considering that the phytotoxicity of vulpia residues declined after 60 days of decomposition (An et al., 1997), the alleviation of residue phytotoxicity by soil may be attributable to an alteration in

TABLE 3. DYNAMICS OF ALLELOCHEMICALS IN COMPOSITION AND QUANTITY IN SOIL-ALONE TREATMENT

	Quantity dynamics of allelochemicals (μg/g soil)					
	Decomposition time (days)					
Chemical name	1	3	7	10	14	21
Benzoic acid	0.44	0.93	0.39		0.61	
Succinic acid	0.49	1.66	0.80	7.32	4.67	0.15
Catechol	0.02	0.01	0.02	0.01	0.01	0.04
Hydrocinnamic acid	0.05	0.05	0.06	0.15	0.12	0.21
α-Hydroxybenzenepropanoic acid		0.02	0.03	0.04	0.03	0.07
p-Hydroxybenzoic acid		1.37		0.14	0.17	0.31
p-Hydroxyphenylacetic acid	0.06	0.08	0.09	0.12	0.08	0.44
p-Hydroxybenzenepropanoic acid				0.28	0.49	
Vanillic acid	0.10	3.01		0.38	0.42	0.36
Protocatechuic acid		0.44				
Syringic acid		0.68		0.27	0.49	
Coniferyl alcohol		0.07	0.04	0.00	0.05	0.13
p-Coumaric acid		0.72				
Ferulic acid	0.02	1.10	0.03	0.06	0.06	0.14

the dynamic pattern of toxin development, driven by biotic and abiotic processes in soil (Bhowmik and Doll, 1979, 1984; Weston and Putnam, 1986; Blum et al., 1999). It may be that as the ratio of soil to residues increases, such an alteration of phytotoxicity pattern would not be obvious. Nevertheless, the finding that residue + soil still exerted strong inhibition at an early stage of the decomposition strongly indicated the existence of such a pattern change. This was certainly important in applying the findings to field practice. For example, a management strategy that used cultivation and then the delay of sowing for about three weeks avoided the peak inhibitory period from vulpia residues but took advantage of the noninhibitory period later, and thereby allowed successful pasture establishment, which had failed earlier owing to heavy infestation by vulpia (An et al., 1996b).

The results of the allelochemical dynamics agreed with the phytotoxicity kinetics, confirming the strong correlation expected between the phytotoxicity of residue extracts and their total phenolic contents. There exists a significant linear correlation ($r^2 = 0.99^{**}$) between vulpia extract concentrations and their total phenolic contents and between vulpia extract concentrations and their phytotoxicity (An, 1995). A causal relationship between these allelochemicals and phytotoxicity has been previously documented (Lovett and Ryuntyu, 1992; An et al., 1993). The present results further enhance the proposition that the identified phenolic compounds were the responsible agents for vulpia phytotoxicity.

The data presented here show that the behavior of individual allelochemicals and the composition of allelochemicals are different, depending on the

degree of residue decomposition. In the residue alone treatment, even though the sum of individual allelochemicals generally increased over time, compounds such as protocatechuic and hydrocinnamic acids kept relatively constant levels, while compounds such as ferulic and vanillic acids showed an increase, a decrease, and then another increase. The appearance of hydroquinone and salicylic acid fluctuated, and hydrocaffeic acid only appeared after seven days of decomposition. Such behavior of phenolic compounds was also observed by Wójcik-Wojtkowiak et al. (1990) and Shindo and Kuwatsuka (1975b) in rye and rice residues, respectively. Chou and Patrick (1976) reported that the concentration of toxins from corn residues reached a maximum at 15–25 days of decomposition and declined thereafter. As it is known that allelopathic effects are often the result of synergistic activity by a cluster of allelochemicals and that synergistic effects are dependent upon the composition of allelochemicals, a better understanding of such compositional changes may be required for effective field application of allelopathy.

The decomposition period had no effect on the phenolic content in the soil-alone treatment. This may indicate that soil itself contained a certain level of phenolic compounds that was not high enough to have effects upon plants and that these phenolics were at an equilibrium level among microbes and various environmental factors such as moisture and temperature. Phenolic acids, such as *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic, have been commonly found in soil: their quantities varied, depending upon plant species growing on the soil, soil type, and extraction method (Dalton, 1999). In the soil alone treatment, allelochemical quantities ranged between 1 and 10 μg/g soil. Biological tests in our previous paper (An et al., 2001) have shown that the activity threshold concentrations of those individual allelochemicals present in soil averaged 150 ppm, but with respect to an artificial 20-compound mixture, the average was only about 9 ppm. In the present study, only 14 compounds were detected in the soil treatment, and the average threshold concentration of those individuals in this mix would be likely greater than 9 ppm, far higher than their actual levels present in the soil. This may explain why the soil alone did not have any effect on test plants.

With the residue + soil treatment, low levels of allelochemicals, as well as fewer allelochemicals, were found. The dynamics pattern of allelochemicals from vulpia residues was altered by the incorporation of soil. In terms of the behavior of individual allelochemicals, it was similar to the residue-alone treatment. The persistence of most compounds was not changed, but their levels decreased over the decaying process. Allelochemicals are subject to various biotic and abiotic processes that reduce their concentrations and availability after their release into the soil. Such processes include utilization by soil-borne microorganisms (Blum and Shafer, 1988; Blum et al., 1998, 1999), sorption by soil organic matter (Makino et al., 1996) and clay minerals (Huang et al., 1977),

polymerization (Wang et al., 1986), chemical transformation into other toxic or nontoxic compounds (Blum, 1998; Okumura et al., 1999), and the degree of contact with plant roots (Blum et al., 1999).

To exert allelopathic effects on target plants, allelochemicals, after release into the environment, need to persist in the soil, undergo biotic and abiotic processes, and reach sufficient concentration for effect. The production, transformation, and destruction of phytotoxic compounds from decaying plant residues occur simultaneously in the soil (Blum, et al., 1999). The effective quantity is the difference between the amount produced and the amount inactivated. Thus, retention and the effective quantity of allelochemicals in soil and their ultimate allelopathic effects will vary, depending upon the relative rates of addition of allelochemicals to the environment, their decomposition or inactivation, the soil properties, and physicochemical conditions. Data from residue + soil showed that the addition of residue to soil increased phytotoxin production in the soil, even though allelochemicals were adsorbed, decomposed, inactivated, and/or polymerized to some extent by the soil. The levels of allelochemicals produced were generally discounted in soil, but sufficient concentration for inhibition still occurred for a short time, as evidenced by the severe phytotoxicity at an early stage of decomposition. In reality, such short-term inhibition could affect a susceptible organism's ability to compete with a nonsusceptible organism (Rice, 1984). As the decomposition period was prolonged, the amount of allelochemical inactivation could not be balanced by new production. Phytotoxin levels soon declined to plant-tolerant levels, which were compatible with the levels in soil. This was reflected in phytotoxic terms as no significant detrimental effects on test plants.

Acknowledgments—The financial support of the Grains Research & Development Corporation, Australia, and technical support from Mr. Steve Boyd and Mr. Philip Hatfield, Environmental & Analytical Laboratories, Charles Sturt University, are gratefully acknowledged.

REFERENCES

AN, M. 1995. Allelopathy in vulpia residues. PhD thesis. Charles Sturt University, Wagga Wagga, Australia.

AN, M., PRATLEY, J. E., and HAIG, T. 1993. The effect of soil on the phytotoxicity of residues of Vulpia myuros, pp. 162–164, *in* Proceedings of 7th Australian Agronomy Conference, Adelaide, South Australia.

AN, M., JOHNSON, I., and LOVETT, J. 1996a. Mathematical modeling of allelopathy: I. Phytotoxicity caused by plant residues during decomposition. *Allelopathy J.* 3:33–42.

AN, M., PRATLEY, J. E., and HAIG, T. 1996b. Field application of *Vulpia* phytotoxicity management: A case study, 616, *in* Proceedings of the 8th Australian Agronomy Conference, Toowoomba, Queensland.

AN, M., PRATLEY, J. E., and HAIG, T. 1997. Phytotoxicity of vulpia residues: I. Investigation of aqueous extracts. *J. Chem. Ecol.* 23:1979–1995.

AN, M., HAIG, T., and PRATLEY, J. E. 2000. Phytotoxicity of vulpia residues: II. Separation, identification and quantitation of allelochemicals from *Vulpia myuros. J. Chem. Ecol.* 26:1465–1476.

AN, M., PRATLEY, J. E., and HAIG, T. 2001. Phytotoxicity of vulpia residues: III. Biological activity of identified allelochemicals from *Vulpia myuros. J. Chem. Ecol.* 27:381–392.

BHOWMIK, P. C., and DOLL, J. D. 1979. Evaluation of allelopathic effects of selected weed species on corn and soybeans. *Proc. North Cent. Weed Control Conf.* 34:43–45.

BHOWMIK, P. C., and DOLL, J. D. 1984. Allelopathic effects of annual weed residues on growth and nutrient uptake of corn and soybean. *Agron. J.* 76:383–388.

BLUM, U. 1998. Effects of microbial utilization of phenolic acids and their phenolic acid breakdown products on allelopathic interactions. *J. Chem. Ecol.* 24:685–708.

BLUM, U., and SHAFER, S. R. 1988. Microbial populations and phenolic acids in soil. *Soil Biol. Biochem.* 20:793–800.

BLUM, U., AUSTIN, M. F., and SHAFER, S. R. 1998. The fate and effects of phenolic acids in a plant-microbial-soil model system, pp. 159–166, *in* F. A. Macias, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds.). Recent Advances in Allelopathy: Vol. 1. A Science for the Future. Cadiz University Press, Cadiz, Spain.

BLUM, U., SHAFER, S. R., and LEHMAN, M. E. 1999. Evidence for inhibitory allelopathic interactions involving phenolic acids in field soils: Concepts vs. an experimental model. *Crit. Rev. Plant. Sci.* 18(5):673–693.

CHOU, C. H., and LIN, H. J. 1976. Autointoxication mechanism of *Oryza sativa.* I. Phytotoxic effects of decomposing rice residues in soil. *J. Chem. Ecol.* 2:353–367.

CHOU, C. H., and PATRICK, Z. A. 1976. Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. *J. Chem. Ecol.* 2:369–387.

DALTON, B. R. 1999. The occurrence and behavior of plant phenolic acids in soil environments and their potential involvement in allelochemical interference interactions: methodological limitations in establishing conclusive proof of allelopathy, pp. 57–74, *in* Inderjit, K. M. M. Dakshini, and C. L. Foy (eds.). Principles and Practices in Plant Ecology: Allelochemical Interactions. CRC Press, Boca Raton, Florida.

HUANG, P. M., WANG, T. S. C., WANG, M. K., WU, M. H., and HSU, N. W. 1977. Retention of phenolic acids by non-crystalline hydroxy-aluminium and -iron compounds and clay minerals of soil. *Soil Sci.* 123:213–219.

LOVETT, J. V., and RYUNTYU, M. Y. 1992. Allelopathy: broadening the context, pp. 11–20, *in* S. J. H. Rizvi and V. Rizvi (eds.). Allelopathy: Basic and Applied Aspects. Chapman & Hall, London.

LOWE, L. E. 1993. Water-soluble phenolic materials, pp. 409–412, *in* M. R. Carter (ed.). Soil Sampling and Methods of Analysis. Canadian Society of Soil Science, Lewis Publishers, Boca Raton, Florida.

MAKINO, T., TAKAHASHI, T., SAKURAI, Y., and NANZYO, M. 1996. Influence of soil chemical properties on adsorption and oxidation of phenolic acids in soil suspension. *Soil Sci. Plant Nutr.* 42:867–879.

MASON-SEDUN, W., and JESSOP, R. S. 1988. Differential phytotoxicity among species and cultivars of the genus *Brassica* to wheat. II. Activity and persistence of water-soluble phytotoxins from residues of the genus *Brassic. Plant Soil* 107:69–80.

OKUMURA, M., FILONOW, A. B., and WALLER, G. R. 1999. Use of ^{14}C-labeled alfalfa saponins for monitoring their fate in soil. *J. Chem. Ecol.* 25:2575–2583.

PATRICK, Z. A., TOUSSOUN, T. A. and SNYDER, W. C. 1963. Phytotoxic substances in arable soils associated with decomposition of plant residues. *Phytopathology* 53:152–161.

PUTNAM, A. R., and TANG, C. S. 1986. The Science of Allelopathy. John Wiley & Sons, New York.

RICE, E. L. 1984. Allelopathy, 2nd ed. Academic Press, New York.

SHINDO, H., and KUWATSUKA, S. 1975a. Behavior of phenolic substances in the decaying process of

plants: II. Changes of phenolic substances in the decaying process of rice straw under various conditions. *Soil Sci. Plant Nutr.* 21:215–225.

SHINDO, H., and KUWATSUKA, S. 1975b. Behavior of phenolic substances in the decaying process of plants: III. Degradation pathway of phenolic acids. *Soil Sci. Plant Nutr.* 21:227–238.

SHINDO, H., and KUWATSUKA, S. 1977. Behavior of phenolic substances in the decaying process of plants: VI. Changes in quality and quantity of phenolic substances in the decaying process of rice straw in a soil. *Soil Sci. Plant Nutr.* 23:319–332.

TANG, C. S., and WAISS, A. C. 1978. Short-chain fatty acids as growth inhibitors in decomposing wheat straw. *J. Chem. Ecol.* 4:225–232.

WANG, T. S. C., HUANG, P. M., CHOU, C. H., and CHEN, J. H. 1986. The role of soil minerals in the abiotic polymerization of phenolic compounds and formation of humic substances, pp. 251–281, *in* P. M. Huang and M. Schnitzer (eds.). Interactions of Soil Minerals with Natural Organics and Microbes. SSSA Special Publication Number 17, Soil Science Society of America, Madison Wisconsin.

WESTON, L. A., and PUTNAM, A. R. 1986. Inhibition of legume seedling growth by residues and extracts of quackgrass (*Agropyron repens*). *Weed Sci.* 34:366–372.

WÓJCIK-WOJTKOWIAK, D., POLITYCKA, B., SCHNEIDER, M. and PERKOWSKI, J. 1990. Phenolic substances as allelopathic agents arising during the degradation of rye (*Secale cereale*) tissues. *Plant Soil* 124:143–147.

Journal of Chemical Ecology, Vol. 27, No. 2, 2001

DESCRIBING PHYTOTOXIC EFFECTS ON CUMULATIVE GERMINATION

L. S. DIAS

Departamento de Biologia, Centro de Ecologia Aplicada
Universidade de Évora
Ap. 94, 7002-554 Évora, Portugal

(Received January 26, 2000; accepted October 7, 2000)

Abstract—Phytotoxic studies strongly depend on evaluation of germination responses, which implies the need for adequate procedures to account for distinct aspects of the germinative process. For this, indices, comparisons among treatments at various times, and model fitting have been proposed. The objective of this work is to compare the three approaches and select the one providing the greatest insight and precision. Speed of germination, speed of accumulated germination, the coefficient of the rate of germination, comparisons at each determination time, including final germination, and the parameters of the Weibull function were examined. The Weibull function proved the best approach to describe the germination process, providing not only the same type of information about the speed of germination, with greater precision, but also additional information about the initiation and shape of the germination response curve.

Key Words—Allelopathy, *Cistus ladanifer*, germination, indices, phenolics, phytotoxicity, *Trifolium subterraneum*, Weibull function.

INTRODUCTION

Allelopathic and phytotoxic studies often involve the assessment of chemical effects on germination, requiring adequate description of the germinative process in addition to data on final germination alone. Attempts to describe germination responses in phytotoxic studies include the use of indices such as the speed of accumulated germination (Einhellig et al., 1982), coefficient of the rate of germination (Dias, 1991), and speed of germination (Wardle et al., 1996), the fit of Richard's function (Lehle and Putnam, 1982), or the test of differences in cumulative germination separately for each time of counts (An et al., 1996).

More recently, Chiapusio et al. (1997) compared four indices and compar-

0098-0331/01/0200-0411$19.50/0

isons between the control and treatments at each time of counts for their ability to test physiological hypotheses in allelopathic studies. The indices were: (1) total germination (G_T) expressed as proportion or percentage, by far the most commonly used germination descriptor; (2) speed of germination (S), usually known as Maguire's (1962) speed of germination; (3) speed of accumulated germination (AS), which can be traced back to Kendrick and Frankland (1969) and Bouton et al. (1976); and (4) coefficient of the rate of germination (CRG), presented by Kotowski (1926), its reciprocal being in use for more than a century (Edwards, 1934).

The results suggest that data interpretation depends on the index, that all indices fail to reflect adequately all allelochemical effects on germination, and that single comparisons between control and treatments at each time would be more appropriate (Chiapusio et al., 1997).

The option for comparisons between the control and treatments at each time germinated seeds were counted (observation time) raises a number of practical and theoretical questions. First, if different phytotoxic studies are to be compared, some kind of standard should be accepted for the observation time. This is a practical objection that raises questions as to the definition of observation time, its adequacy for different species, and the possibility that data interpretation might depend upon observation time; in fact, if different time intervals are chosen, different patterns of effects could emerge, as a close look at the report of Chiapusio et al. (1997) of *p*-hydroxybenzoic acid effects suggests. Finally, there is the more theoretical question of the real meaning, physiological or allelopathic, of making comparisons at 6-hr intervals or any other equally or unequally spaced interval.

An alternative approach would consider the whole process of germination, with observation time depending primarily on the species used. Therefore, observation time would provide point estimates, meaningless per se, allowing the fit of a general model that should be flexible enough to accommodate the widest possible range of germination patterns and with parameters meaningful in terms of germination. Additionally, it would be advantageous to separate the end of the process, expressed by total germination, from the path taken to attain it.

A number of models have been proposed to describe and compare germination, including orthogonal polynomials (Goodchild and Walker, 1971), cumulative normal distributions (Janssen, 1973), logistic functions (Schimpf et al., 1977; Hsu et al., 1984), gamma distributions (Thornley, 1977), Richard's function (Lehle and Putnam, 1982), and Weibull function (Bonner and Dell, 1976; Brown, 1987). Objections to many can be found in Brown and Mayer (1988), and include the failure of orthogonal polynomials to asymptote, the sensitivity to late-germinating seeds in gamma distributions, and the unreasonable assumption of symmetry in normal and logistic functions. In addition, when these and other models were compared in the same set of data by Brown and Mayer (1988), the

Weibull function (Weibull, 1951) proved the most suitable on theoretical and practical grounds (better fit and less sensitivity to initial estimates), accommodating a wide range of germination patterns (Brown, 1987).

The three-term Weibull function can be expressed by

$$Y = 1 - \exp - \{[(X - l)/k]^c\}$$

where Y is the cumulative proportion of germinated seeds at time X, l is a location parameter estimating the latest time at which the germination is zero, k is a scale parameter with $l + k$ estimating the time at which the germination is 0.63, and c is a shape parameter that evaluates the symmetry of the distribution ($3.26 \leq c \leq 3.60$ showing symmetry, $c < 3.26$ positive, $c > 3.60$ negative asymmetry).

Therefore, the objectives of the present study were to compare the adequacy of the Weibull function to reflect phytotoxic effects on germination with comparisons between cumulative germination at each observation time and the indices examined by Chiapusio et al. (1997). The experimental procedure involved testing the effects on germination of complete and phenolics-removed water extracts of vegetative shoot tops of *Cistus ladanifer* L., a spontaneous, aromatic shrub, known for its allelopathic activity (Dias et al., 1999). No attempt was made to fully consider the single or combined contribution of osmotic pressure or pH to the effects of water extracts.

METHODS AND MATERIALS

Materials. Shoot tops of *C. ladanifer* were collected near Évora (southern Portugal) in early summer. Cuttings included up to 50 mm of lignified stem, but only the three upper nodes were used. Seeds of subterranean clover (*Trifolium subterraneum* L. cv. Clare) were provided by the Estação Nacional de Melhoramento de Plantas.

Extract Preparation. Intact shoot tops were soaked in distilled water for 24 hr at 35°C, continuous light, filtered through Whatman No. 1 paper, and the extract adjusted to a concentration of 50 mg/ml (fresh weight volume). Phenolics were not quantified but their occurrence was ascertained by using Folin-Ciocalteau reagent (E. Merck). Half of the extract was repeatedly treated with Polyclar AT (BDH Chemicals, Ltd.), stirred for 15 min at room temperature, and filtered through Whatman No. 1 paper until no reaction with Folin-Ciocalteau reagent was detected by spectrophotometry. Both extracts were centrifuged for 30 min at 5200g, and the supernatant was used. Osmotic pressure and pH of the treatments, control included, were determined with a semimicroosmometer (Knauer type M) and a pH-meter (Metrohm E-520).

Germination Bioassay. Four replicated 10-cm Petri dishes were fitted with

Whatman No. 1 paper, sown with 25 seeds of subterranean clover, and wetted with 5 ml of the appropriate extract. The control was prepared similarly with distilled water. Seeds were incubated under a 20°C/30°C, 16/8-hr cycle, continuous dark, and were considered germinated if the radicle was at least as long as the greater dimension of the seed (Rietveld, 1975). Germinated seeds were regularly counted and discarded for eight days.

Germination Indices and Modeling. The selected indices, S, AS, and CRG, were calculated from the original data. For each observation time, cumulative germination (G_D) was expressed as proportion of sown seeds. The three-parameter Weibull function was fitted by least squares nonlinear regression with the Marquardt method, with data expressed as the cumulative proportion of seeds that actually germinated. Estimates of l were accepted only when their values were between the last time without and the first with observed germination. The same applies for k, considering that $l+k$ estimates the number of days at which the proportion of seeds that actually germinated is 0.63.

Statistical Analysis. Comparison of indices, G_D, and Weibull's parameters were made with a comparison-wise error rate of 0.05 by two-tailed Student's t or Mann-Whitney U tests.

RESULTS AND DISCUSSION

Results are summarized in Table 1. According to them, osmotic pressure and pH relevance can not be completely discarded, implying that, as usually happens in this type of study, the phytotoxic interpretation may include effects other than those of the biological activity of chemicals present in the extracts.

Nevertheless, the same information is obtained from the three indices S, AS, and CRG, namely that the complete extract and phenolics-removed extract differ from the control but not from each other. Because of the higher values of the indices in the control, water extracts of *C. ladanifer* reduce the speed at which germination develops, and because no differences were found between the extracts, that reduction can be attributed to the nonphenolic fraction.

When observation times are examined, there is a clear inhibition of germination associated with the two extracts at days 1.9 and 2.7, the intensity strongly decreasing from day 1.9 to day 2.7. At day 3.6, conclusions are prevented by the lack of "transitivity" (Chew, 1976), i.e., the complete extract and the control are the same, the complete extract and the phenolics-removed extract are also the same, but the control and the phenolics-removed extract are different. Results are clear again at days 4.7 and 7.7 ($=G_T$), when no significant differences were found among the control, complete extract, and phenolics-removed extract. Therefore, no effects of water extracts of *C. ladanifer* on final germination of subterranean clover were found. As detected by the three indices, both

TABLE 1. OSMOTIC PRESSURE (OP), pH, AND VALUES (MEAN ± SE) G_D (D FOR DAYS), INDICES (S, AS, AND CRG), AND WEIBULL PARAMETERS (l, k AND c) IN CONTROL (C), PHENOLICS-REMOVED EXTRACT (RE) AND COMPLETE EXTRACT (CE) TREATMENTS[a]

	C	RE	CE
OP	0	30	25
pH	5.0	5.5	3.6
S	7.802 ± 0.653 a	5.327 ± 0.531 b	4.590 ± 0.275 b
AS	22.134 ± 1.975 a	13.229 ± 1.977 b	11.033 ± 0.820 b
CRG	0.416 ± 0.021 a	0.266 ± 0.042 b	0.259 ± 0.016 b
$G_{1.0}$	0	0	0
$G_{1.9}$	0.551 ± 0.048 a	0.194 ± 0.086 b	0.094 ± 0.010 b
$G_{2.7}$	0.613 ± 0.050 a	0.376 ± 0.048 b	0.249 ± 0.025 b
$G_{3.6}$	0.623 ± 0.049 a	0.440 ± 0.047 b	0.448 ± 0.061 ab
$G_{4.7}$	0.675 ± 0.048 a	0.539 ± 0.047 a	0.532 ± 0.050 a
$G_{7.7}$	0.696 ± 0.035 a	0.712 ± 0.035 a	0.626 ± 0.038 a
l	1.020 ± 0.026 a	1.224 ± 0.238 a	1.172 ± 0.169 a
k	0.597 ± 0.099 a	2.175 ± 0.450 b	2.282 ± 0.407 b
c	0.785 ± 0.011 a	0.986 ± 0.141 a	1.703 ± 0.230 b

[a] OP is expressed as mosmol/kg, S and AS as number of germinated seeds per day, CRG as day^{-1}, G_D as proportion, l and k as day. In the same line, means with different letters are significantly different at $P = 0.05$ ($N = 4$, except for Weibull parameters of control, where $N = 3$).

treatments significantly delayed germination, an effect that had disappeared by day 4.7 and should, as before, be attributed to the nonphenolic fraction of the extracts.

With the exception of one replicate of the control, the Weibull function could always be fitted, with the coefficient of determination ranging between 0.751 and 0.999 (average of 0.960).

No significant differences in l were found among the treatments and the control, suggesting that the ability of subterranean clover seeds to start germination is not affected by either the phenolic or nonphenolic fraction.

Conversely, significant differences in k were found between the control and extracts, but not between extracts. Thus, after the onset of the process, the rate of germination ($1/k$) is equally reduced by the complete and phenolics-removed extract (Figure 1). The nonphenolic fraction might be responsible for this effect, since no differences were found between extracts.

The same conclusion resulted when indices and single comparisons were examined. Modeling the germination process by the Weibull function, however, allows a more precise estimate of this delay, which can be expressed by the time needed to attain 0.5 of final germination (G_{50}). In this case, G_{50} is 1.395 days in the control, 2.724 days in the phenolics-removed extract, and 3.012 days in

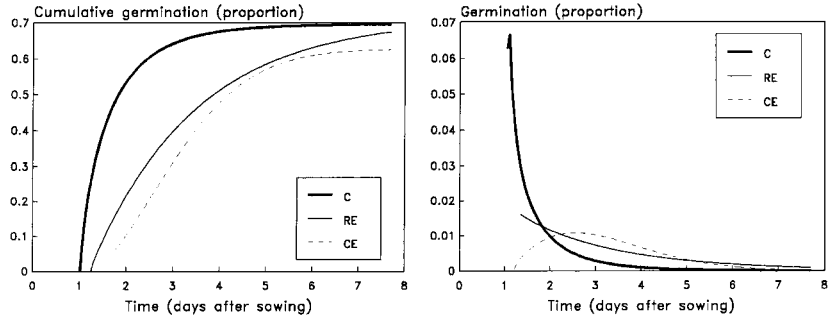


FIG. 1. Cumulative and noncumulative germination of subterranean clover in the control (C), phenolics-removed (RE), and complete (CE) extract, expected from fitted Weibull functions.

the complete extract if the start of the experiment is considered, or 0.375, 1.500, and 1.840 days, respectively, if the beginning of the germination, evaluated by l, is considered.

Finally, the shape of the germination is expressed by c. Given the mean value of the control, the germination of subterranean clover is clearly positively asymmetric with a pronounced tail to the right, as can be seen in Figure 1. This would be expected in such a species. The same occurs with the complete and phenolics-removed treatments. However, no differences were found between the control and phenolics-removed extract, with both differing from the complete extract. This implies that the strong asymmetry of the frequency distribution of germinated seeds over time is reduced by phenolic compounds extracted from *C. ladanifer*, but is insensitive to the nonphenolic fraction.

CONCLUSIONS

According to the data, (1) total germination and (2) time needed for its start are not affected by extracts; (3) the rate at which the germination proceeds from then on is equally reduced by the extracts, with the nonphenolic fraction being the cause; while (4) a less asymmetric distribution of germinated seeds is to be found when phenolic compounds of *C. ladanifer* are tested in addition to the nonphenolic fraction.

Conclusion 1 can only be obtained from single comparisons of final germination, which should always be considered in addition to Weibull modeling. Conclusion 3 can be obtained from indices, comparisons at determination times, or Weibull modeling, with the later allowing a greater precision. Finally, conclusions 2 and 4 only emerge when the Weibull function is fitted to the data,

providing a more complete and insightful picture of phytotoxic effects on germination.

Therefore, the use of the Weibull function appears to be a better approach to describe and analyze germination processes in phytotoxic studies, compared to cumulative germination at each observation time or the use of common indices.

Acknowledgments—I thank Gertrudes Mariano (Departamento de Biologia, Universidade de Évora) for technical assistance, Manuel Barradas (Director of Estação Nacional de Melhoramento de Plantas) for seed provision, and Alexandra S. Dias (Departamento de Biologia, Universidade de Évora) and two anonymous reviewers for comments on the manuscript.

REFERENCES

AN, M., PRATLEY, J. E., and HAIG, T. 1996. Differential phytotoxicity of *Vulpia* species and their plant parts. *Allelopathy J.* 3:185–194.

BONNER, F. T., and DELL, T. R. 1976. The Weibull function: A new method of comparing seed vigor. *J. Seed Technol.* 1:96–103.

BOUTON, J. H., DUDECK, A. E., and SMITH, R. L. 1976. Germination in freshly harvested seed of centipedegrass. *Agron. J.* 68:991–992.

BROWN, R. F. 1987. Germination of *Aristida armata* under constant and alternating temperatures and its analysis with the cumulative Weibull distribution as a model. *Aust. J. Bot.* 35:581–591.

BROWN, R. F., and MAYER, D. G. 1988. Representing cumulative germination. 2. The use of the Weibull function and other empirically derived curves. *Ann. Bot.* 61:127–138.

CHEW, V. 1976. Comparing treatment means: A compendium. *HortScience* 11:348–357.

CHIAPUSIO, G., SÁNCHEZ, A. M., REIGOSA, M. J., GONZÁLEZ, L., and PELLISSIER, F. 1997. Do germination indices adequately reflect allelochemical effects on the germination process? *J. Chem. Ecol.* 23:2445–2453.

DIAS, L. S. 1991. Allelopathic activity of decomposing straw of wheat and oat and associated soil on some crop species. *Soil Tillage Res.* 21:113–120.

DIAS, L. S., PEREIRA, I. P., and DIAS, A. S. 1999. Research on allelopathy in Portugal, pp. 89–98, *in* S. S. Narwal (ed.). Allelopathy Update. 1. International Status. Science Publishers, Inc., Enfield, New Hampshire.

EDWARDS, T. I. 1934. Relations of germinating soy beans to temperature and length of incubation time. *Plant Physiol.* 9:1–30.

EINHELLIG, F. A., SCHON, M. K., and RASMUSSEN, J. A. 1982. Synergistic effects of four cinnamic acid compounds on grain sorghum. *J. Plant Growth Regul.* 1:251–258.

GOODCHILD, N. A., and WALKER, M. G. 1971. A method of measuring seed germination in physiological studies. *Ann. Bot.* 35:615–621.

HSU, F. H., NELSON, C. J., and CHOW, W. S. 1984. A mathematical model to utilize the logistic function in germination and seedling growth. *J. Exp. Bot.* 35:1629–1640.

JANSSEN, J. G. M. 1973. A method of recording germination curves. *Ann. Bot.* 37:705–708.

KENDRICK, R. E., and FRANKLAND, B. 1969. Photocontrol of germination in *Amaranthus caudatus. Planta (Berlin)* 85:326–329.

KOTOWSKI, F. 1926. Temperature relations to germination of vegetable seed. *Proc. Am. Soc. Hortic. Sci.* 23:176–181.

LEHLE, F. R., and PUTNAM, A. R. 1982. Quantification of allelopathic potential of sorghum residues by novel indexing of Richard's function fitted to cumulative cress seed germination curves. *Plant Physiol.* 69:1212–1216.

MAGUIRE, J. D. 1962. Speed of germination—aid in selection and evaluation for seedling emergence and vigor. *Crop Sci.* 2:176–177.

RIETVELD, W. J. 1975. Phytotoxic grass residues reduce germination and initial root growth of ponderosa pine. USDA Forest Service, RP RM-153, 15 pp.

SCHIMPF, D. J., FLINT, S. D., and PALMBLAD, I. G. 1977. Representation of germination curves with the logistic function. *Ann. Bot.* 41:1357–1360.

THORNLEY, J. H. M. 1977. Germination of seeds and spores. *Ann. Bot.* 41:1363–1365.

WARDLE, D. A., NICHOLSON, K. S., and RAHMAN, A. 1996. Use of a comparative approach to identify allelopathic potential and relationship between allelopathy bioassays and "competition" experiments for ten grassland and plant species. *J. Chem. Ecol.* 22:933–948.

WEIBULL, W. 1951. A statistical distribution function of wide applicability. *J. Appl. Mech.* 18:293–297.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

HIGH LIGHT INTENSITY: A CRITICAL FACTOR IN THE WIND-TUNNEL FLIGHT OF TWO SCARABS, THE ROSE CHAFER AND JAPANESE BEETLE

JEREMY J. HEATH,* ROGER N. WILLIAMS, and P. LARRY PHELAN

Department of Entomology
Ohio Agricultural Research and Development Center/Ohio State University
1680 Madison Avenue
Wooster, Ohio 44691

(Received July 6, 2000; accepted October 31, 2000)

Abstract—We analyzed the flight of the rose chafer *Macrodactylus subspinosus* (F.) (Melolonthinae: Scarabaeidae) and the Japanese beetle *Popillia japonica* Newman (Rutelinae: Scarabaeidae) in a wind tunnel with controlled humidity, temperature, light, and airflow. The data indicate that an optimum combination of light and temperature dramatically improves their response to lures. Both species took off upwind, oriented to, and contacted the odor source well (40–60%) when light intensity was >50% of a clear day, temperature was 26–27°C, and relative humidity was 65–75%.

Key Words—Coleoptera, Scarabaeidae, Melolonthinae, *Macrodactylus subspinosus*, Rutelinae, *Popillia japonica*, wind-tunnel flight, semiochemicals.

INTRODUCTION

The most discriminating bioassays for characterizing semiochemicals are those that utilize the entire natural response and restrict the animal as little as possible (Miller and Roelofs, 1978; Baker and Cardé, 1984). "Because the responses made in-flight are so highly integrated, bioassays utilizing flight in wind are probably the most discriminating in pheromone research" (Baker and Linn, 1984). The wind tunnel has proven an invaluable tool in the identification of attractants for many insect species.

Attempts to conduct wind-tunnel bioassays with Scarabaeidae, such as the Japanese beetle, *Popillia japonica* Newman, and the rose chafer, *Macrodacty-*

* To whom correspondence should be addressed.

0098-0331/00/0300-0419$19.50/0

lus subspinosus (F.) have met with limited success. This difficulty is evident in the fact that most sex pheromones and other attractants for scarabs have been developed using olfactometers or field tests. Although field-testing is important, it is most efficiently used as the ultimate test of the efficacy or accuracy of the synthetic blend. The advantages of using a wind tunnel over field studies in the initial stages of identification include greater control of variables that modulate response, higher discriminating power, and the ability to conduct experiments throughout the year or during inclement weather (Baker and Linn, 1984).

M. subspinosus is attracted to an equal blend of α-ionone, (*E*)-2-nonenol, hexanoic acid, pentanoic acid, and octyl butyrate (Williams et al., 1993). *P. japonica* is attracted to a 3 : 7 : 3 blend of 2-phenylethyl propionate, eugenol, and geraniol (PEG) (Ladd and McGovern, 1980). The sex attractant pheromone of *P. japonica* is (*R*,*Z*)-5-dec-1-enyloxacyclopentan-2-one (Tumlinson et al., 1977). In the field, traps baited with these compounds will catch large numbers of individuals in a short time (Williams et al., 1990; Klein et al., 1981), but in the laboratory, it is difficult to elicit upwind flight to either of these blends (Klein, Linn, and Robbins personal communication). Leal (1995, 1998) has successfully flown the scarab, *Anomala daimiana*, in a wind tunnel (Leal, 1995) and has demonstrated that it is possible to overcome the lack of a discriminating bioassay by using his "short-cut" techniques, but agrees that "one of the main barriers in the task of identifying sex pheromones from scarab beetles is the lack of a uniform and consistent bioassay" (Leal, 1995). Successful indoor wind-tunnel experiments with the green June beetle, *Continis nitida*, (Domek et al., 1990) have suggested that increased light intensity may be important. In their experiments, the wind tunnel was lit with sixteen 40-W cool white fluorescent tubes.

Scarabs include some of the world's most significant insect pests (Vittum et al., 1999), but pesticidal control of scarabs has been inconsistent since the loss of cyclodiene insecticides (Facundo et al., 1994). Cost-effective and discriminating laboratory bioassays would speed development of behavior-modifying chemicals as economically viable alternatives for control.

METHODS AND MATERIALS

Insects. M. subspinosus adults were obtained from field traps in Castalia, Ohio (41°22′52″N, 82°49′23″W) baited with the aforementioned five-component blend on June 5, 1999. The location was a sandy field of mainly tall fescue, *Festuca arundinacea*; orchard grass, *Dactylis glomerata*; and several Asteraceae members. *P. japonica* adults were obtained from field traps in Wooster, Ohio (40°46′47″N, 81°55′12″W) baited with PEG lure and the sex pheromone on June 22, 1999. *M. subspinosus* were kept cool and transported from the field in a plastic drink cooler. They were stored in said container near a window at

room temperature until needed and fed sections of musk thistle (*Carduus nutans*) collected from the Castalia site. *P. japonica* were also transported and stored in this manner, but were fed store-bought sliced red delicious apples. Beetles were stored up to five days before use in assays.

Chemicals. The equal five-component blend used in trials with *M. subspinosus* consisted of α-ionone and (*E*)-2-nonenol (Bedoukian Research Inc., Danbury, Connecticut); hexanoic and pentanoic acid (Aldrich Chemical Co., Milwaukee, Wisconsin); and octyl butyrate (Penta Manufacturing, Livingston, New Jersey). Neat PEG lure was obtained from Michael G. Klein, USDA-ARS, Japanese Beetle Laboratory, Wooster, Ohio.

Wind Tunnel. The flight chamber was a 50-cm × 50-cm × 200-cm pine frame painted gray. It had heat-shrink plastic windows and eight aluminum mixing screens mounted over the upwind end of the tunnel to reduce turbulence. Airflow was created with a window fan placed upwind of the mixing screens. Airspeed was measured by timing the flight of a pulse of wood smoke and maintained at 30 cm/sec for all experiments by varying the distance between the fan and the mixing screens.

The wind tunnel was housed in a 3.2-m × 2.4-m × 2.1-m high environmental chamber (Conviron model BDW80) that contained 16 metal halide lamps (MH) (General Electric, Multi-Vapor, MV-400), 16 high-pressure sodium (HPS) lamps (General Electric, Lucalox, LU-400), two 200-W incandescent bulbs (IB), chrome walls, and humidity and temperature controls. The light from all the lamps passed through a Plexiglas ceiling. In *M. subspinosus* trials, the temperature and relative humidity were constant at 26°C and 75% ± 5 ($\overline{X} \pm s$), respectively. Four light levels were used: 10 (2 IB), 480 (6 MH, 6 HPS, and 2 IB), 800 (10 MH, 10 HPS, and 2 IB), and 1260 μmol photons/sec/m^2 (16 MH, 16 HPS, and 2 IB). We used a fixed, factorial design in trials with *P. japonica* to test interactions between light and temperature. Relative humidity was maintained at 66% ± 7 ($\overline{X} \pm s$). Light levels were as above (with the omission of level 480) and crossed with the following four temperatures: 21, 24, 27, and 30°C. Light intensity was measured with a Li-Cor Quantum sensor, which measured photosynthetically active radiation (PAR, 400–700 nm), and a Li-Cor (LI-185B) display. The spectral quality of the light was also measured with a Li-Cor scanning spectroradiometer (model LI-1800), which measured radiation from 300 to 1100 nm at 2-nm intervals.

Experimental Protocol. Experiments were conducted in a randomized complete block with blocking over time. In both experiments, treatments within a block were presented randomly over time. *M. subspinosus* experiments were conducted in five blocks over one day (June 6, 1999) between 14:00 and 21:00 hours. *P. japonica* experiments were conducted in three blocks with one block conducted per day between 12:00 and 22:00 hr (June 23–25, 1999). The peak activity of *P. japonica* and *M. subspinosus* is between 13:00 and 15:00 hr (Lacey

et al., 1994) and 10:00 and 14:00 hr (Heath, unpublished), respectively. In all experiments, we conducted 10 trials per treatment replication and recorded the proportion responding.

Beetles were removed from their storage cage and transferred to 4.5-cm × 3.5-cm diam. cylindrical screen acclimation cages. They were then allowed to acclimate in the environmental chamber under treatment conditions for at least 5 min before trials. Each beetle was coerced to crawl from the acclimation cages onto a rolled section of screening and held about 15 cm into the end of the wind tunnel and at the same height as the chemical source (25 cm). Beetles were tested individually and given up to 1 min to respond. Three responses were recorded: take-off upwind, orientation to the odor plume (locking on), and source contact. Orientation was observed as a directed, relatively slow, flight upwind with very little horizontal casting. This response contrasted with seemingly random flight upwind often colliding with the wind-tunnel walls, which was scored only as take-off upwind. As in Facundo et al. (1994), those that took flight but did not contact the source were given a maximum of three chances within the 1-min period. The most complete response was recorded. Lures were placed 20 cm downwind of the mixing screens and at a height of 25 cm in glass vials near a crumpled white paper towel (flower mimic). The *M. subspinosus* five-component lure was presented at 10 mg/ml in ethanol and *P. japonica* PEG lure was presented at 100 mg/ml in ethanol. We did not determine the sex in *M. subspinosus* trials and to reduce variability only used males in *P. japonica* trials.

Statistical Procedures. Take-off upwind, orientation to the odor plume, and source contact were analyzed first with an ANOVA and then with polynomial contrasts using SYSTAT (Wilkinson et al., 1989). *M. subspinosus* source contact proportions were arcsin ($p^{1/2}$) transformed, and the transformed data met assumptions of homogeneity of variance and normality of errors. *P. japonica* source contact proportions met assumptions of homogeneity of variance and normality of errors without transformation. In both experiments, adults were used only once and chosen from a random section of the holding container.

RESULTS

Light intensity inside the wind tunnel was marginally reduced by the plastic windows, but the light quality (that is, the proportion of radiant energy devoted to each wavelength) was nearly unchanged. Likewise, among the three highest levels of light, the quality did not differ. However, the lowest level (10 μmol photons/sec/m^2) differed both in quality and quantity (Figure 1), since only the incandescent bulbs were used.

In both species, positive photoaxis was more obvious at the lower light levels. When light levels were increased, they reduced their flight toward the

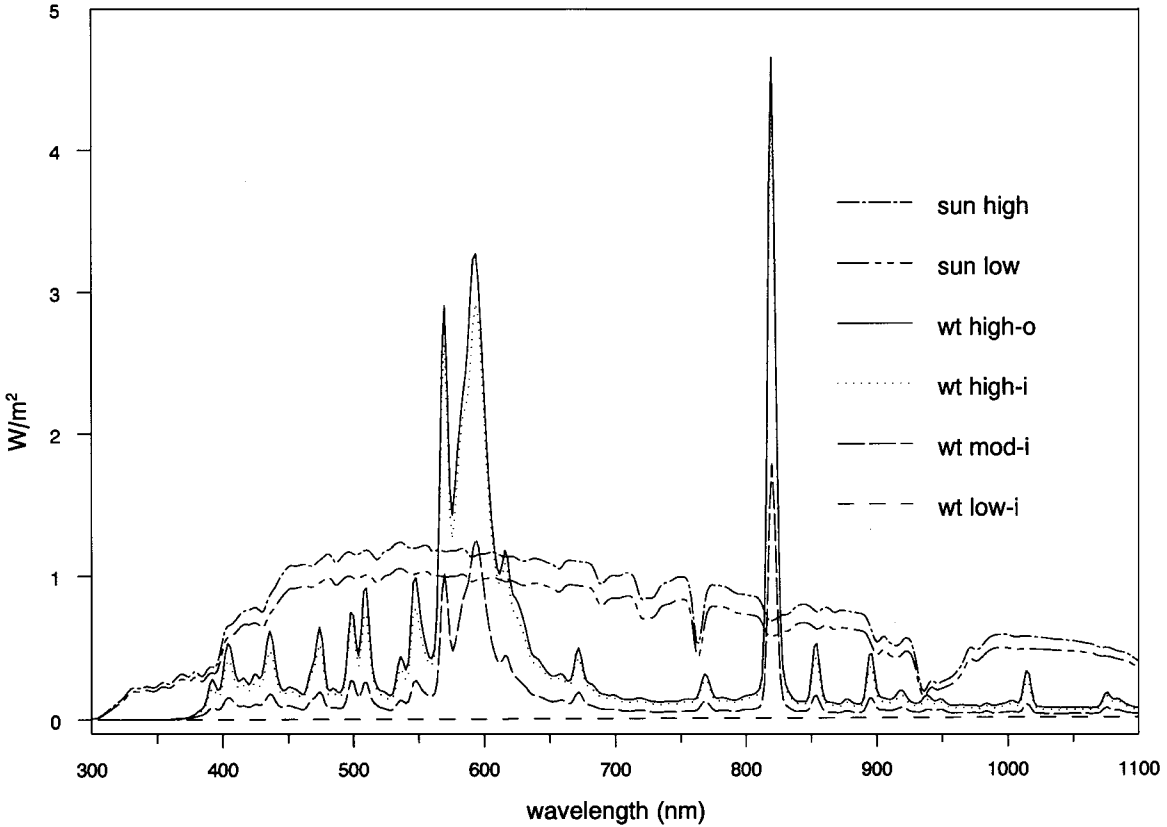


FIG. 1. Spectral quality of light used in the current experiments compared to that of natural light in Peoria, Illinois, in September. "Sun high" and "sun low" are at 12:36 hr and 11:36 hr, respectively; "wt high," "wt mod," and "wt low" in the legend refer to the 1260-, 480- and 10-μmol/sec/m^2 light levels, respectively. The suffixes refer to the location of the spectroradiometer sensor, which was either inside (-i) the wind tunnel or outside (-o) to illustrate the filtering effects of the wind-tunnel windows on light quality and quantity. Note that the "wt low-i" line runs close to the *x*-axis.

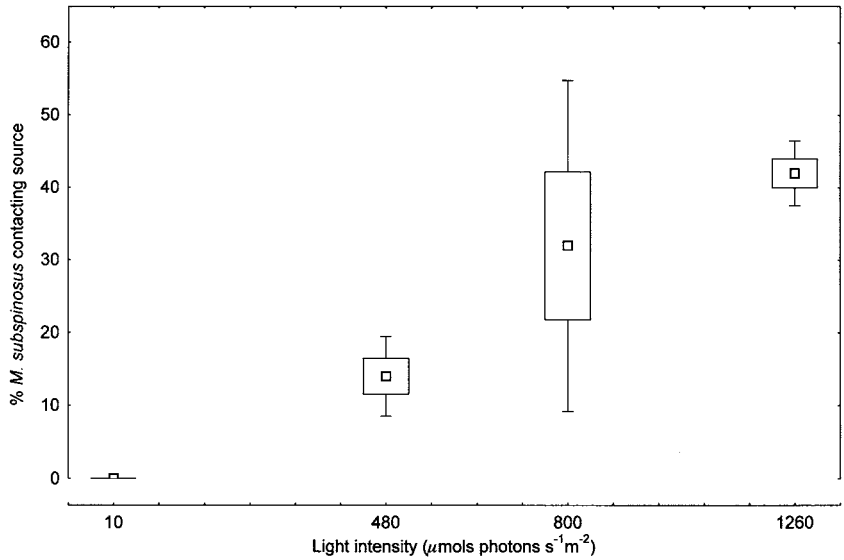


FIG. 2. Percentage of *M. subspinosus* adults contacting the source often ($N = 5$) with respect to light intensity (PAR, 400–700 nm). Mean, ±1 standard error, and ±1 standard deviation are represented by the point, box, and whiskers, respectively.

wind-tunnel ceiling after take-off and began orienting to the odor plume more readily. Details for the take-off-upwind and orientation-to-the-plume responses have been omitted for simplicity, but the profiles were generally very similar to those of the source-contact response analyzed below and presented in Figures 2 and 3.

In trials with *M. subspinosus*, the effect of light intensity on source contact was significant ($F = 13.6$; $df = 3, 12$; $P < 0.001$), but there was no significant block (time) effect. As light intensity increased, the ability of *M. subspinosus* adults to contact the odor source also increased linearly (Figure 2) (first-order polynomial contrast, $F = 39.0$; $df = 1, 12$; $P < 0.001$). Higher order (second and third) polynomial coefficients were not significantly different from zero.

In trials with *P. japonica*, the main effects of blocking ($F = 6.3$; $df = 2, 22$; $P = 0.007$), light intensity ($F = 26.5$; $df = 2, 22$; $P < 0.001$), and temperature ($F = 5.9$; $df = 3, 22$; $P = 0.004$) were all significant. However, there was a significant interaction between light and temperature ($F = 6.5$; $df = 6, 22$; $P < 0.001$, Figure 3). For instance, at 27 and 30°C, the highest light intensity inhibited response (only second-order contrasts were significant; 27°C: $F = 17.1$, $df = 1, 4$, $P = 0.014$; 30°C: $F = 28.0$, $df = 1, 4$, $P = 0.006$); but at 21 and 24°C, the effect of light was positive (only first-order contrasts were significant; 21°C: $F = 8.6$, *df*

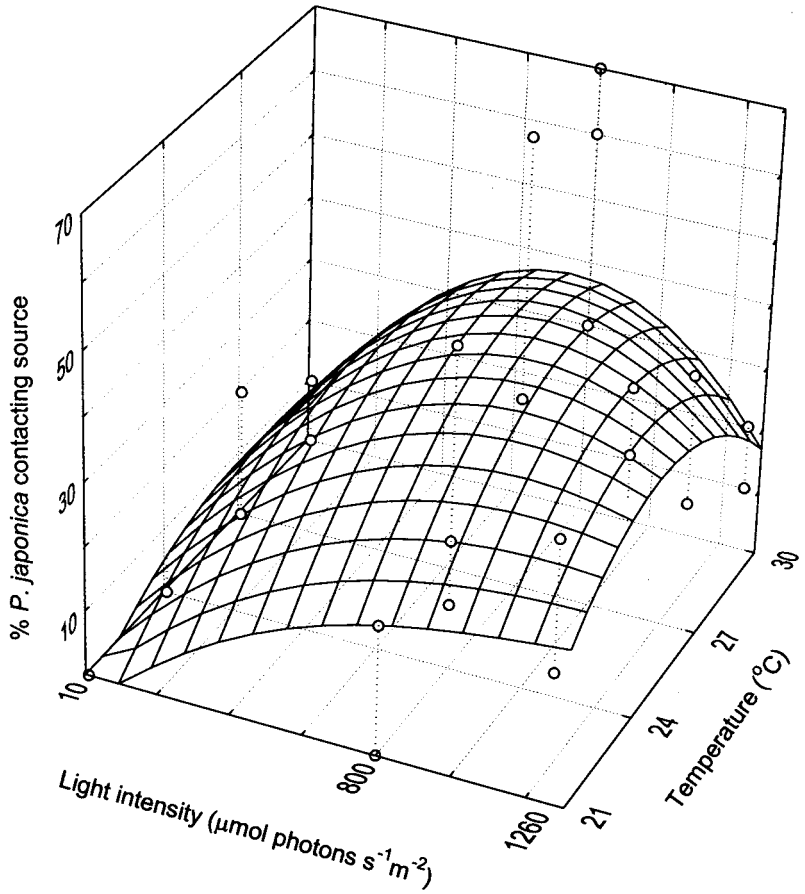


FIG. 3. Response surface and raw data for the percentage of 10 *P. japonica* males contacting the odor source with respect to light intensity (PAR, 400–700 nm) and temperature. Note several points are identical and thus overlapped ($N = 3$).

= 1, 4, $P = 0.043$; 24°C: $F = 17.2$, $df = 1, 4$, $P = 0.014$). Temperature × light polynomial contrasts revealed that increased temperature inhibited response at the highest light level (second-order contrast, $F = 8.7$, $df = 1, 6$, $P = 0.026$; first- and third-order contrasts were not significant). At the second light level, the effect of temperature on source contacts increased linearly (first-order contrast, $F = 22.9$, $df = 1, 6$, $P = 0.003$; higher-order contrasts were not significant). At the first light level, increasing the temperature did not significantly increase the response (all order contrasts were not significant).

DISCUSSION

We submit that identification of diurnal scarab semiochemicals has been slowed by the lack of a discriminating long-range laboratory bioassay. This deficiency is evident in the relatively large percentage of studies conducted under field conditions. These data suggest that the lack of high light intensities in past attempts with wind tunnels may have contributed to the failure to obtain a discriminating response. Lacey et al. (1994) found that *P. japonica* are most active in the field when wind speed is moderate and light intensity and temperature are maximized. Most wind-tunnel studies with beetles have used light intensities <80 μmol photons/sec/m^2 (Visser, 1976; Salom and McLean, 1990; Zhang et al., 1994; Prokopy et al., 1995; Evans and Allen-Williams, 1998) and only one (Domek et al., 1990) has used higher light intensities on scarabs. The light intensities in the current study were 10 times higher than in most studies and the data indicate that this is necessary to obtain a quantifiable response in these scarabs.

In trials with *P. japonica*, the response was inhibited at higher levels of temperature and light (Figure 3). Given that the quantity of stimulus presented was comparable to natural levels, the inhibition is likely due to differences in light quality. Figure 1 shows qualitative differences between sunlight and our artificial lighting with respect to wavelength. Although the total radiation, even at our highest level, was lower than a sunny day in September, the yellow–orange band at ca. 600 nm and the near-infrared band at ca. 825 nm were considerably higher than natural light (Figure 1). These qualitative differences could have their effect in a number of ways, of which spectral sensitivity is the most likely. The spectral sensitivity of *P. japonica* may be such that higher-than-natural levels of yellow–orange light may be inhibitory even when the total radiation is less than would occur naturally. *Liocola brevitarsis* (Scarabaeidae) are sensitive to light below 600 nm (Lin and Wu, 1991); if *P. japonica* possesses similar sensitivities, the high levels at 600 nm may inhibit them. Since beetles were stored in a relatively dark container prior to bioassays, their eyes would have been dark-adapted. This could have increased their sensitivity and thus indirectly decreased their threshold of inhibition.

The evolutionary advantage of flying to plant compounds only when the light levels are high is not obvious. However, Furutani and Arita (1990) have illustrated that total carbohydrate content of leaves is linked to light intensity and to Chinese rose beetle (Scarabaeidae) feeding preference. Furthermore, Wardlaw (1990) indicated that production and translocation of carbohydrates in plants varies diurnally. In this scenario, beetles that respond to plant volatiles when light intensity is high would increase their probability of finding plants high in carbohydrates. Further, if sight is important in recognizing hosts or mates, they may gain visual acuity with additional light. Further studies should consider whether

the effect exists for pheromones, which portion of the electromagnetic spectrum is responsible (i.e., UV, visible light, or infrared), where the modulating radiation is received, where in the nervous system the impulse is integrated to modulate behavior, and what benefit beetles gain from flying only when light levels are high.

These results have implications for conducting laboratory bioassays with other beetle families. Prystupa et al. (1988) conducted wind-tunnel studies with *Diabrotica* spp. in a greenhouse; results were more discriminating those those of an earlier experiment carried out in the laboratory (Dobson and Teal, 1987). However, Guss et al. (1982) report failed orientation experiments with *Diabrotica* in greenhouse studies. Since glass effectively filters out most infrared radiation (Lazzari and Nunez, 1989), it would be interesting to compare the greenhouse window materials in the aforementioned studies to gain insight into the importance of infrared radiation in modulating orientation. Bartelt and Zilkowski (1998) and Phelan and Lin (1991) have had considerable success flying nitidulids in wind tunnels, but the beetles often take several minutes to hours to respond (Zilkowski et al., 1999)—perhaps light is important.

Our finding that high light intensity dramatically increases diurnal scarab response to semiochemicals in a wind-tunnel bioassay should make future scarab semiochemical identifications faster and more cost-effective since workers will have the added advantage of a discriminating laboratory bioassay. It also highlights a potential model system for the study of how encoded olfactory information is modulated by other stimuli in the nervous system.

Acknowledgments—This project would not have been possible without the funding from the Ohio Grape Industry Committee. We thank Michael R. McGuire, USDA, Peoria, Illinois, for providing sunny day data, equipment, and assistance with the spectroradiometer measurements; Michael G. Klein, USDA-ARS, Japanese Beetle Laboratory, Wooster, Ohio, for providing the JB lures and helpful comments; and Jennifer L. Heath for assistance with the bioassays.

REFERENCES

BAKER, T. C., and CARDÉ, R. T. 1984. Techniques for behavioral bioassays, pp. 45–73, *in* H. E. Hummel and T. A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

BAKER, T. C., and LINN, C. E., JR. 1984. Wind tunnels in pheromone research, pp. 75–110, *in* H. E. Hummel and T. A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

BARTELT, R. J., and ZILKOWSKI, B. W. 1998. A versatile and quantitative volatile-delivery system for laboratory bioassays. *J. Chem. Ecol.* 24:535–558.

DOBSON, I. D., and TEAL, P. E. A. 1987. Analysis of long-range reproductive behavior of male *Diabrotic virgifera virgifera* Leconte and *D. barberi* Smith and Lawrence to stereoisomers of 8-methyl-2-decyl propanoate under laboratory conditions. *J. Chem. Ecol.* 13:1331–1341.

DOMEK, J. M., TUMLINSON, J. H., and JOHNSON, D. T. 1990. Response of male green June beetles

Cotinis nitida (L.) (Coleoptera: Scarabaeidae) to female volatiles in a flight tunnel. *J. Insect Behav.* 3:271–276.

EVANS, K. A., and ALLEN-WILLIAMS, L. J. 1998. Response of cabbage seed weevil (*Ceutorhynchus assimilis*) to baits of extracted and synthetic host-plant odor. *J. Chem. Ecol.* 24:2101–2114.

FACUNDO, H. T., ZHANG, A., ROBBINS, P. S., ALM, S. R., LINN, C. E., JR., VILLANI, M. G., and ROELOFS, W. L. 1994. Sex pheromone responses of the Oriental beetle (Coleoptera: Scarabaeidae). *Environ. Entomol.* 23:1508–1515.

FURUTANI, S. C., and ARITA, L. H. 1990. Effect of light exposure and carbohydrate content of snap bean leaves on Chinese rose beetle (Coleoptera: Scarabaeidae) feeding. *J. Econ. Entomol.* 83:2022–2025.

GUSS, P. L., TUMLINSON, J. H., SONNET, P. E., and PROVEAUX, A. T. 1982. Identification of a female-produced sex pheromone of the western corn rootworm *Diabrotica virgifera virgifera. J. Chem. Ecol.* 8:545–556.

KLEIN, M. G., TUMLINSON, J. H., LADD, T. L., JR., DOOLITTLE, R. E. 1981. Japanese beetle *P. japonica* (Coleoptera: Scarabaeidae): response to synthetic sex attractant plus phenethy proprionate: Eugenol. *J. Chem. Ecol.* 7:1–7.

LACEY, L. A., AMARAL, J. J., COUPLAND, J., and KLEIN, M. G. 1994. The influence of climatic factors on the flight activity of the Japanese beetle (Coleoptera: Scarabaeidae): Implications for use of a microbial control agent. *Biol. Control* 4:298–303.

LADD, T. L., and MCGOVERN, T. P. 1980. Japanese beetle *Popillia japonica*: A superior attractant, phenylethyl propionate + eugenol + geraniol, 3 : 7 : 3. *J. Econ. Entomol.* 73:689–691.

LAZZARI, C. R., and NUNEZ, J. A. 1989. The response to radiant heat and the estimation of the temperature of distant sources in *Triatoma infestans. J. Insect Physiol.* 35:525–529.

LEAL, W. S. 1995. Sex pheromones of plant-feeding scarab beetles. *Korean J. Appl. Entomol.* 34:9–14.

LEAL, W. S. 1998. Chemical ecology of phytophagous scarab beetles. *Annu. Rev. Entomol.* 43:39–61.

LIN, J. T., and WU, C. Y. 1991. A comparative study on the color vision of four coleopteran insects. *Bull. Inst. Zool., Acad. Sin.* 31:81–88.

MILLER, J. R. and ROELOFS, W. L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

PHELAN, P. L., and LIN, H. 1991. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:1253–1272.

PROKOPY, R. J., COOLEY, S. S., and PHELAN, P. L. 1995. Bioassay approaches to assessing behavioral responses of plum curculio adults (Coleoptera: Curculionidae) to host fruit odor. *J. Chem. Ecol.* 21:1073–1084.

PRYSTUPA, B., ELLIS, C. R., and TEAL, P. E. A. 1988. Attraction of adult *Diabrotica* (Coleoptera: Chrysomelidae) to corn silks and analysis of the host-finding response. *J. Chem. Ecol.* 14:635–651.

SALOM, S. M., and MCLEAN, J. A. 1990. Flight and landing behavior of *Trypodendron lineatum* (Coleoptera: Scolytidae) in response to different semiochemicals. *J. Chem. Ecol.* 16:2589–2604.

TUMLINSON, J. H., KLEIN, M. G., DOOLITTLE, R. E., LADD, T. L., and PROVEAUX, A. T. 1977. Identification of the female Japanese beetle sex pheromone: Inhibition of male response by an enantiomer. *Science* 197:789–792.

VISSER, J. H. 1976. The design of a low-speed wind tunnel as an instrument for the study of olfactory orientation in the Colorado beetle (*Leptinotarsa decemlineata*). *Entomol. Exp. Appl.* 20:275–288.

VITTUM, P. J., VILLANI, M. G., and TASHIRO, H. 1999. Turfgrass insects of the United States and Canada. Comstock Pub. Associates, Ithaca, New York.

WARDLAW, I. F. 1990. Tansley review no. 27. The control of carbon partitioning in plants. *New Phytol.* 116:341–381.

WILKINSON, L. 1989. SYSTAT: The system for statistics. SYSTAT, Evanston, Illinois.

WILLIAMS, R. N., MCGOVERN, T. P., KLEIN, M. G., and FICKLE, D. S. 1990. Rose chafer (Coleoptera Scarabaeidae) improved attractants for adults. *J. Econ. Entomol.* 83:111–116.

WILLIAMS, R. N., FICKLE, D. S., and MCGOVERN, T. P. 1993. Attractants for the rose chafer, *Macrodactylus subspinosus* (F.), containing alpha-ionone. United States Department of Agriculture, Patent 5,202,124; April 13.

ZHANG, A., FACUNDO, H. T., ROBBINS, P. S., LINN, C. E., JR., HANULA, J. L., VILLANI, M. G., and ROELOFS, W. L. 1994. Identification and synthesis of female sex pheromone of Oriental beetle, *Anomala orientalis* (Coleoptera: Scarabaeidae). *J. Chem. Ecol.* 20:2415–2427.

ZILKOWSKI, B. W., BARTELT, R. J., BLUMBERG, D., JAMES, D. G., and WEAVER, D. K. 1999. Identification of host-related volatiles attractive to pineapple beetle *Capophilus humeralis. J. Chem. Ecol.* 25:229–252.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

DOES PHEROMONE BIOLOGY OF *Lambdina athasaria* AND *L. pellucidaria* CONTRIBUTE TO THEIR REPRODUCTIVE ISOLATION?

CAMERON M. DUFF,[1,6] GERHARD GRIES,[1,*] KENJI MORI,[2] YASUO SHIRAI,[2] MASANORI SEKI,[2] HIROSATO TAKIKAWA,[2] TAO SHENG,[3] KEITH N. SLESSOR,[3] REGINE GRIES,[1] CHRIS T. MAIER,[4] and DOUGLAS C. FERGUSON[5]

[1]*Center for Environmental Biology, Department of Biological Sciences*
[3]*Department of Chemistry*
Simon Fraser University
Burnaby, British Columbia V5A 1S6 Canada

[2]*Department of Chemistry, Faculty of Science*
Science University of Tokyo
Kagurazaka 1-3, Shinjuku-ku
Tokyo 162-8601, Japan

[4]*Department of Entomology*
The Connecticut Agricultural Experiment Station
New Haven, Connecticut, 06504

[5]*Systematic Entomology Laboratory*
Agricultural Research Service, USDA
c/o National Museum of Natural History
Washington, DC, 20560-0168

(Received March 8, 2000; accepted November 7, 2000)

Abstract—Recently, 7-methylheptadecane and 7,11-dimethylheptadecane have been reported as sex pheromone components of both spring hemlock looper (SHL), *Lambdina athasaria*, and pitch pine looper (PPL), *Lambdina pellucidaria*. Our objective was to test the hypothesis that SHL and PPL are reproductively isolated, in part, through species specificity in: (1) absolute configuration of pheromone components, (2) diel periodicity of pheromonal communication, and/or (3) seasonal flight period. In coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of stereoselectively synthesized (7*S*)- and (7*R*)-7-methylheptadecane [7*S*; 7*R*]

* To whom correspondence should be addressed.
[6] Current address: Canadian Food Inspection Agency, Plant Protection Division, Nepean, Ontario K1A DY9, Canada.

0098-0331/00/0300-0431$19.50/0

as well as (7*S*,11*S*)-, (7*R*,11*R*)-, and (*meso*-7,11)-7,11-dimethylheptadecane [7*S*,11*S*; 7*R*,11*R*; *meso*-7,11], only 7*S* and *meso*-7,11 elicited responses by male SHL and PPL antennae. In field experiments, male SHL and PPL were attracted only to lures containing 7*S* plus *meso*-7,11. In hourly recordings of trap-captured males, SHL and PPL in their respective habitats were trapped between 24:00 and 03:00 hr. Capture of both SHL and PPL in pheromone-baited traps throughout June indicated overlapping seasonal flight periods. These findings of identical absolute configuration of pheromoal components, diel periodicity of pheromonal communication, and overlap of seasonal flight periods support synonymy of SHL and PPL. Finite taxonomic classification of PPL and SHL must await careful assessment of further criteria, such as morphometrics, molecular comparisons and ecological analyses.

Key Words—Geometridae, 7-methylheptadecane, 7,11-dimethylheptadecane, pheromone chirality, diel periodicity of pheromonal communication, seasonality of flight, reproductive isolating mechanisms, synonomy.

INTRODUCTION

The spring hemlock looper (SHL), *Lambdina athasaria*[7] (Walker), and pitch pine looper (PPL), *L. pellucidaria* (Grote and Robinson), have remarkably similar life histories (summarized in Duff, 1998). Although they prefer hemlock (SHL) and pine (PPL) forests, they are neither spatially nor temporally completely isolated. Both SHL and PPL overwinter as pupae in the forest duff. Adults begin to emerge in mid to late May and are present until the end of June or early July. With identical sex pheromone blends (7-methylheptadecane and 7,11-dimethylheptadecane) identified in both species (Gries et al., 1994; Maier et al., 1998), alternative mechanisms of reproductive isolation needed to be investigated. Here we tested the hypotheses that PPL and SHL are reproductively isolated, in part, through species specificity in: (1) absolute configuration of pheromone components; (2) diel periodicity of pheromonal communication; and/or (3) seasonal flight period.

METHODS AND MATERIALS

Syntheses. (7*S*)-7-Methylheptadecane, (7*R*)-7-methylheptadecane, (7*S*,11*S*)-7,11-dimehylheptadecane, (7*R*,11*R*)-7,11-dimethylheptadecane and (*meso*-7,11)-7,11-dimethylheptadecane, hereafter referred to as 7*S*, 7*R*, 7*S*,11*S*, 7*R*,11*R*, and *meso*-7,11 (Figure 1), were synthesized as previously described (Sheng,

[7] Univoltine *L. athasaria* investigated in this study is not to be confused with the bivoltine oak feeder, *L. fervidaria athasaria* (Walker), which is illustrated by Covell (1984). Larvae of *L. athasaria* considered here are restricted to eastern hemlock.

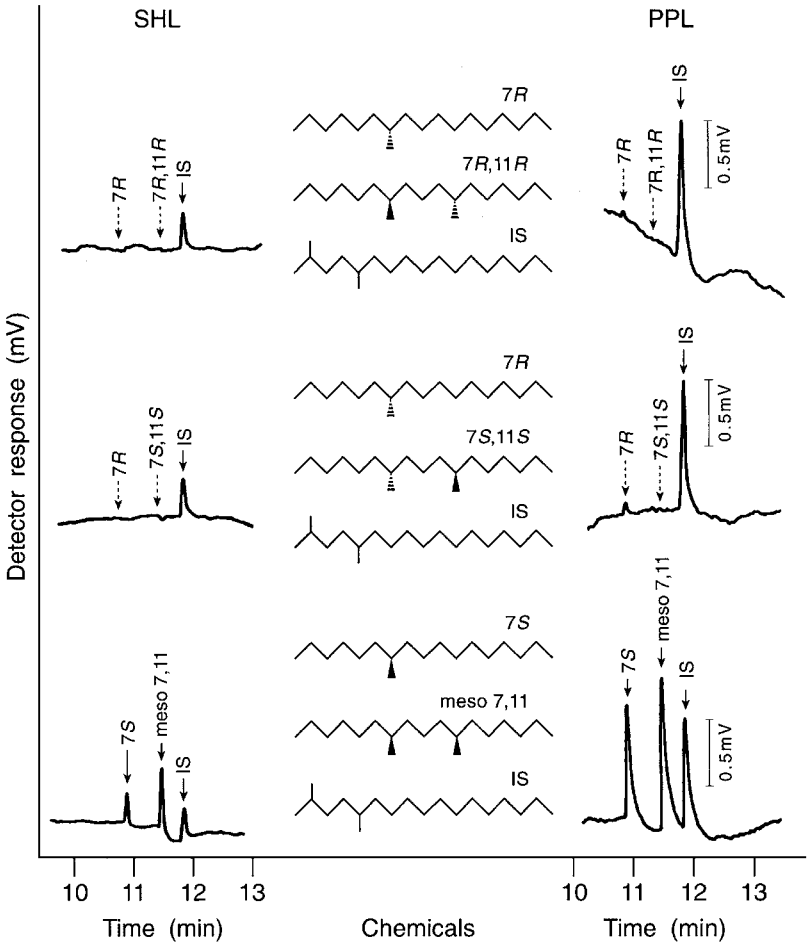


FIG. 1. Representative GC-EAD recordings from male spring hemlock looper (SHL) and from male pitch pine looper (PPL) antennae, responding to 1000 pg of 2,5-dimethylheptadecane [internal standard (IS)] and to 2 pg of optical isomers of pheromone components. Antennal recordings conducted in consecutive order (top to bottom), employing the same antenna. Flame ionization detector (FID) recordings omitted. Compound nomenclature: 7*R* = (7*R*)-7-methylheptadecane; 7*S* = (7*S*)-7-methylheptadecane; 7*R*,11*R* = (7*R*,11*R*)-7,11-dimethylheptadecane; 7*S*,11*S* = (7*S*,11*S*)-7,11-dimethylheptadecane; meso 7,11 = (*meso*-7,11)-7,11-dimethylheptadecane. Chromatography: Hewlett Packard 5890 equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-5 (J&W Scientific, Folsom, California); splitless injection, temperature of injection port and FID detector: 250°C; temperature program: 120° for 1 min, then 10°C/min to 280°C.

1996; Shirai, 1997; Shirai et al., 1999). Based on gas chromatographic analyses, all compounds used in field experiments were >94% chemically pure. Compounds were 99–100% enantiomerically pure as estimated by the enantiomeric excess (99.8–100%) of the starting material (enantiomers of methyl 3-hydroxy-2-methyl propanoate).

Laboratory Analyses. Adult male SHL and PPL were collected at night in Peoples State Forest, Barkhamsted, Litchfield County, Connecticut, and Myles Standish State Forest, Plymouth, Plymouth County, Massachusetts, respectively. Moths were cooled (8°C) overnight and couriered to Simon Fraser University the next day for coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975). Each antenna tested (four and five for male SHL and PPL, respectively) was subjected in the same order to three mixtures of candidate pheromone components at 2 pg each. Mixtures 1–3 contained 7*R* plus 7*R*,11*R*, 7*R* plus 7*S*,11*S*, and 7*S* plus *meso*-7,11, respectively. An internal standard, 1 ng of 2,5-dimethylheptadecane, was coinjected with each mixture, to monitor possible deterioration of antennal responses over the 30–40 min test period for each antenna.

Experimental Sites. Experiments with SHL and PPL were conducted at Peoples State Forest and Myles Standish State Forest, respectively. In Peoples State Forest 120 km inland, the transition-zone forest of gymno- and angiosperm trees forms a dense canopy with hemlock, *Tsuga canadensis* (L.) Carr., as the dominant species. The forest lies at 300 m in elevation and receives 306 mm of rain from June to August (Owenby and Ezell, 1992). The pine forest at Myles Standish State Forest exhibits an open forest canopy with pitch pine, *Pinus rigida* Miller, as the dominant species. Directly on the Atlantic coast, the pine forest experiences a moderate, ocean-influenced climate, with little summer rain and prolonged periods of drought (Schweitzer and Rawinski, 1988). More detailed characteristics about experimental sites and their locations are provided in Duff (1998).

General Design of Field Experiments. Experiments of identical or compatible design, conducted concurrently for both SHL and PPL (except experiment 7), were set up in randomized, complete blocks with traps and blocks at 15- to 20-m intervals. Green Unitraps (Phero Tech Inc., Delta, British Columbia, Canada) were suspended from *T. canadensis* (SHL) and *P. rigida* (PPL) at 1.5–2 m above ground and 2–5 m within the forest margin. Traps were baited with grey rubber septa (The West Company, Lionville, Pennsylvania) impregnated with stereoisomers of synthetic pheromone components in HPLC-grade hexane. Each trap contained a dichlorvos cube (Bio-Strip, Inc., Reno, Nevada) to kill captured males. Moths were removed and counted every two days.

Field Test of Optical Isomers of Pheromone Components. Experiment 1 (SHL) and experiment 2 (PPL) tested stereoisomeric 7,11 alone and in combination with either 7*S*, 7*R* or both. The 7*S* (experiment 3, SHL) or 7*S* plus 7*R*

(experiment 4, PPL) were tested alone and in binary and quaternary combinations with 7*S*,11*S*, 7*R*,11*R*, and *meso*-7,11. Experiment 5 (SHL) tested 7*S* alone and in binary combination with *meso*-7,11 and all possible ternary and quaternary combinations with 7*S*,11*S*, 7*R*,11*R*, and *meso*-7,11. Experiment 6 (PPL) was identical to experiment 5, but replaced 7*S* with 7*S* + 7*R*. Experiment 7 was conducted only with PPL and tested 7*S* plus *meso*-7,11 alone and in combination with 7*R* at three different ratios. Experiment 8 (SHL) and experiment 9 (PPL) tested 7*S* alone and in combination with *meso*-7,11 at three different ratios. Experiment 10 (SHL) and experiment 11 (PPL) tested *meso*-7,11 alone and in combination with 7*S* at three different ratios. Experiment 12 (SHL) and experiment 13 (PPL) tested 7*S* plus *meso*-7,11 at increasing doses.

Height-Dependent Trap Captures of Male SHL and PPL. For experiment 14 and 15, ten trees >200 m apart were selected at each experimental site. Unitraps baited with grey rubber septa impregnated with 50 μg 7*S* and 16 μg *meso*-7,11 were hung at 1.5, 3, and 5 m above ground in each tree. Captured male SHL (experiment 14) or PPL (experiment 15) were recorded two days after experiment initiation.

Diel Periodicity and Seasonality of Trap Captures. Experiments 16 and 17 recorded the diel periodicity of captures of male SHL and PPL, respectively. Ten trees >100 m apart were selected at each experimental site. One Unitrap baited with grey rubber septa impregnated with 50 μg 7*S* and 16 μg *meso*-7,11 was hung 1.5 m above ground and 2–5 m within the forest margin in each tree. Captured males were recorded hourly for 24 hr. Experiments 18 and 19 recorded seasonal flight periods of male SHL and PPL, respectively. Selection of trees and trap placement were the same as in experiments 16 and 17. Traps were baited with grey rubber septa impregnated with 50 μg racemic 7 and 16 μg stereoisomeric 7, 11. Captured males were removed and counted every two days from May 28, to June 24, 1997.

Statistical Analyses. Despite transformation, most trap catch data were not normally distributed and were therefore subjected to nonparametric analyses of variance by ranks (Friedman's test) followed by comparison of means (Bonferroni test) (Zar, 1984; SAS/STAT 1988 user guide, release 6.03 edition, SAS Institute, Cary, North Carolina).

RESULTS

In GC-EAD recordings, 7*S*, but not 7*R*, and *meso*-7,11, but not 7*S*,11*S* or 7*R*,11*R*, elicited responses from male SHL and PPL antennae (Figure 1). In field experiments 1 and 2, 7*S* in combination with stereoisomeric 7,11 attracted male SHL and PPL (Figure 2). For both species, 7*R* was inactive with 7,11, but doubled captures of male PPL when added to 7*S* plus 7,11 (Figure 2, experiment 2). For

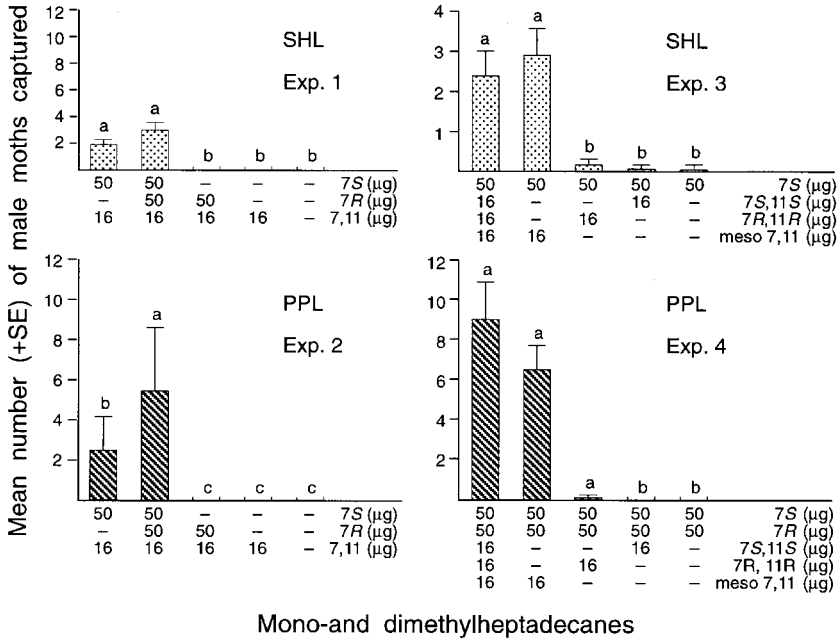


FIG. 2. Left: Captures of male spring hemlock loopers (SHL) (experiment 1; June 10–12, 1997; 10 replicates) and pitch pine loopers (PPL) (experiment 2; May 30–June 3, 1997; 10 replicates) in Unitraps baited with stereoisomeric dimethylheptadecane alone and in combination with 7*S*, 7*R*, or both. Right: Captures of male SHL (experiment 3; June 12–14, 1997; 10 replicates) and male PPL (experiment 4; June 3–5, 1997; 10 replicates) in Unitraps baited with 7*S* alone (SHL) or 7*S* plus 7*R* alone (PPL), and in binary and quaternary combinations with 7*S*,11*S*, 7*R*,11*R*, and *meso*-7,11. For each experiment, bars with the same letter are not significantly different, $P > 0.05$. Compound nomenclature as in Figure 1 legend. Note that when apparent behavioral activity of 7*R* in experiment 2 was retested in a subsequent experiment, it was inactive (Duff, 1998).

both SHL and PPL, the synergistic stereoisomer of 7,11 proved to be *meso*-7,11 (Figure 2, experiments 3, 4; Figure 3, experiments 5, 6); 7*S*,11*S* and 7*R*,11*R* were benign. Retesting 7*R* as a potential pheromone component for PPL in experiment 7 failed to disclose any behavioral activity associated with this compound (Duff, 1998). The blend of 7*S* plus *meso*-7,11 at a 50 : 16-µg ratio was more attractive to male SHL and PPL than blend ratios with reduced amounts of either *meso*-7,11 (Figure 4, experiments 8 and 9) or 7*S* (Figure 4, experiments 10 and 11). When 7*S* plus *meso*-7,11 were tested at five doses separated by orders of magnitude, only traps baited with the highest dose captured significant numbers of males (Figure 5, experiments 12 and 13).

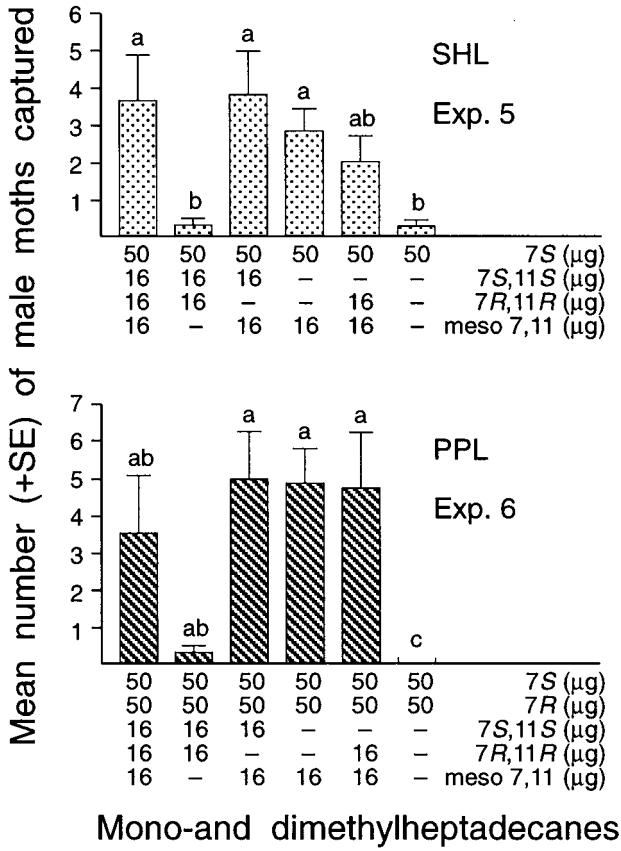


FIG. 3. Captures of male spring hemlock loopers (SHL) (experiment 5; June 14–16, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 6; June 5–7, 1997; 10 replicates) in Unitraps baited with 7*S* alone (SHL) or 7*S* plus 7*R* alone (PPL), in binary combination with *meso*-7,11), and in all ternary and quaternary combinations with 7*S*,11*S*, 7*R*,11*R*, and *meso*-7,11. For each experiment, bars with the same letter are not significantly different, $P > 0.05$. Compound nomenclature as in Figure 1 legend.

Traps suspended at 1.5 m above ground captured significantly fewer male SHL than those at 5 m (Figure 6, experiment 14) and significantly fewer male PPL than those at 3 and 5 m (Figure 6, experiment 15). In hourly recordings of captured males, seven SHL and five PPL were captured between 24 : 00 hr and 0300 hr (Figure 6, experiments 16 and 17). The seasonal flight periods of SHL and PPL overlapped (Figure 6, experiments 18 and 19), but few SHL were captured until mid-June.

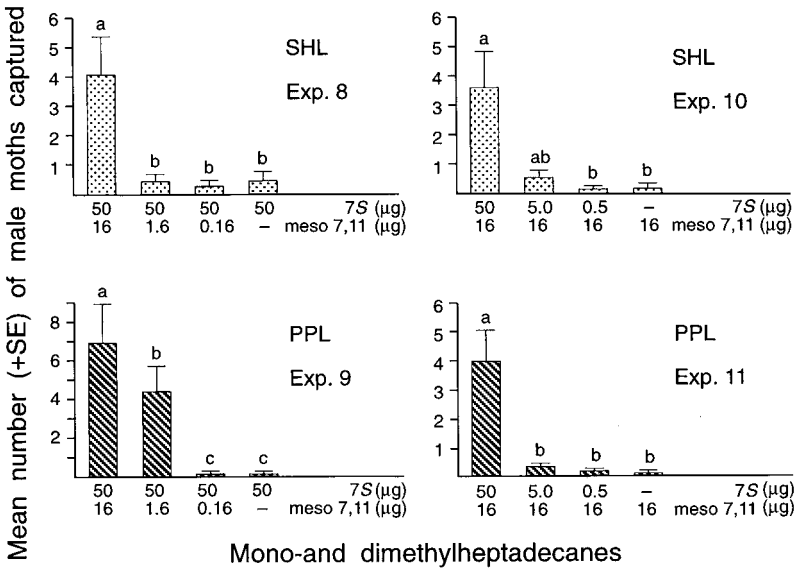


FIG. 4. Left: Captures of male spring hemlock loopers (SHL) (experiment 8; June 16–18, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 9; June 11–13, 1997; 10 replicates) in Unitraps baited with 7*S* alone and in combination with (*meso*-7,11) at three different ratios. Right: Captures of male SHL (experiment 10; June 18–20, 1997; 10 replicates) and male PPL (experiment 11; June 13–15, 1997; 10 replicates) in Unitraps baited with *meso*-7,11 alone and in combination with 7*S* at three different ratios. For each experiment, bars with the same letter superscript are not significantly different, $P > 0.05$. Compound nomenclature as in Figure 1 legend.

DISCUSSION

Electrophysiological recordings (Figure 1) and field experiments (Figures 2–6) indicate that SHL and PPL lack specificity of their pheromone blends. Antennae from males of both species discriminated between optical isomers of pheromone components (mono- and dimethylheptadecanes), but invariably responded only to 7*S* and *meso*-7,11 (Figure 1). Attraction of male SHL and male PPL only to lures containing 7*S* + *meso*-7,11 (Figures 2–4) confirms enantio- and stereospecific recognition of pheromone components, and demonstrates that SHL and PPL use chiral pheromone components of identical absolute configuration. Because 7*R*, 7*S*,11*S*, or 7*R*,11*R* elicited neither antennal (Figure 1) nor behavioral responses (Figures 2–4), they can not be employed by female SHL or PPL to inhibit or reduce cross-attraction of heterospecific males. Moreover, different blend ratios of 7*S* + *meso*-7,11 had similar effects on captures of male

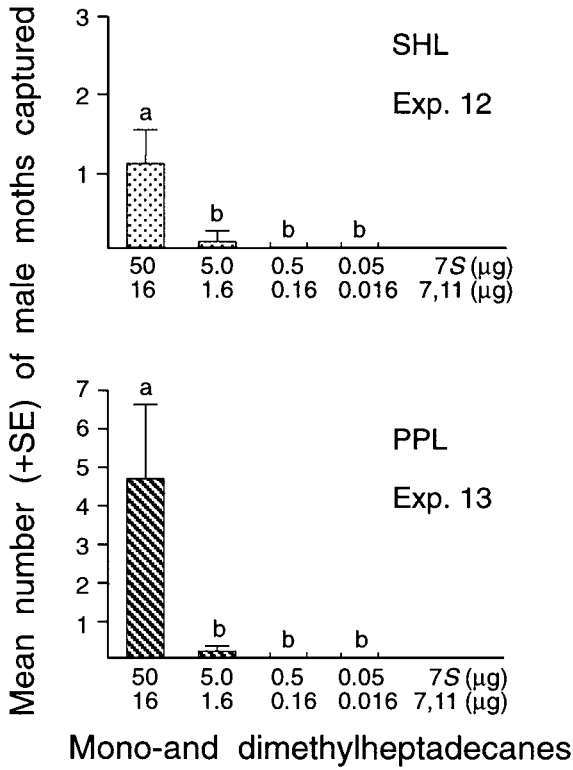


FIG. 5. Captures of male spring hemlock loopers (SHL) (experiment 12; June 20–22, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 13; June 15–17, 1997; 10 replicates) in Unitraps baited with 7*S* + *meso*-7,11 at four different doses. For each experiment, bars with the same letter are not significantly different, $P > 0.05$. Compound nomenclature as in Figure 1 legend.

SHL and PPL (Figure 4, experiments 8–11), eliminating blend ratios as a means of pheromonal specificity.

Enantiospecific perception of pheromone components by males implies enantioselective biosyntheses by females. Confirmation that female SHL and PPL stereoselectively produce 7*S* and *meso*-7,11, however, must await development of a chiral GC column that separates optical isomers of methyl- and dimethylheptadecane.

With identical sex pheromones, SHL and PPL could maintain reproductive isolation through temporal (diel and/or seasonal) separation of sexual communication. Sympatric and coseasonal artichoke plume moths, *Platyptilia carduidactyla* (Riley) and *P. williamsii* (Grinnell), for example, use identical sex

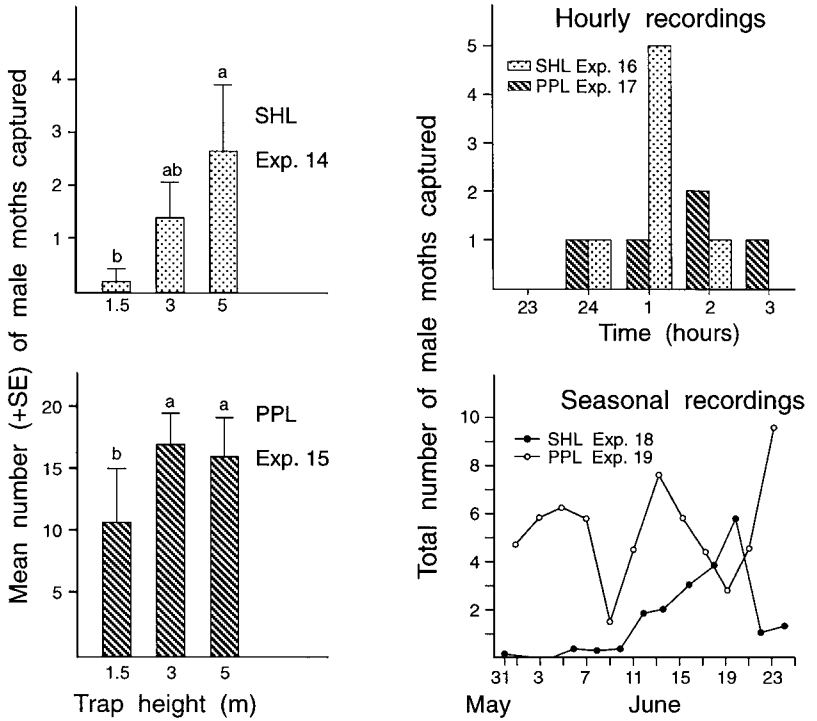


FIG. 6. Left: Captures of male spring hemlock loopers (SHL) (experiment 14; June 19–22, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 15; June 17–21, 1997; 10 replicates) in Unitraps suspended from trees at 1.5, 3, and 5 m above ground and baited with 50 μg 7*S* plus 16 μg *meso*-7,11. For each experiment, bars with the same letter are not significantly different, $P > 0.05$. Top right: Hourly recordings of trap captured male SHL (experiment 16; June 19–22, 1997; 10 replicates) and male PPL (experiment 17; June 17–18, 1997; 10 replicates) in Unitraps baited with 50 μg 7*S* plus 16 μg *meso*-7,11. Bottom right: Seasonal recordings of trap captured male SHL (experiment 18; May 31–June 26, 1997; 10 replicates) and male PPL (experiment 19; May 31–June 26, 1997; 10 replicates) in Unitraps baited with enantiomeric 50 μg 7 and stereoisomeric 50 μg 7,11. Compound nomenclature as in Figure 1 legend.

pheromones but release them at different times of the night (Haynes and Birch, 1986). Diel periods of pheromone communication in SHL and PPL, in contrast, seem to overlap (Figure 6). Even though the seasonal flight period of SHL may commence ~7–10 days earlier than that of PPL (Maier and Lemmon, 1996), capture of male SHLs and PPLs in pheromone-baited traps throughout June (Figure 6) eliminates seasonality of flight as a reproductive isolating mechanism.

Spatial separation (between or within habitats) of sexual communication represents a third potential isolating mechanism. The SHL occurs in moist areas within forests of eastern hemlock. The PPL, in contrast, typically inhabits dry sandy areas with mainly pitch and other hard pines. While SHL and PPL are mostly allopatric in eastern North America (Maier and Lemmon, 1996), their habitats are not far apart and areas of sympatry may exist. Captures of male SHL and PPL mainly in traps suspended at 3 and 5 m (Figure 6) suggest that females of both species call in similar locations within the forest canopy, and that spatial separation of sexual communication would fail in coinhabited forests.

In summary, our study did not reveal discernible differences in the pheromone biology of PPL and SHL, supporting the contention that they should be synonymized (Zhang, 1994). Finite taxonomic classification of PPL and SHL, however, must await careful assessment of other criteria (Roelofs and Brown, 1982; Roelofs and Comeau, 1969), such as morphometrics, molecular comparisons, and ecological analyses.

Acknowledgments—We thank Gale Ridge O'Connor (Connecticut Agricultural Experiment Station, New Haven, Connecticut) for assistance in locating field sites and coordinating field experiments; Austin Mason and the staff at Myles Standish State Forest (Plymouth, Massachusetts) for permission to use their forest as experimental site; Paul Froese and Al Oliver (formerly of Agriculture and Agri-Food Canada) for allowing C.M.D. to carry out his work with the Plant Protection Division of Agriculture and Agri-Food Canada (now the Canadian Food Inspection Agency) in a reduced capacity; Dale Schweitzer and John H. Borden for review of the manuscript.

This research was supported, in part, by a Grant-in-Aid for Scientific Research to K.M. by the Japanese Ministry of Education, Science, Sports and Culture, by Agriculture and Agri-Food Canada, and by research grants from the Natural Sciences and Engineering Research Council of Canada to G.G. and to K.N.S.

REFERENCES

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c:722–725.

COVELL, C. V., JR. 1984. A field guide to the moths of eastern North America. Petersen Field Guide Series. Houghton Mifflin, Boston.

DUFF, C. M. 1998. Does pheromone biology of spring hemlock looper, *Lamdina athasaria*, and pitch pine looper, *Lambdina pellucidaria* (Lepidoptera: Geometridae) contribute to their reproductive isolation? Master's thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

GRIES, R., GRIES, G., LI, J., MAIER, C. T., LEMMON, C. R., and SLESSOR, K. N. 1994. Sex pheromone components of the spring hemlock looper, *Lambdina athasaria* (Walker) (Lepidoptera: Geometridae). *J. Chem. Ecol.* 20:2501–2511.

HAYNES, K. F., and BIRCH, M. C. 1986. Temporal reproductive isolation between two species of plume moths (Lepidoptera: Pterophoridae). *Ann. Entomol. Soc. Am.* 79:210–215.

MAIER, C. T., and LEMMON, C. R. 1996. Life cycle of *Lambdina athasaria* (Walker) (Lepidoptera: Geometridae) developing on hemlock in Connecticut. *Can. Entomol.* 128:995–1003.

MAIER, C. T., GRIES, R., and GRIES, G. 1998. Sex pheromone components of the pitch pine looper,

Lambdina pellucidaria (Grote and Robinson) (Lepidoptera: Geometridae). *J. Chem. Ecol.* 24:491–500.

OWENBY, J. R., and EZELL, D. S. 1992. Monthly station normals of temperature, precipitation, heating and cooling degree days 1961–90 Massachusetts, Connecticut. US Department of Commerce, National Oceanic and Atmospheric Administration National Climactic Center.

ROELOFS, W. L., and BROWN, R. L. 1982. Pheromone and evolutionary relationships of Tortricidae. *Annu. Rev. Ecol. Syst.* 12:395–422.

ROELOFS, W. L., and COMEAU, A. 1969. Sex pheromone specificity: Taxonomic and evolutionary aspects in Lepidoptera. *Science* 217:657–659.

SHENG, T. 1996. Synthesis of the pheromone components of the milkweed bug, *Oncopeltus fasciatus* and the spring hemlock looper, *Lambdina athasaria*. MSc thesis. Simon Fraser University, Burnaby, British Columbia, Canada.

SHIRAI, Y. 1997. Synthesis of optically active alkanes as insect pheromones and optical resolution of 2,6-dimethylheptane-1,7-diol. MSc thesis. Science University of Tokyo, Tokyo.

SHIRAI, S., SEKI, M., and MORI, K. 1999. Pheromone synthesis, CXCIX: Synthesis of all the stereoisomers of 7-methylheptadecane and 7,11-dimethylheptadecane, the female sex pheromone components of the spring hemlock looper and pitch pine looper. *Eur. J. Org. Chem.* 1999:3139–3145.

SCHWEITZER, D. F., and RAWINSKI, T. J. 1988. Northeastern pitch pine/scrub oak barrens, Eastern Heritage Task Force, The Nature Conservancy, 294 Washington, St., Boston, Massachusetts, 21 pp.

ZHANG, B.-C. 1994. Index of economically important Lepidoptera. CAB International, Wallingford, UK.

ZAR, J. H. 1984. Biostatistical Analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

PUTATIVE STEROIDAL PHEROMONES IN THE ROUND GOBY, *Neogobius melanostomus*: OLFACTORY AND BEHAVIORAL RESPONSES

CHERYL A. MURPHY,[1,*] NORM E. STACEY,[1] and LYNDA D. CORKUM[2]

[1]*Department of Biological Sciences*
University of Alberta
Edmonton, Alberta T6G 2E9, Canada

[2]*Department of Biological Sciences*
University of Windsor
Windsor, Ontario, N9B 3P4, Canada

(Received July 7, 2000; accepted November 14, 2000)

Abstract—To identify potential hormonal pheromones of the round goby (*Neogobius melanostomus*), a species recently introduced to the Great Lakes, we used electro-olfactogram (EOG) recording to examine olfactory responsiveness to more than 100 steroids and prostaglandins. *Neogobius* detected free and conjugated 18-, 19- and 21-carbon steroids, but did not detect prostaglandins. EOG cross-adaptation, used to determine if *Neogobius* can discriminate the detected compounds at the sensory level, suggested that the detected steroids act on four classes of olfactory receptor mechanisms named (according to the most potent ligand for each): estrone, 17β-estradiol-3β-glucuronide, etiocholanolone, and dehydroepiandrosterone-3-sulfate. Although none of the detected steroids induced reproductive behaviors, exposure to steroids from three of the four receptor classes (estrone, 17β-estradiol-3β-glucuronide, or etiocholanolone) increased ventilation rate in males, whereas only etiocholanolone increased ventilation rate in females. Using the ventilation increase as a behavioral bioassay of steroid detection, behavioral cross-adaptation studies in males demonstrated that steroids discriminated at the sensory level are also discriminated behaviorally. These findings suggest the round goby may use steroids as putative pheromones.

Key Words—Round goby, *Neogobius melanostomus*, sex pheromones, steroid, electro-olfactogram, cross-adaptation, ventilation, sexual dimorphism.

* To whom correspondence should be addressed at current address: Department of Oceanography and Coastal Sciences, Coastal Fisheries Institute, Wetlands Resources Building, Louisiana State University, Baton Rouge, Louisiana 70803.

0098-0331/00/0300-0443$19.50/0

INTRODUCTION

Water-borne steroids, prostaglandins, and their metabolites are potent stimulators of reproductive physiology and behavior in a number of fish species (Sorensen and Caprio, 1998; Sorensen and Stacey, 1999). Moreover, electro-olfactogram (EOG) recording studies (e.g., Sorensen et al., 1995), which seek evidence for hormonal pheromones by examining olfactory response to numerous steroid and prostaglandin compounds, indicate that these fish pheromones are widespread among freshwater fishes and are present in some euryhaline marine forms (Stacey and Cardwell, 1995, 1997). The majority of studies on fish hormonal pheromones have focused on ostariophysin species from the orders Cypriniformes, e.g., goldfish, *Carassius auratus* (Sorensen and Stacey, 1999); common carp, *Cyprinus carpio* (Irvine and Sorensen, 1993; Stacey et al., 1994a); tinfoil barb, *Puntius schwanenfeldi* (Cardwell et al., 1995); loach, *Misgurnus anguillicaudatus* (Kitamura et al., 1994), and Siluriformes, e.g., African catfish, *Clarias gariepinus* (van den Hurk and Resink, 1992), but there also is evidence for hormonal pheromones in several Salmoniformes, e.g., Atlantic salmon, *Salmo salar* (Waring and Moore, 1997); arctic char, *Salvelinus alpinus* (Sveinsson and Hara, 1995); brook trout, *Salvelinus fontinalis*, and brown trout, *Salmo trutta* (Essington and Sorensen, 1996). In contrast, with the exception of the pioneering work of Colombo et al. (1977, 1982) in the black goby (*Gobius jozo*), reports of pheromonal androgens in the urine of male yellowfin Baikal sculpins (*Cottocomephorus grewingki*) (Katsel et al., 1992), a brief description of EOG responsiveness to steroids in a cichlid (*Haplochromis burtoni*) (Robison et al., 1998), and indications that preovulatory Eurasian ruffe (*Gymnocephalus cernuus*) release a novel conjugated steroid with behavioral actions on males (Murphy et al., 2000), nothing is known of hormonal pheromone systems in perciform fishes, a diverse and ecologically important group both in freshwater and marine ecosystems.

In this study, we report the results of EOG and behavioral studies indicating the use of steroidal pheromones by the round goby, *Neogobius melanostomus*, a native of the Caspian and Black seas, which recently has been introduced to the Great Lakes in ship ballast (Crossman et al., 1992; Jude et al., 1992). The objectives of our study were twofold. First, we wished to increase the understanding of perciform pheromones by focusing on a species from a diverse and widely distributed perciform group (Family Gobiidae) that contains many marine taxa and that is suitable for laboratory-based reproductive studies (Moiseyeva and Rudenko, 1976). Second, we hoped that an understanding of hormonal pheromones of *Neogobius* might suggest methods for control of this species in the Great Lakes.

Spawning in *Neogobius* appears to be typical of many gobiids (Miller, 1984). Males migrate from deeper waters to spawning areas in the spring, estab-

lish territories prior to arrival of females, defend a nest site to which females are attracted for spawning, and care for single or multiple batches of eggs (Moiseyeva and Rudenko, 1976; MacInnis and Corkum, 2000). As with other male gobiids [e.g., *Bathygobius soporator* (Tavolga, 1956)], male round gobies use visual displays (coloration changes and posturing) and acoustical signals when courting females (Protosov et al., 1965; Moiseyeva and Rudenko, 1976). In particular, evidence that pheromones play important reproductive roles in other gobies (Tavolga, 1956) and that a conjugated steroid, 5β-androstan-3α-ol-17-one-3-glucuronide (etiocholanolone-glucuronide; ETIO-g), has pheromonal function in *G. jozo* (Colombo et al., 1980, 1982) suggested that, as in many other fish (Stacey and Cardwell, 1995, 1997; Sorensen and Stacey, 1999), EOG studies would be useful in determining if *Neogobius* detects hormonal pheromones.

The round goby has established thriving populations in all of the Great Lakes and might invade the Mississippi River drainage system, with potentially detrimental effects on many North American native fish species and ecosystems (Charlebois et al., 1997). Great Lakes *Neogobius* are of concern because there is evidence they have contributed to population declines of native mottled sculpin (*Cottus bairdi*) and other benthic fishes (Dubs and Corkum, 1996). Round gobies displace native benthic species from optimal spawning habitats, eat their eggs and young, and have a relatively high fecundity since they spawn a number of times in a season (Dubs and Corkum, 1996; Charlebois et al., 1997; MacInnis and Corkum, 2000). Additionally, because round gobies eat zebra mussels (*Dreissena polymorpha*), which can accumulate polychlorinated biphenyls and in turn are preyed upon by sport fish, they might facilitate introduction of toxins into Great Lakes food webs (Ray and Corkum, 1997). Understanding sex pheromones of the round goby could lead to techniques for population monitoring or disruption of spawning.

METHODS AND MATERIALS

Experimental Fish. Sexually mature *Neogobius* were collected between May and August, 1994–1996 by angling and bottom trawl from the St. Clair and Detroit Rivers (Windsor, Ontario, Canada), flown to Edmonton within three days of capture, and maintained in mixed-sex groups (four to six fish) under constant photoperiod (16L : 8D). Fish were held in 70-liter flow-through aquaria supplied with dechlorinated tap water that varied seasonally from 9 to 18°C. Aquaria contained gravel, an air stone, floating artificial plants, and clay flower pots and PVC pipes for shelter. A variety of live, frozen and flake food was provided ad libitum.

Fish were sexed by the shape of the urogenital papilla: the females have a broad, truncated tip with a large pore, and that of the male is long and slen-

der with a minute opening. Both males and females used in the EOG recording and behavior experiments were caught in Windsor in breeding condition. Most females had distended abdomens at capture and discharged eggs into aquaria within two weeks of captivity. Males kept in mixed sex groups periodically exhibited courtship behavior (color changes and territoriality), and occasionally fertilized eggs were found on the ceiling of the PVC tubes, indicating that holding conditions did not inhibit reproductive behavior. Only larger fish (>8 cm in length) were used in both EOG and behavior experiments. All experimental procedures involving fish were in accordance with Canadian Council of Animal Care guidelines.

Electro-olfactogram (EOG) Recording Technique. Sexually mature male and female *Neogobius* were tested for olfactory sensitivity to a variety of odors using EOG recording procedures similar to those described by Cardwell et al. (1995). Larger males and females (>8.0 cm in length) were chosen because it was too difficult to obtain a recording from smaller fish; the incurrent pore was too small for the size of electrodes used. Larger fish were sexually mature at the time of capture (see above) but may have undergone some gonadal recrudescence while maintained in the laboratory. Immediately prior to recording, fish were anesthetized by orally perfusing the gills with dechlorinated tap water containing 0.05% 2-phenoxyethanol (2-PE; Sigma, St. Louis, Missouri), wrapped in wet tissue and secured to a stand placed in an electrically grounded water bath. Fish were fitted with a polyethylene mouth tube that delivered aerated, dechlorinated water (containing 0.05% 2-PE) throughout the entire recording procedure. The temperature of the water bath and the anesthetic water (9–18°C) approximated that of the holding aquaria at the time of testing.

A glass capillary tube (70- to 120-μm tip diameter) filled with gelatin (8% in 0.6% NaCl) bridged Ag–AgCl electrodes filled with 3 M KCl to the olfactory tissue. The tip of the reference electrode was placed in the water bath. The odor delivery tube was positioned next to the excurrent pore of one naris, creating a reversed water flow (in the excurrent pore and out the incurrent pore); initial recordings failed to generate any EOG response if the odor tube was placed near the incurrent pore and the recording electrode was placed in the excurrent pore. In an attempt to facilitate recording, the naris was dissected to expose olfactory receptor cells. However, we were unable to obtain any measurable EOG response from the exposed naris; therefore the naris was left intact for all recordings.

Throughout a recording session (maximum duration of 3 hr), the naris was continuously perfused with dechlorinated tap water (background) until a computer-controlled solenoid switched the background solution to a test solution for 2 sec. The amplified signals (Grass P-18 DC amplifier) were digitized (National Instruments Lab-PC A/D converter), and the response was recorded for 10 sec from initiation of the odor pulse. After recording was completed, the gills were perfused with tap water without anesthetic until the fish began venti-

lating and could be returned to its aquarium. A number of fish were retested in a maximum of two additional EOG recording sessions (allowing at least one week between sessions), each of which was for a different cross-adaptation experiment (see EOG Cross-Adaptation below). There was no evidence that the EOG response was affected by prior recordings, insofar as response magnitudes remained equivalent on subsequent tests.

When an anesthetized fish had been mounted in the water bath for 30–45 min, recording began by determining olfactory response to 10^{-5} M L-alanine. If the response to L-alanine was greater than 3 mV (prestimulus voltage to peak stimulus voltage), testing with hormonal compounds was initiated. If the response was less than 3 mV, the recording electrode and/or odor tube were repositioned until a minimum 3-mV response was obtained or the recording was terminated and the fish recovered.

During the course of a recording, the sensitivity by 10^{-5} M L-alanine was monitored frequently to ensure the stability of the recording. As well, to determine if mechanical artifacts contributed to the EOG recording, the naris was exposed occasionally to 2-sec pulses of the same dechlorinated tap water (background water) that chronically irrigated the naris and was used for dilution of test odors (see Tested Odors below). Data (base to peak voltage differences) are presented as an absolute response in mV with response to background water (if any) subtracted. Sensory adaptation during recording was minimized by using a short exposure time (2 sec) and allowing 1–2 min between exposures to test solutions.

Tested Odors. The olfactory epithelium of *Neogobius* was exposed to 10^{-8} M solutions of eight prostaglandins (prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, and $F_{3\alpha}$; 15-keto-prostaglandin E_2; 15-keto-prostaglandin $F_{2\alpha}$; 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$) and 114 steroids (Table 1) to determine compounds that consistently induced EOG response. Steroids and prostaglandins selected for testing included all those reported to be olfactory stimulants or to have pheromonal activity in other fish (Stacey and Cardwell, 1995; Sorensen and Stacey, 1999), as well as a number of related compounds, metabolites, and conjugates. Steroids and steroid conjugates were purchased from Sigma (St. Louis, Missouri) and Steraloids (Newport, Rhode Island) or received as gifts from Dr. A. P. Scott (Lowestoft), and Dr. J. G. D. Lambert (Utrecht). Prostaglandins were purchased from Cayman Chemical Company (Ann Arbor, Michigan). Because, in initial tests, prostaglandins failed to induce an EOG response in any fish, they will not be considered further in this section.

The 10^{-5} M L-alanine (Sigma, St. Louis, Missouri) used to monitor EOG recording preparations was prepared as a 10^{-2} M stock solution in double-distilled deionized water, stored in glass scintillation vials at 4°C and diluted in background water at the time of EOG recording. Olfactory response to 10^{-6} M L-alanine was also recorded, as this concentration was used in behavior tests.

TABLE 1. STEROIDS TESTED IN EOG RECORDING, GROUPED ACCORDING TO STRUCTURAL SIMILARITY AND INCLUDING ABBREVIATIONS

5α-Androstan (unconjugated): 5α-androstan-3,17-dione; 5α-androstan-3β-ol-17-one (epiandrosterone; EPIANDR); 5α-androstan-17β-ol-3-one.

5α-Androstan (conjugated): 5α-androstan-3β-ol-17-one-3β-glucuronide (epiandrosterone-glucuronide; EPIANDR-g); 5α-androstan-3α-ol-17-one-3α-SO_4 (androsterone-sulfate; ANDR-s).

5β-Androstan (unconjugated): 5β-androstan-3,17-dione (etiocholan-3,17-dione; ETIO-3,17-dione); 5β-androstan-3α-ol-17-one (etiocholanolone; ETIO); 5β-androstan-3β-ol-17-one.

5β-Androstan (conjugated): 5β-androstan-3α,17β-diol-3α-glucuronide; 5β-androstan-3α,17β-diol-17β-glucuronide; 5β-androstan-3α,11β-diol-17-one-3α-glucuronide (etiocholanolone-glucuronide; 11β-ETIO-g); 5β-androstan-3α-ol-17-one-3α-glucuronide (etiocholanolone-glucuronide; ETIO-g)

4-Androsten (unconjugated): 4-androsten-11β,17β-diol-3-one; 4-androsten-3,17-dione (androstenedione; AD); 4-androsten-11-keto-17β-ol-3-one (11-ketotestosterone); 4-androsten-11-keto-3,17-dione; 4-androsten-11α-ol-3,17-dione; 4-androsten-11β-ol-3,17-dione; 4-androsten-17α-ol-3-one; 4-androsten-17β-ol-3-one (testosterone; T).

4-Androstene (conjugated): 4-androsten-17β-ol-3-one-17β-glucuronide (testosterone glucuronide); 4-androsten-17β-ol-3-one-17β-SO_4 (testosterone sulfate).

5-Androsten (unconjugated): 5-androstene-3β,17β-diol; 5-androstene-3β-ol-17-one (dehydroepiandrosterone; DHEA).

5-Androsten (conjugated): 5-androstene-3β-ol-17-one-3β-glucuronide (dehydroepiandro sterone-glucuronide; DHEA-g); 5-androstene-3β-ol-17-one-3β-SO_4 (dehydroepiandrosterone-sulfate; DHEA-s).

Estratriens (unconjugated): 1,3,5(10)-estratrien-3α,17α-diol (17α-estradiol); 1,3,5 (10)-estratrien-3α,17β-diol (17β-estradiol; E2); 1,3,5 (10)-estratrien-3α-ol-17-one (estrone; E1); 1,3,5 (10)-estratrien-3α,16α,17β-triol (estriol; E3).

Estratriens (conjugated): 1,3,5 (10)-estratrien-3α,17β-diol-3α-glucuronide (17β-estradiol-3α-glucuronide; E2-3g); 1,3,5 (10)-estratrien-3α,17β-diol-17β-glucuronide (17β-estradiol-17β-glucuronide; E2-17g); 1,3,5 (10)-estratrien-3α,17β-diol-3α-SO_4; 1,3,5 (10)-estratrien-3α,17β-diol-17β-SO_4); 1,3,5 (10)-estratrien-3α,17β-diol-di-SO_4; 1,3,5 (10)-estratrien-3α-glucuronide-17β-SO_4; 1,3,5 (10)-estratrien-3α-SO_4-17β-glucuronide

Estrens (unconjugated): 4-estren-17β-ol-3-one

5α-Pregnan (unconjugated): 5α-pregnan-3,20-dione; 5α-pregnan-3β,17α-diol-20-one; 5α-pregnan-3β,20α-diol; 5α-pregnan-3β,20β-diol; 5α-pregnan-17,21-diol-3,20-dione; 5α-pregnan-17-α-ol-3,20-dione; 5α-pregnan-3β-ol-20-one; 5α-pregnan-3β,11β,17α,21-tetrol-20-one; 5α-pregnan-3α,17α,20β-triol; 5α-pregnan-3β,17α,20β-triol; 5α-pregnan-11β,17,21-triol-3,20-dione; 5α-pregnan-3α,17α,21-triol-20-one; 5α-pregnan-3β,17α,21-triol-20-one

5β-Pregnan (unconjugated): 5β-pregnan-3α,20β-diol (3α,20β-5βP); 5β-pregnan-3β,20β-diol; 5β-pregnan-17α,21-diol-3,20-dione (3β,20β-5βP); 5β-pregnan-3α,17α-diol-20-one (3α,17α-5βP); 5β-pregnan-3β,17α-diol-20-one; 5β-pregnan-3,20-dione (5βP); 5β-pregnan-17α-ol-3,20-dione; 5β-pregnan-3α,11β,17α,21-pentol; 5β-pregnan-3α,11β,17α,20β,21-pentol; 5β-pregnan-3α,17α,20α,21-tetrol; 5β-pregnan-3α,17α,20β,21-tetrol; 5β-pregnan-3α,11β,17α,21-tetrol-20-one; 5β-pregnan-3α,17α,20β,21-tetrol-11-one; 5β-pregnan-3α,17α,20α-triol; 5β-pregnan-3α,17α,20β-triol (3α,17α,20β-5βP); 5β-pregnan-3α,17α,21-triol-11,20-dione; 5β-pregnan-11β,17α,21-triol-3,20-dione; 5β-pregnan-3α,17α,21-triol-20-one; 5β-pregnan-3β,17α,21-triol-20-one

TABLE 1. CONTINUED

5β-Pregnan (conjugated): 5β-pregnan-3α,17α-diol-20-one-3α-glucuronide (3α,17α-5βP-3g)
4-Pregnen (unconjugated): 4-pregnen-11β,17α-diol-3,20-dione; 4-pregnen-11β,21-diol-3,20-dione; 4-pregnen-16α,17β-diol-3,20-dione; 4-pregnen-17α,21-diol-3,20-dione; 4-pregnen-17α,20α-diol-3-one; 4-pregnen-17α,20β-diol-3-one; 4-pregnen-20β,21-diol-3-one; 4-pregnen-17α,21-diol-3,11,20-trione; 4-pregnen-3,20-dione (progesterone); 4-pregnen-11α-ol-3,20-dione; 4-pregnen-11β-01,-3,20-dione; 4-pregnen-17α,-ol-3,20-dione; 4-pregnen-21-ol-3,20-dione; 4-pregnen-20α-ol-3-one; 4-pregnen-20β-ol-3-one; 4-pregnen-11β,17α,20β,21-tetrol-3-one; 4-pregnen-11β,17α,21-triol-3,20-dione; 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol); 4-pregnen-14α,17β,21-triol-3,20-dione; 4-pregnen-17α,20β,21-triol-3,11-dione; 4-pregnen-17α,20α,21-triol-3-one; 4-pregnen-17α,20β,21-triol-3-one; 4-pregnen-3,11,20-trione
4-Pregnen (conjugated): 4-pregnen-11β,21-diol-3,20-dione-21-SO_4; 4-pregnen-17α,21-diol-3,20-dione-21-SO_4; 4-pregnen-17α,20β-diol-3-one-20β-glucuronide; 4-pregnen-17α,20α-diol-3-one-20α-SO_4; 4-pregnen-17α,20β-diol-3-one-20β-SO_4; 4-pregnen-21-ol-3,20-dione-21-glucuronide; 4-pregnen-20β-ol-3-one-20β-SO_4; 4-pregnen-11β,17α,21-triol-3,20-dione-21-glucuronide; 4-pregnen-11β,17α,21-triol-3,20-dione-21-SO_4; 4-pregnen-17α,20β,21-triol-3-one-21-SO_4; 4-pregnen-17α,20β-diol-3-one-20β-PO_4
5-Pregnen (unconjugated): 5-pregnen-3β,17α-diol-20-one; 5-pregnen-3β,21-diol-20-one; 5-pregnan-3β-ol-20-one (pregnenolone); 5-pregnen-3β,17α,21-triol; 5-pregnen-3β,17α,21-triol-20-one
5-Pregnen (conjugated): 5-pregnen-3β,21-diol-20-one-3β-SO_4; 5-pregnen-3β,21-diol-20-one-21-SO_4; 5-pregnen-3β-ol-20-one-3β-glucuronide; 5-pregnen-3β-ol-20-one-3β-SO_4

All other test odors were first prepared as 10^{-3} M stock solutions in 99% ethanol (free and sulfated steroids, prostaglandins) or 50 : 50 ethanol–water (glucuronated steroids), and stored at −20°C. Working solutions (10^{-6} M) were prepared in duplicate by diluting stock solutions in double-distilled, deionized water and stored in glass scintillation vials at 4°C. Test solutions were prepared immediately before recording by diluting appropriate amounts of the working solutions in 100 ml of background water (Cardwell et al., 1995). Ethanol control solutions were prepared in the same manner but contained no steroid or prostaglandin.

In initial recordings, five gobies (two males, three females) were exposed to 10^{-8} M test solutions of all the compounds listed above. Compounds that did not elicit a response from any fish were considered undetectable and were not tested again. A compound was considered detectable if, in more than one fish, it induced a voltage change greater than that induced by the background solution. Additionally, to ensure that a response to a compound was not due to contamination of the test solution, each detected steroid was tested on the same fish with a new test solution. Following these intial recordings, only detected steroids were used in further studies. After additional fish were tested with detectable steroids, male and female EOG responses to detected steroids were compared (*t*-test) (Wilkinson, 1994).

EOG Concentration–Response Studies. To compare olfactory potency of detected steroids, concentration–response relationships were determined for six detected steroids (estrone, E1; estradiol-3-glucuronide, E2-3g; etiocholan-3α-ol-17-one, ETIO; androsterone-sulfate, ANDR-s; dehydroepiandrosterone-sulfate, DHEA-s; dehydroepiandrosterone-glucuronide, DHEA-g) that, at 10^{-8} M, elicited consistent and large responses (greater than 20% of the response to 10^{-5} M L-alanine) and were thought likely to operate through separate olfactory receptor mechanisms. Criteria for determining compounds that may act through separate olfactory receptor mechanisms were arbitrary and based on studies in goldfish showing that 19- and 21-carbon steroids act through different receptor mechanisms and that some free and conjugated steroids also act through separate mechanisms (Sorensen et al., 1995). The concentration–response relationship of ETIO-g also was determined because, even though at 10^{-8} M it induced an EOG response less than 20% of that induced by 10^{-5} M L-alanine, it has been proposed to have pheromonal function in *G. jozo* (Colombo et al., 1980, 1982).

Concentration–response tests began at 10^{-12} M and increased by log molar increments to 10^{-8} M, with 10-min intervals between each exposure. Concentration–response tests for each selected steroid were performed on six fish (four females, two males). Response magnitudes (millivolts) were analyzed by ANOVA and Tukey multiple comparison test ($\alpha = 0.05$) (Wilkinson, 1994).

EOG Cross-Adaptation Studies. These studies compared the EOG response to a test compound before and during adaptation to an adapting compound, under the assumption that, if the EOG response to the test compound is unaffected by adaptation, it is acting through an olfactory receptor mechanism separate from that mediating response to the adapting compound (e.g., Caprio and Byrd, 1984; Sorensen et al., 1995). The design of the standard cross-adaptation procedure makes the assumption that a brief exposure to an odor will not influence EOG response to that odor when presented a short time later. However, interpretation of a cross-adaptation experiment will be confounded if a tested odor induces a smaller response on the second presentation, even in the absence of a tonically delivered adapting odor. The extent of this phenomenon in *Neogobius* was evaluated by a sequential exposure test.

Six fish (four females, two males) were exposed sequentially to 2-sec 10^{-8} M pulses of all steroids used for the cross-adaptation studies, and then retested with 2-sec 10^{-8} M pulses of the same steroids 30 min later: response magnitude to 10^{-5} L-alanine remained constant throughout. Differences between response magnitudes induced by the first and second exposures were analyzed by Wilcoxon matched-pairs test (Wilkinson, 1994). As well, for each steroid, the magnitude of the EOG response induced by the second exposure was converted to a percentage of the response induced by the first exposure (% IR = percentage of initial response), and the mean % IR used in analysis of the cross-adaptation experiment (see below). Steroids that failed to induce a second response in this

sequential exposure test were eliminated from all cross-adaptation studies (see Results).

Our cross-adaptation procedure was similar to that described by Sorensen et al. (1995). The naris first was exposed sequentially to 2-sec 10^{-8} M solutions of all of the detected compounds to establish initial preadaptation responses. Then, during adaptation to a 10^{-7} M solution of the adapting compound, the 10^{-8} M test steroids were administered again and in the same sequence. Test steroids were used at 10^{-8} M because many of them did not induce an EOG response at lower concentrations.

Five adapting steroids (E1, E2-3g, DHEA-g, ETIO, and DHEA-s) were chosen based on structure and detection threshold and on the results of preliminary cross-adaptation experiments (data not shown) that indicated which compounds operate through separate olfactory receptor mechanisms. Cross-adaptation experiments for each adapting steroid were conducted on six fish (four females, two males).

To monitor the quality of the EOG recording during the cross-adaptation procedure, the naris was exposed to 10^{-5} M L-alanine at the beginning and end of each cross-adaptation test, and after every fourth steroid exposure. As it was difficult to maintain a recording on *Neogobius* for the time required for a cross-adaptation experiment (>3 hr), only results of cross-adaptation tests in which L-alanine responses remained relatively stable throughout the recordings (approximately 50% of the tests) were used for analysis and presentation.

To reduce the potential for adaptation during the preadaptation testing, the compounds known to have the smallest response magnitudes were tested first. As well, compounds of similar structure were not tested in sequence to reduce the possibility of adaptation by sequential exposure to compounds that might operate through the same receptor mechanism. Some steroids that induced small responses at 10^{-8} M in initial testing (see Detected Odors in Results) failed to induce a response in the pretest of the cross-adaptation, and therefore, were not tested during the cross-adaptation phase.

Two liters of 10^{-7} M adapting steroid solution were prepared immediately before beginning cross-adaptation pretesting, held in a running bath of background water to maintain temperature, and used both to adapt the naris and to prepare dilutions of test steroids for cross-adaptation testing. Immediately following preadaptation application of 2-sec pulses of all the test steroids, the naris was exposed to the adapting steroid until the induced response reached a stable plateau (usually within 3 min) and then exposed to 2-sec pulses of the test steroids dissolved in adapting solution. The magnitude of the response induced during adaptation was converted to %IR and, for each steroid, the difference between %IR in cross-adaptation and %IR in the sequential exposure test was analyzed by Mann-Whitney *U* test.

Odor-Induced Ventilation Increase. Individual male or nonovulated female

gobies displayed no overt reproductive response to bolus addition of single detected steroids (final water concentration 10^{-8} M). However, a number of detected steroids did induce a transitory increase in ventilation frequency (opercular movements per minute) that formed the basis of a consistent and quantitative behavioral bioassay. Although the biological significance of odor-induced ventilation increases in *Neogobius* is unclear, the fact that in some benthic fish water flow through the naris changes in synchrony with ventilation (Nevitt, 1991) suggests increased ventilation might facilitate odor detection or processing.

Ventilation frequency was monitored in aerated 100-liter, flow-through test aquaria (90 cm long × 30 cm wide × 38 cm high) that were opaque on the ends and one side, with one side left clear for observation; a gravel substrate was provided and one PVC tube (12 cm length; 6 cm diameter) was placed with one end facing the observation side. Fish usually remained within the PVC tube, enabling ventilation frequency to be monitored readily. To minimize visual disturbance during observations, fish were observed through a small hole cut in an opaque cloth curtain that blocked the fish's view of the experimenter. Test odors and control solutions were injected into the test aquaria (at the water surface directly over the PVC tube) through silastic tubing fed through a hole in the cloth curtain and anchored to each aquarium. Injections of dye showed that an odor plume should reach the PVC tube within one min and disperse evenly throughout the aquarium within 3 min.

A single male or nonovulated female (>8 cm in length) was placed in each test aquarium at least five days before testing and exposed to different odors on consecutive days. On the morning of each test day, water flow was turned off 2 hr before an experiment, the fish was tested with only one odor, and water flow was resumed. In each test, a fish was observed for 12 min prior to odor addition (pre-exposure period), a test solution was then injected, and the fish was observed for an additional 24 min (test period). Ventilations were counted continuously for 1 min during every 3-min interval before and after addition of an odor, yielding 4 min of pre-exposure ventilation and 8 min of test ventilation. Six males and six females were tested with each odor.

Ventilation frequency of individual males and females was monitored in response to addition of ethanol vehicle (1 ml), eight steroids that induced an EOG response (E1, E2, E2-3g, ETIO, ETIO-g, DHEA-s, DHEA-g, and ANDR-s), one steroid that did not induce an EOG response (testosterone-glucuronide; T-g), and L-alanine. Steroid odors were tested either individually or in combinations by injecting either 100 μl or 1 ml of a 10^{-3} M steroid-ethanol solution to create 10^{-9} and 10^{-8} M concentrations after dispersal. L-Alanine (10 ml of a 10^{-2} M aqueous solution) was added to create a 10^{-6} M final concentration.

To determine if basal (pre-exposure) ventilation of males and females differed, pre-exposure ventilation frequencies of males and females were compared by unpaired *t*-test (Wilkinson, 1994), using for each fish the grand mean

of pre-exposure frequencies from all odor tests. Ventilation frequency differences between pre-exposure and test periods were analyzed separately by sex and odor, comparing the mean (per minute) frequency of the four pre-exposure samples and the mean of the frequencies observed during minutes 4 and 7 of the test period (Wilcoxon test) (Wilkinson, 1994). All ventilation data from the test period are presented as a percentage of the mean basal (pre-exposure) ventilation frequency.

Ventilation Concentration–Response Studies. Concentration–response relationships were examined for those steroids (E1, E2-3g, and ETIO) that increased ventilation frequency of male fish when tested at 10^{-8} M. The protocol was identical to that already described, except that the final concentrations in the test aquaria ranged from 10^{-12} M to 10^{-8} M and were prepared by diluting appropriate volumes of 10^{-3} M steroid solutions into 100 ml of double-distilled, deionized water, and then adding 1 ml of this solution to the test aquarium. Testing of each steroid began at 10^{-12} M, and increased in log molar increments on consecutive days. Six males were tested, all receiving the same treatment each day.

For each steroid, differences in test ventilation frequency due to odor concentration were analyzed by Friedman's nonparametric ANOVA (Wilkinson, 1994), using for each fish the increase in ventilation during the test period (mean of minutes 4 and 7 after odor addition). Dunnett's test (Zar, 1984) was used to determine threshold by comparing the response (mean of minutes 4 and 7 after odor addition) of the lowest concentration tested (10^{-12} M) to each of the higher concentrations.

Behavioral Cross-Adaptation Studies. Behavioral cross-adaptation using ventilation as the behavioral response was conducted to determine if *Neogobius* can behaviorally discriminate among steroids that might act through separate olfactory receptor mechanisms, as indicated by the results of our EOG cross-adaptation studies (see EOG Cross-Adaptation Studies in Results). Unlike EOG cross-adaptation, behavioral cross-adaptation did not involve addition of the test odor prior to applying the adapting odor, because there was no means for rapidly removing the test odor prior to applying the adapting odor. Thus, the purpose of the behavioral cross-adaptation experiment was to determine if adaptation eliminates the ventilatory response to the test steroid, rather than to compare responses to a test steroid prior to and during adaptation.

In behavioral cross-adaptation, a fish was observed for a 12-min pre-exposure period, immediately exposed to an adapting steroid (either 10^{-8} M E1 or ETIO), observed for a 24-min adapting period, immediately exposed to a test steroid (10^{-9} M), and observed for a further 24-min test period. Ventilation frequency was monitored continuously every third minute; six male fish were tested in each treatment group. The adapting steroid was expected to increase ventilation, whereas the test steroid was expected to increase ventilation only if it acts

through a different receptor mechanism than the adapting steroid (Murphy and Stacey, 1999).

Differences in ventilation frequencies in the pre-exposure, adapting, and test periods were analyzed by Friedman's nonparametric ANOVA (Wilkinson, 1994) and pairwise differences were analyzed by Dunnett's test (Zar, 1984) using mean frequencies for the pre-exposure period and for minutes 4 and 7 of the adapting and test periods.

RESULTS

Response to Test Odors. L-Alanine consistently induced an EOG response in *Neogobius* both at 10^{-5} M (Figure 1) and 10^{-6} M (mean ± SEM = 3.1 ± 0.6 mV; $N = 8$). Response magnitude to 10^{-5} M L-alanine was similar ($P > 0.05$; t test) in males and females (Figure 1). There were no significant mechanical artifacts in the EOG recordings, responses to background water being <0.1 mV in all cases. Neither the ethanol solvent in 10^{-8} M test solutions (up to 0.001%) nor any of the eight tested prostaglandins induced EOG responses in any fish. However, of 114 tested steroids (Table 1), 19 met the criterion of detection by inducing an EOG response >0.1 mV in more than one fish (Figure 1). Because there was no indication that EOG responses of males and females differed in magnitude (Figure 1), EOG results from both genders were pooled in concentration–response and cross-adaptation studies.

At a concentration of 10^{-8} M (the highest concentration tested), a diverse array of conjugated and unconjugated 18-, 19-, and 21-carbon steroids induced EOG responses; those induced by the 18-carbon steroids generally were the largest (Figure 1). The detected 19-carbon steroids form a diverse group, including the 4-androstene (AD), 5-androstene (DHEA-g, and DHEA-s), 5α-androstan (ANDR-s, and EPIANDR-g), and 5β-androstan (ETIO, ETIO-g, 11β-ETIO-g, and ETIO-3,17-DIONE) configurations. In contrast, all the detected 21-carbon steroids were variations and conjugates of the 5β-pregnan moiety.

EOG Concentration–Response Studies. All concentration–response profiles increased in magnitude with concentration once detection threshold was reached (Figure 2). Although it was clear that some fish responded to 10^{-11} M E1, E2-3g, ETIO, and ETIO-g, group responses to steroids were not significantly different ($P < 0.05$) than responses to background water until 10^{-9} M for E1, E2-3g, and ETIO, and 10^{-8} M for DHEA-g, ANDR-s, ETIO-g, and DHEA-s (Figure 2).

EOG Cross-Adaptation Studies. When steroids intended for use in the cross-adaptation studies were delivered as 2-sec 10^{-8} M pulses 30 min apart (sequential exposure study), there was a trend for many steroids to induce smaller EOG responses on the second presentation, although the reduction was significant ($P < 0.05$; Wilcoxon test) only for DHEA-g (Figure 3A). Although few

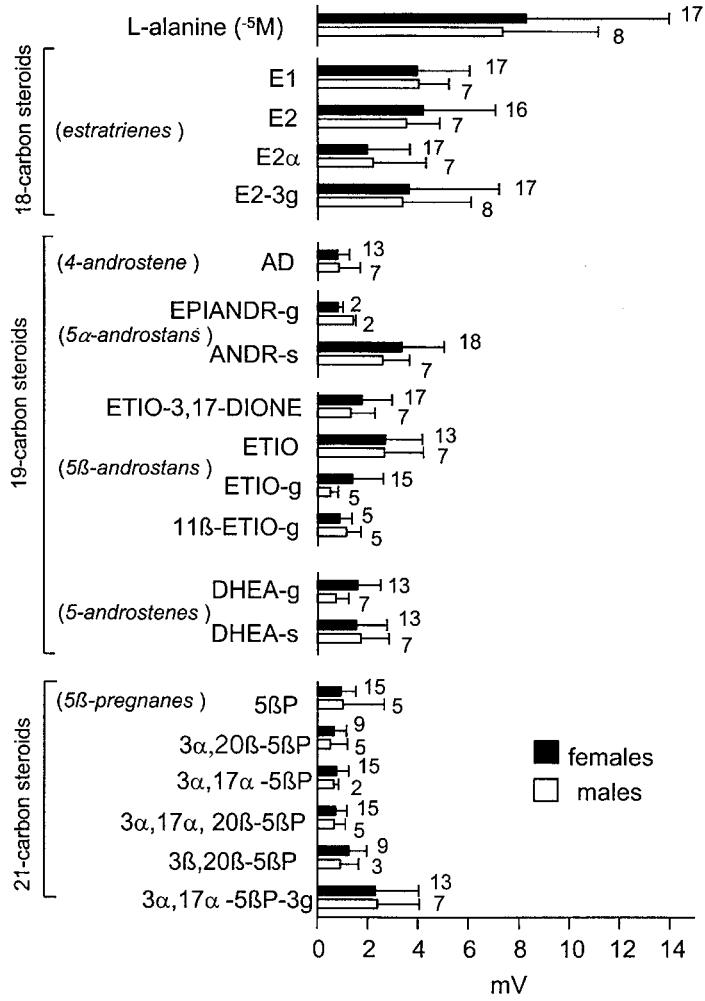


FIG. 1. Comparison of male and female EOG response magnitudes (mean + SE) to detected steroids (10^{-8} M) and amino acid standard. Sample size indicated beside bars. See text and Table 1 for full names of steroids.

replicates were conducted, three of the 5β-pregnan compounds (5βP; 3α,20β-5βP; and 3β,20β-5βP) which induced small EOG responses in initial tests (Figure 1) did not induce EOG response when given the second time in the sequential exposure test, and therefore were omitted from subsequent cross-adaptation studies. For each of the remaining 16 detected steroids, the mean % IR during

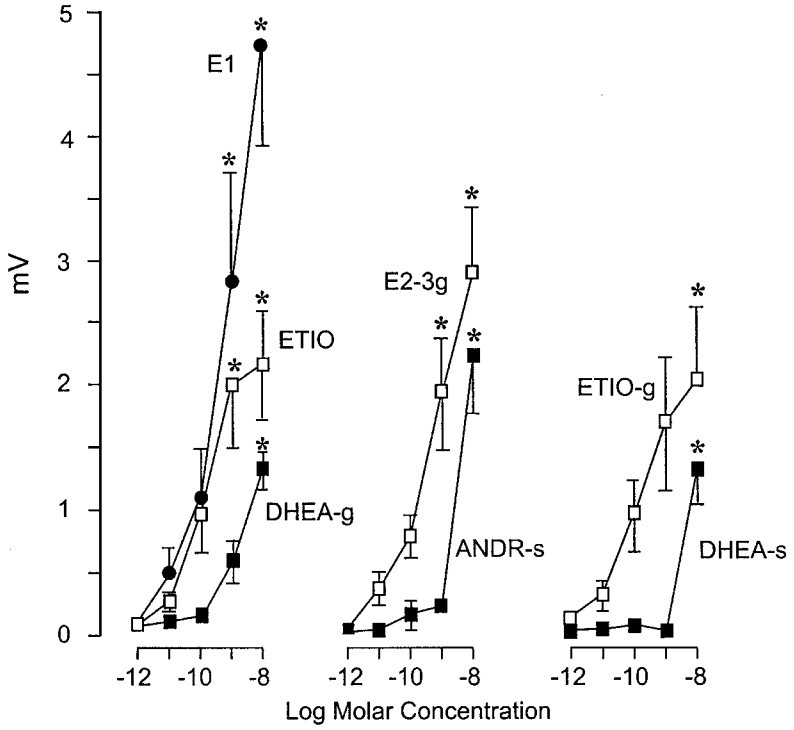


FIG. 2. Concentration response profiles (mean ± SE) for selected steroids. EOG responses expressed in millivolts. $N = 6$ for each steroid. *Response was significantly ($P < 0.05$) greater than response to background water.

the sequential exposure test (Figure 3A) was compared (Mann-Whitney *U* test) to the mean %IR during cross adaptation (Figure 3B–F).

Adaptation to 10^{-7} M ETIO significantly reduced EOG responses to 2-sec 10^{-8} M pulses of a diversity of 4-androsten (AD), 5α-androstan (ANDR-s), 5β-androstan (ETIO; ETIO-3,17-DIONE; and ETIO-g), and 5β-pregnan steroids (3α,17α-5βP; 3α,17α,20β-5βP; 3α,17α-5βP-g) (Figure 3B). Although adaptation to 10^{-7} M DHEA-s also significantly reduced response to ANDR-s (a 5α-androstan), it did not reduce responses to 5β-androstan steroids (e.g., ETIO, and ETIO-g) and produced a different pattern of adaptation of 5β-pregnan steroids than did adaptation to ETIO (Figure 3C).

In marked contrast to the pattern of adaptation to DHEA-s (Figure 3C), adaptation to DHEA-g (Figure 3D) did not affect responses to 5β-pregnanes, significantly reduced response to E2-3g and, rather than reducing response to ANDR-s, reduced response to its 3β isomer, EPIANDR-s (Figure 3D). This pat-

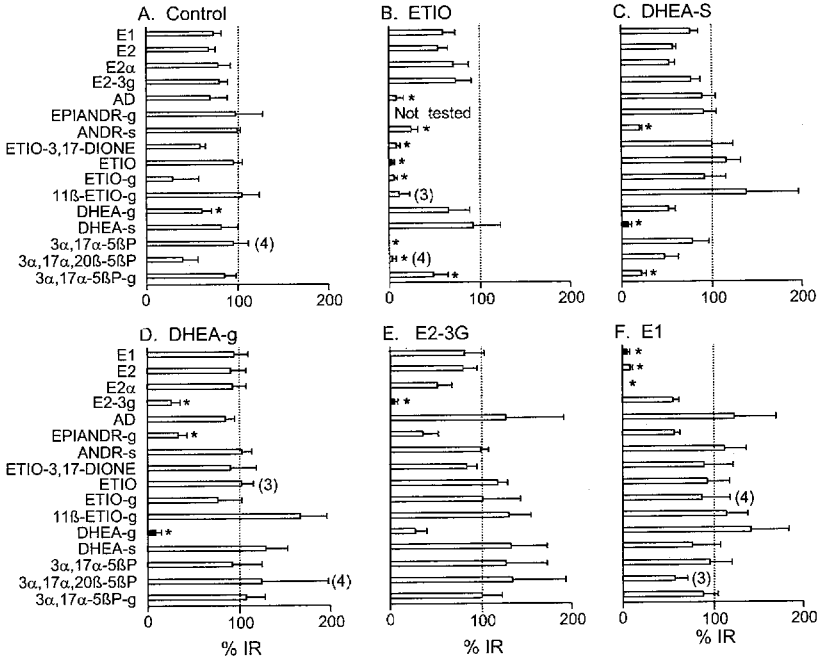


FIG. 3. EOG response to a 2-sec pulse of 10^{-8} M steroid expressed as percent of initial response (% IR) (mean + SE) to either (A) a 10^{-8} M of the same steroid delivered 30 min previously or during adaptation to (B) 10^{-7} M etiocholanolone (ETIO), (C) 10^{-7} M dehydroepiandrosterone sulfate (DHEA-s), (D) 10^{-7} M dehydroepiandrosterone-glucuronide (DHEA-g), (E) 10^{-7} M estradiol-glucuronide (E2-3g), or (F) 10^{-7} M estrone (E1). In B–F, response to adapting steroid indicated by solid bars. $N = 6$ except where indicated beside bars. See text and Table 1 for full names of steroids and text for calculation of % IR. *Response was reduced significantly ($P < 0.05$) by prior exposure (A) or by adaptation (B–F).

tern of adaptation to DHEA-g was similar to that seen during adaptation to E2-3g (Figure 3E), although in the latter case the apparent reductions in response to EPIANDR-s and DHEA-g were not significant.

Finally, adaptation to E1 significantly reduced responses only to the estratriene compounds E1, E2, and E2α (Figure 3F).

Although the results of our cross-adaptation studies (Figure 3) have not fully characterized the olfactory interactions of the steroids detected by *Neogobius*, they indicate the presence of at least four olfactory receptor mechanisms, which, for convenience, we term ETIO, DHEA-s, E2-3g, and E1.

Odor-Induced Ventilation Increase. *Neogobius* did not change their ventila-

tion rate in response to addition of 10^{-6} M L-alanine, the ethanol steroid solvent, 10^{-8} M T-g (a steroid that does not induce EOG response), or 10^{-8} M DHEA-s (a steroid that does induce EOG response) (Figures 4A and B and 5). In contrast, ventilation rate consistently increased in response to ETIO, E1, E2-3g, ANDR-s, and E2 (Figures 4C–E and 5).

Addition of ETIO increased ventilation in both male and female *Neogobius*, the response commencing within the first minute of steroid addition and persisting for approximately 10 min, after which ventilation quickly returned to basal rates (Figure 4C). Similar increases in ventilation were seen in both males and females in response to ETIO-g and ANDR-s (Figure 5), steroids in which EOG responses are significantly reduced during adaptation to ETIO (Figure 3B).

In contrast to the effect of ETIO, addition of E1 and E2-3g increased ventilation of males but not of females (Figure 4D, E). E2, for which EOG response is significantly reduced during adaptation to E1 (Figure 3F), also increased ventilation in males (Figure 5); the effect of E2 on ventilation was not examined in females. Addition of DHEA-g failed to affect ventilation, the apparent trend to increased ventilation being due to an increase in one fish of each sex (Figure 5).

Addition of a mixture of 10^{-9} M E1, E2-3g, ETIO, and DHEA-s induced a significant increase in ventilation, in which the magnitude (Figure 5) and duration (data not shown) were similar to those of its active components.

Behavioral Concentration–Response Studies. The threshold of the ventilation rate response was 10^{-10} M for ETIO and E1 and 10^{-9} M for E2-3g (Figure 6). Ventilation did not increase further at suprathreshold odor concentrations.

Behavioral Cross-Adaptation. In all four behavioral cross-adaptation experiments that used 10^{-8} M ETIO as the adapting steroid, male *Neogobius* significantly increased ventilation rate when exposed to the adapting odor (Figures 7 and 8A). During adaptation to ETIO, these males did not increase ventilation in response to addition of either 10^{-9} M ETIO or 10^{-9} M ANDR-s (Figures 7A and B and 8A), a steroid in which EOG response is significantly reduced by adaptation to ETIO (Figure 3B). In contrast, males adapted to ETIO significantly increased ventilation in response to addition of 10^{-9} M E1 and 10^{-9} M E2-3g (Figures 7C and D and 8A), steroids in which EOG response magnitude is not reduced by adaptation to ETIO (Figure 3B).

Similar results were obtained in behavioral cross-adaptation experiments that used 10^{-8} M E1 as the adapting steroid (Figure 8B): adapted males did not respond to E2, whose induced EOG response is significantly reduced by E1 adaptation (Figure 3F), but significantly increased ventilation rate in response to steroids (ETIO, E2-3g) whose induced EOG responses are unaffected by E1 adaptation (Figure 3F) (Murphy and Stacey, 1999).

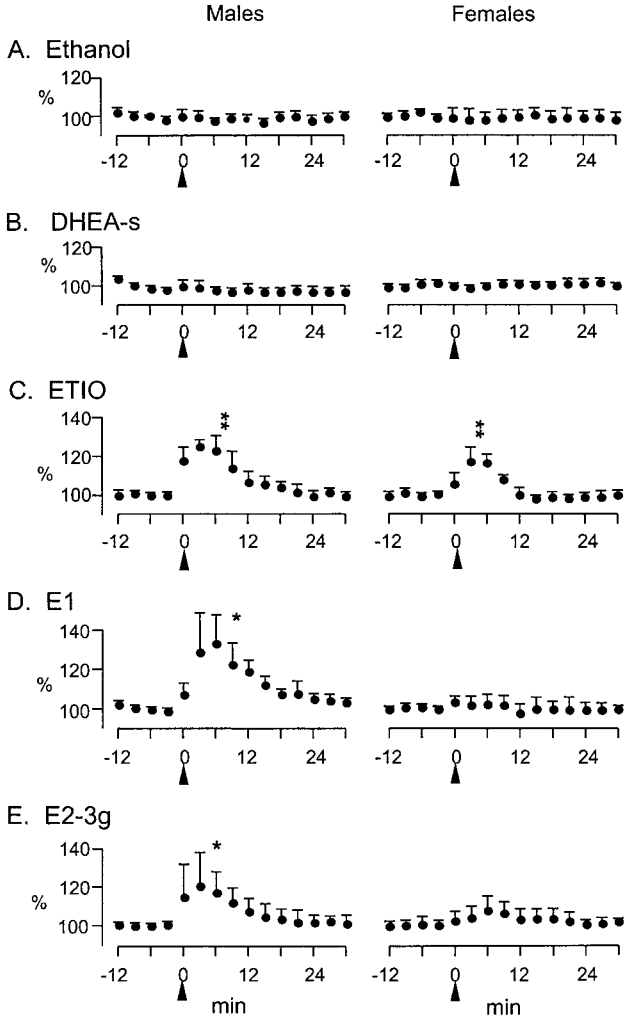


FIG. 4. Percent change in basal ventilation rate (mean + SE) of male and female gobies after addition (arrow) of (A) 1 ml ethanol or exposure to (B) 10^{-8} M DHEA-s, (C) 10^{-8} M ETIO, (D) 10^{-8} M E1, or (E) 10^{-8} M E2-3g. Ventilation rates following addition of test substances were calculated as a percentage of the basal ventilation rate (i.e., the mean ventilation rate in the 12 min preceding additions). $N = 6$ for all groups. Mean ventilation rate during minutes 4 and 7 following test substance addition is significantly different from the basal ventilation rate ($^{*}P < 0.05$; $^{**}P < 0.01$).

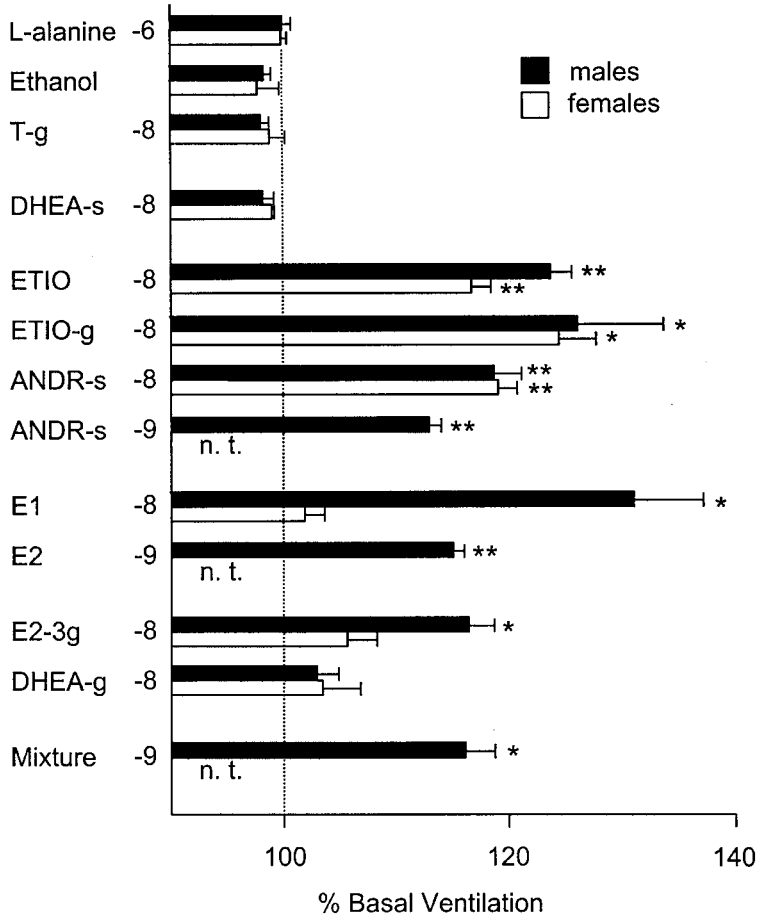


FIG. 5. Percent change in basal ventilation rate (mean + SE) of male and female gobies during minutes 4 and 7 after the addition of single test substances or a mixture consisting of four 10^{-9} M steroids (E1, E2-3g, ETIO, and DHEA-s). Ventilation responses to test substances were calculated as in Figure 4. See text and Table 1 for full names of steroids. $N = 6$ for all groups. n.t. = not tested in females. Mean ventilation rate after addition of a test substance is significantly different from basal ventilation rate ($^{*}P < 0.05$; $^{**}P < 0.01$).

DISCUSSION

The results of this study demonstrate that the round goby, *Neogobius melanostomus*, exhibits olfactory and behavioral responses to steroids, and thus may use released steroids as sex pheromones. The olfactory epithelium of *Neo-*

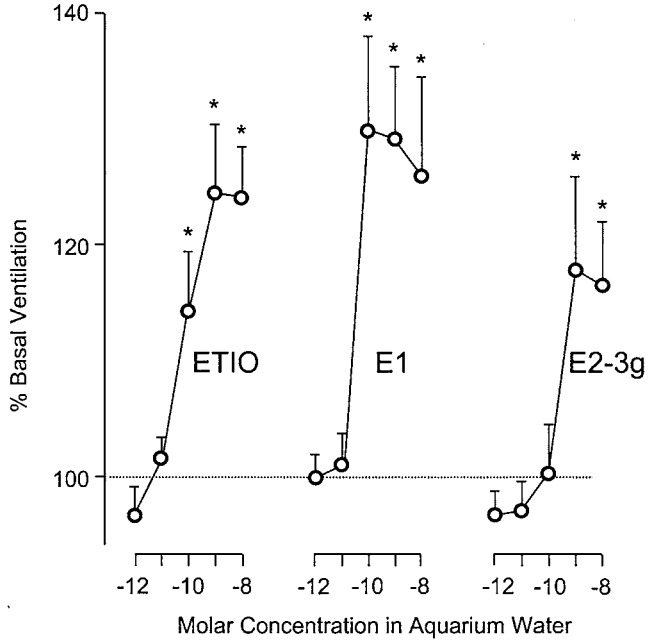


FIG. 6. Percent change in basal ventilation rate (mean + SE) of male gobies during the minutes 4 and 7 after exposure to ETIO, E1, and E2-3g. Ventilation responses to test substances were calculated as in Figure 4. $N = 6$ for all groups. *Mean ventilation rate after addition of a test substance is significantly different from basal ventilation rate ($P < 0.05$).

gobius exhibits EOG responses to a diverse array of steroids (Figure 1), but not to prostaglandins. These findings are consistent with the small number of EOG studies conducted in other perciform species that report a lack of olfactory responsiveness to a range of commercially available prostaglandins and responsiveness to a variety of steroidal compounds (Robison et al., 1998; Stacey et al., 1994b; Stacey and Cardwell, 1995, 1997; Murphy et al., 2000). Our EOG recording studies in *Neogobius* also indicate a lack of sexual dimorphism in detection of steroids (Figure 1). Although the precise reproductive status of the fish used in the recording (i.e., level of gonadal recrudescence) was not determined, other studies on fish report that gender and gonadal maturity have a very minor effect [common carp (Irvine and Sorensen, 1993)] or no effect at all [goldfish (Sorensen et al., 1995), tinfoil barb, *Puntius schwanenfeldi* (Cardwell et al., 1995)] on EOG detection and response magnitude of steroidal odors. By using ventilation increase as a behavioral bioassay, we also find that some detected steroids are perceived by *Neogobius* (Figure 4); unlike olfactory responses, however, behavioral responses to detected steroids is sexually dimorphic (Figure 5).

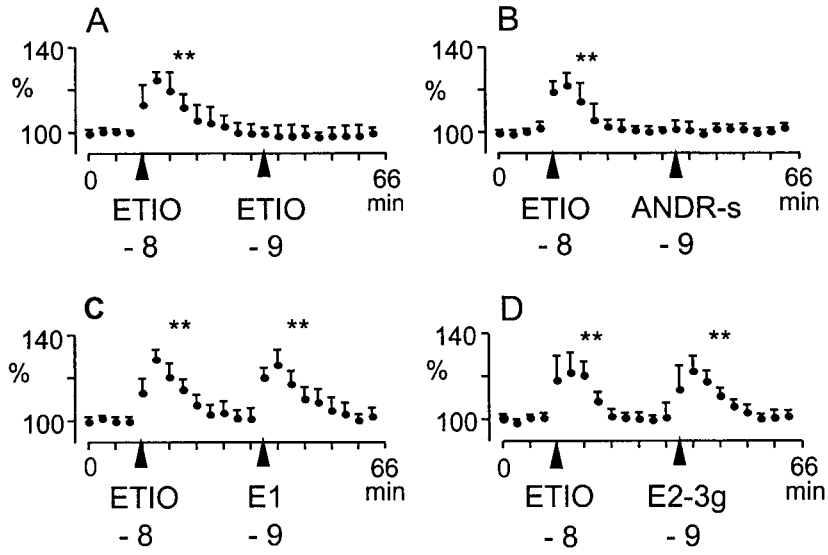


FIG. 7. Percent change in basal ventilation rate (mean + SE) of male gobies in behavioral cross-adaptation studies using 10^{-8} M ETIO (first arrow) as the adapting steroid and 10^{-9} M (A) ETIO, (B) ANDR-s, (C) E1, or (D) E2-3g (second arrow) as the test steroid. Ventilation responses to adapting and test substances were calculated as in Figure 4. $N = 6$ for all groups. **Mean ventilation rate after addition of an adapting or test steroid is significantly different from basal ventilation rate ($P < 0.01$).

Finally, our finding that ETIO-g induces olfactory and behavioral responses in *Neogobius* is consistent with biochemical and behavioral studies (Colombo et al., 1977, 1980, 1982) that indicate a pheromonal function for this steroid conjugate in the black goby, *G. jozo*.

Colombo et al. (1980) showed that ovulated female *G. jozo* are attracted to an ETIO-g source and often induced to oviposit in the absence of a male, whereas preovulatory females, or ovulated females that had completed oviposition, are not. In contrast, we observed no reproductive behavioral responses when isolated *Neogobius* or male–female pairs were exposed to ETIO-g or other detectable steroids (Murphy, 1998). Absence of reproductive behavior response in our studies could have been due to at least four factors. First, absence of female response to ETIO-g and related steroids was likely due to the fact that we were unable to obtain ovulated females for testing. Second, behavioral response to some steroid(s) might require the presence of nonolfactory cues that were not present in our test aquaria. Third, some detected steroids might induce only physiological responses. Fourth, although female *G. jozo* exhibit complex behavioral responses to ETIO-g alone, reproductive behavioral response in *Neogob-*

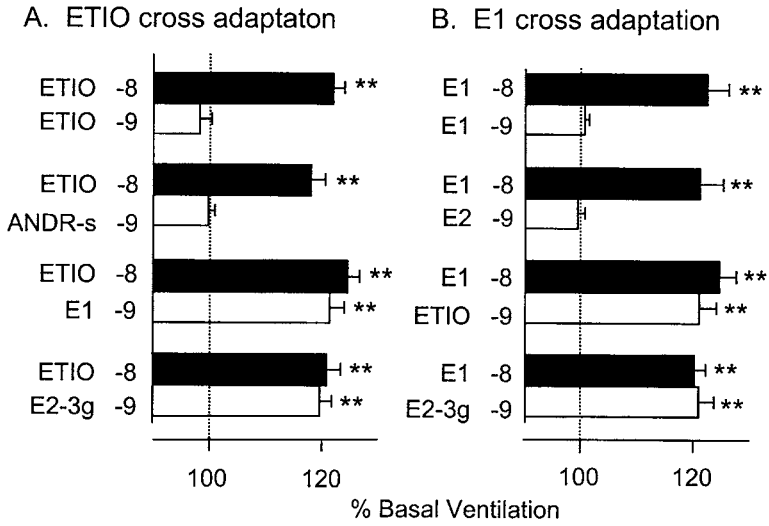


FIG. 8. Percent change in basal ventilation rate (mean + SE) of male gobies in behavioral cross-adaptation experiments using (A) 10^{-8} M ETIO and (B) 10^{-8} M E1 as the adapting steroids, as in Figure 7. Ventilation responses to adapting and test substances were calculated as in Figure 4. $N = 6$ for all groups. **Mean ventilation rate after addition of an adapting or test steroid is significantly different from basal ventilation rate ($P < 0.01$). Note (A) summarized from Murphy and Stacey (1999).

ius might require exposure to steroid mixtures. Nonetheless, we feel that the nature of the steroid-induced olfactory and ventilatory responses reported in this study provide evidence that steroidal compounds may function as reproductive pheromones in *Neogobius*.

Although the function of the ventilation responses to steroid odorants is not clear, the behavior may serve as a "sniffing" mechanism to facilitate odor detection by increasing water flow through the olfactory organ as has been proposed in flounder species (*Lepidopsetta bilineata* and *Platichthys stellatus*) (Nevitt, 1991). Ventilation rate increases may be analogous to tongue flicking observed in many lizard species in response to olfactory stimuli; an increase in the number of tongue flicks in response to novel odors is usually measured as an indication of odor detection in squamate reptiles (Cooper, 1994). Our ventilation rate bioassay requires further clarification on mode of action, as we have not ruled out other mechanisms of detection. For example, there is the small possibility that the steroids could be detected through the taste system or interact with gill processes. Despite these limitations, the ventilation rate measurements closely correspond to the olfactory measurements, and we predict that further investigation will show that this ventilation rate increase in response to steroids

is mediated by the olfactory system. Indeed, odor-induced ventilation could be a valuable behavioral bioassay for examining odor perception in other benthic fishes.

Our EOG cross-adaptation studies indicate that steroids detected by the olfactory organ of *Neogobius* (Figure 1) are discriminated by at least four proposed receptor mechanisms (E1, E2-g, ETIO, and DHEA-s); at least two compounds (ANDR-s and 3α,17α-5βP-g) appear to interact with two of these proposed receptor mechanisms (ETIO and DHEA-s) (Figure 3). Based on results of EOG concentration-response studies (Figure 2), we named the four proposed receptor classes after the most potent steroid odorants, assuming these to be likely natural ligands. However, the steroidal compounds released by *Neogobius* are not known.

Although our EOG and behavioral data indicate *Neogobius* possess multiple olfactory receptor mechanisms for steroidal compounds, using EOG cross-adaptation to link odorants with specific olfactory transduction processes (receptor mechanisms) can be problematic. For example, if olfactory receptors possess multiple binding sites for different odorants, an adapting odor that binds to one site could allosterically affect the binding properties of the other site(s), resulting in nonreciprocal cross-adaptation among compounds thought to share the same transduction mechanism (Caprio and Byrd, 1984). As well, if odorants possess more than one odotope (ligand–receptor binding site) (Shepherd, 1987), EOG cross-adaptation results will be complex. Interpretation of our cross-adaptation results also is confounded by the finding that even a brief (2-sec) pulse of steroid odor can reduce response to that odor when delivered 30 min later (Figure 3A). Therefore, due to the possibility that brief exposure to one compound reduces EOG response to all compounds detected via the same receptor mechanism, we maintained the same order of steroid exposure in our EOG cross-adaptation experiments and compared the reduction of test odorant response magnitude during adaptation to the reduction observed in the control (sequential exposure test; Figure 3A).

Despite these theoretical and practical problems inherent in EOG cross-adaptation studies, our interpretation of the cross-adaptation data is supported by results of the ventilation behavioral bioassays conducted with male *Neogobius*. Although males showed no behavioral response to one of the proposed steroid odorant classes (DHEA-s), they increased ventilation when exposed to the other three (E1, E2-3g, ETIO) (Figures 4 and 6), and did not respond to a steroid (T-g) that failed to induce an EOG response (Figure 5). Most importantly, ventilation responses of males clearly show they discriminate steroids that EOG cross-adaptation results indicate are detected by separate receptor mechanisms and do not discriminate steroids that appear to act through the same receptor mechanism (Figures 7 and 8).

Although behavioral assays have been used to examine discrimination of

amino acid odorants in channel catfish (*Ictalurus punctatus*) (Valentincic et al., 1994), the present study appears to be the first to demonstrate a clear relationship between peripheral (EOG) and central (behavioral) discrimination of steroid odorants. We expect that further behavioral cross-adaptation experiments using detected steroids not employed in this study will clarify the ability of *Neogobius* to discriminate steroid odorants.

Statistically, threshold ventilation response to ETIO and E1 (Figure 6) were lower (more sensitive) than threshold EOG responses (Figure 2); however, such a comparison likely has little biological significance. For example, given our small sample sizes for EOG concentration–response studies ($N = 6$), and the shapes of the concentration-response profiles (Figure 2), the statistical EOG thresholds appear to be conservative estimates of olfactory sensitivity. Moreover, the statistically greater sensitivity of ventilation responses to ETIO and E1 may simply have resulted from fish being exposed briefly to the injected steroid prior to its full dispersal throughout the test aquarium. The low detection thresholds to the steroids also suggest that the odors act through the olfactory system rather than the gustatory system (Sorensen and Caprio, 1998).

Unlike EOG responses to steroids (Figure 1), ventilation responses were clearly sexually dimorphic, males responding to three of the proposed steroid odorant types (E1, E2-3g, and ETIO) and females responding only to ETIO (Figure 4). Male androgenic steroid(s) appears to regulate this sexual dimorphism; females implanted with methyl-testosterone exhibit male-typical ventilation responses to E1 and E2-3g (Murphy and Stacey, unpublished results). The possible functional significance for this behavioral dimorphism is considered below.

Together, our EOG and behavioral data indicate the olfactory organ of *Neogobius* possesses at least four receptor mechanisms for steroid odorants. The proposed E1 receptor responded rather nonspecifically to unconjugated 18-carbon (C_{18}) steroids (E1, E2, and E2α), the nature of the oxygen group at C-17 apparently having little effect on olfactory potency. In contrast, addition of a hydroxyl group on C-16 (estriol; 1,3,5[10]-estratrien-3α,16α,17β-triol), or the addition of a glucuronide or sulfate group at C-3 or C-17 rendered the steroid undetectable by the proposed E1 receptor.

The proposed E2-3g receptor mechanism appears to interact with three seemingly diverse steroids (E2-3g, DHEA-g, and EPIANDR-g; Figure 3D,E). E2-3g and DHEA-g adapted each other, and both reduced the response to EPIANDR-g, although only the effect of DHEA-g adaptation on EPIANDR-g response was significant (Figure 3). Given the structure of the steroids adapted by ETIO (Figure 3B), it is expected that EPIANDR-g also interacts with the proposed ETIO receptor, although this was not examined. The structural requirements for ligands interacting with the proposed E2-3g receptor are unclear, given that it appears to interact with 5α-androstan (EPIANDR-g), 5-androstene

(DHEA-g), and estratriene (E2-3g) compounds. However, both a 5β- or planar glucuronide group at C-3 and an unconjugated oxygen group at C-17 appear important, because estratrienes without a C-3 glucuronide (E2), or with a C-3 sulfate (17β-estradiol-3-SO4) or C-17 glucuronide or sulfate (17β-estradiol-3-glucuronide-17β-SO_4), either did not induce EOG response (Table 1) or were unaffected by E2-3g adaptation (Figure 3). Unfortunately, no 5α-androstan-3α-glucuronides (e.g., androsterone-3α-glucuronide) were tested for interaction with the proposed E2-3g receptor, although ETIO-g, a 5β-androstan with a 3α-glucuronide, was detectable but unaffected by E2-3g adaptation (Figure 3E).

Given that E2-3g increased ventilation in male *Neogobius*, it is surprising that DHEA-g did not (Figure 5). The ineffectiveness of DHEA-g could have been due to the fact that it was a less potent olfactory stimulant than E2-3g (Figure 2). This seems unlikely, however, because ANDR-s, which appeared to be equipotent to DHEA-g in EOG concentration-response studies (Figure 2), increased ventilation when tested at a lower concentration than DHEA-g (Figure 5).

As males gobies are not reported to synthesize C_{18} steroids (Colombo et al., 1977; Asahina et al., 1989), we assume it is C_{18} steroids from females that normally interact with the proposed E1 and E2-3g receptors of males, either to signal that local females are vitellogenic, or to signal gender of females approaching the male's nest. The functional significance of separate receptors for conjugated and unconjugated C_{18} steroids is not known. However, studies on rainbow trout (*Oncorhynchus mykiss*) indicate that free steroids are preferentially released across the gills, whereas conjugates are released in the urine and feces (Vermeirssen and Scott, 1996). If this is the case with *Neogobius*, it is possible that E2-3g is released in concentrated urinary pulses and used by males to detect females at a distance, whereas E1 is released tonically at low concentration and used to identify females at the nest site.

The unconjugated C_{18} steroids detected by *Neogobius* are known to be detected by only a few species of the nonperciform fish (Orders Cypriniformes and Characiformes) that have been studied by EOG recording (Stacey et al., 1994b; Stacey and Cardwell, 1995, 1997). EOG studies indicate unconjugated C_{18} steroids are not detected by any of the small number of perciform species examined (Stacey et al., 1994b; Stacey and Cardwell, 1995, 1997; Robison et al., 1998; Murphy et al., 2000).

There is evidence that conjugated (glucuronated and sulfated) C_{18} steroids have pheromonal function in other species. C_{18} sulfates induce EOG responses in a number of characiform species (Stacey and Cardwell, 1995, 1997), and E2-3g and other glucuronated and sulfated forms induce EOG responses in a perciform, *Haplochromis burtoni* (Robison et al., 1998); however, there has been no attempt to determine pheromonal functions of the detected steroids in any of these species. Van den Hurk and Lambert (1983) proposed that male zebra fish (*Danio*

rerio) are attracted to females by a mixture of ovarian steroid glucuronides that contains 17β-estradiol-17β-glucuronide (E2-17g) and testosterone glucuronide (T-g). However, our EOG studies (Stacey and Cardwell, 1995, 1997), using the suite of steroids tested in *Neogobius*, indicate that zebra fish do not detect these steroid glucuronides, and instead detect one conjugated 4-pregnen compound (17,20β-P-20β-SO_4), which functions as a pheromone in goldfish (Sorensen et al., 1995). These EOG responses in zebra fish are consistent with studies of odor-induced olfactory bulb activity in zebra fish showing that prostaglandin $F_{2\alpha}$ and 17,20β-P-20β-SO_4 induce bulbar activity, whereas E2-17g and T-g do not (Friedrich and Korsching, 1998).

Structural requirements for ligands of the proposed DHEA-s receptor are not clear. A conjugate on C3 evidently is important, because DHEA did not induce an EOG response, but it appears the orientation of the conjugate is not critical, given that DHEA-s (a 5β- steroid) adapted the response to ANDR-s (a 5α- steroid; Figure 3C). Surprisingly, DHEA-s also adapted the EOG response to 3α,17α-5βP-3g. DHEA-s failed to increase ventilation in either sex and is reported to induce EOG response in only one other species, the cichlid *Haplochromis burtoni* (Robison et al., 1998).

The proposed ETIO receptor appears to be relatively nonspecific, since it interacted with a variety of C_{19} and C_{21} compounds, two of which (ANDR-s and 3α,17α-5βP-3g) also interacted with the proposed DHEA-s receptor (Figure 3B). However, the proposed ETIO receptor did exhibit specificity insofar as ETIO adaptation did not reduce EOG responses to 5-androsten compounds (DHEA-g, DHEA-g; Figure 3B) or to any steroids believed to interact with the proposed E2-3g and E1 receptors (Figure 3E,F). Both males and females increased ventilation when exposed to several steroids (ETIO, ETIO-g, and ANDR-s) that interact with the proposed ETIO receptor (Figure 5). Interaction of numerous 5β-reduced steroids with the proposed ETIO receptor is consistent with studies of *G. jozo* showing a preponderance of 5β-reduced products in in vitro incubations (Colombo et al., 1977). However, similar studies of the urohaze goby (*Glossogobius olivaceus*) indicate predominance of 5α-reduction in the testes and seminal vesicles (Asahina et al., 1989). Thus, interaction of the proposed ETIO receptor with a 5α-reduced steroid (ANDR-s) raises questions as to the nature of the natural ligands in *Neogobius*.

Olfactory and behavioral response to ETIO-g by the round goby is consistent with earlier work (Colombo et al., 1977, 1980, 1982) suggesting that this compound is released by male *G. jozo* to attract females and stimulate oviposition. However, given the number and variety of steroids interacting with the proposed ETIO receptor, it is likely that any pheromone acting through this receptor in *Neogobius* would be a mixture of C_{19} and C_{21} compounds. The fact that ETIO-g increases ventilation in both male and female *Neogobius* suggests that this and other steroids interacting with the proposed ETIO receptor induce repro-

ductive responses in both genders. It is possible that male *Neogobius* use ETIO to assess the proximity and reproductive status of neighboring males, whereas females use ETIO for mate search and mate selection, as is likely the case in *G. jozo* (Colombo et al., 1980).

In summary, this study of EOG and behavioral responses to steroidal compounds provides the most extensive data set on putative hormonal pheromones in any perciform species. Although the findings suggest that *Neogobius* has evolved a complex sex pheromone system, further research is required to determine the functions of the putative hormonal pheromones and the natural steroid odorants involved. Such research is warranted not only because *Neogobius* is a tractable species that could serve as a model for pheromone studies of paternal nest-guarding fishes, but also because further understanding might reveal pheromonal techniques to control the spread of this species in the Great Lakes and other North American aquatic ecosystems.

Acknowledgments—The authors thank J. R. Cardwell for help with EOG recording, B. Wakeford for help with fish maintenance, W. Ray and A. MacInnis for providing live specimens, and two anonymous reviewers for helpful comments on the manuscript. This research was supported by a grant to N.E.S. from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

ASAHINA, K., SUZUKI, K., HIBIYA, T., and TAMAOKI, B. 1989. Structure and steroidogenic enzymes of the seminal vesicles of the urohaze-goby (*Glossogobius olivaceus*). *Gen. Comp. Endocrinol.* 74:385–391.

CAPRIO, J., and BYRD, R. P. JR. 1984. Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in catfish. *J. Gen. Physiol.* 84:403–422.

CARDWELL, J. R., STACEY, N. E., TAN, E. S. P., MCADAM, D. S. O., and LANG, S. L. C. 1995. Androgen increases olfactory receptor response to a vertebrate sex pheromone. *J. Comp. Physiol. A* 176:55–61.

CHARLEBOIS, P. M., MARSDEN, J. E., GOETTEL, R. G., WOLFE, R. K., JUDE, D. J., and RUDNIKA, S. 1997. The round goby, *Neogobius melanostomus* (Pallus), a review of European and North American literature. Illinois-Indiana Sea Grant Program and Illinois Natural History Survey. Illinois Natural History Survey Special Publication No. 20.

COLOMBO, L., BELVEDERE, P., and PILATI, A. 1977. Biosynthesis of free and conjugated 5β-reduced androgens by the testis of the black goby, *Gobius jozo* L. *Boll. Zool.* 44:131–134.

COLOMBO, L., MARCONOTO, A., BELVEDERE, P., and FRISCO, C. 1980. Endocrinology of teleost reproduction: A testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* 47:355–364.

COLOMBO, L., BELVEDERE, P. C., MARCONOTO, A., and BENTIVEGNA, F. 1982. Endocrinology of teleost reproduction, pp. 84–94, *in* C. J. J. Richter and H. J. T. Goos (eds.). Proceedings of the Second International Symposium on the Reproductive Physiology of Fish. Pudoc, The Netherlands.

COOPER, W. E. 1994. Chemical discrimination by tongue-flicking in lizards: A review with hypotheses on its origin and its ecological and phylogenetic relationships. *J. Chem. Ecol.* 20:439–487.

CROSSMAN, E. J., HOLM, E., CHOLMONDELY, R., and TUININGA, K. 1992. First record for Canada

of the rudd, *Scardinius erythrophthalmus*, and notes on the introduced round goby, *Neogobius melanostomus. Can. Field Nat.* 106:206–209.

DUBS, D. O. L., and CORKUM, L. D. 1996. Behavioral interactions between round gobies (*Neogobius melanostomus*) and mottled sculpins (*Cottus bairdi*). *J. Great Lakes Res.* 22:838–844.

ESSINGTON, T. E., and SORENSEN, P. W. 1996. Overlapping sensitivities of brook trout and brown trout to putative hormonal pheromones. *J. Fish Biol.* 48:1027–1029.

FRIEDRICH, R. W., and KORSCHING, S. I. 1998. Chemotopic, combinatorial, and noncombinatorial odorant representations in the olfactory bulb revealed using a voltage-sensitive axon tracer. *J. Neurosci.* 18:9977–9988.

IRVINE, I. A. S., and SORENSEN, P. W. 1993. Acute olfactory sensitivity of wild common carp, *Cyprinus carpio*, to goldfish sex pheromones is influenced by gonadal maturity. *Can. J. Zool.* 71:2199–2210.

JUDE, D. J., REIDER, R. H., and SMITH, G. R. 1992. Establishment of gobiidae in the Great Lakes Basin. *Can. J. Fish. Aquat. Sci.* 49:416–421.

KATSEL, P. L., DMITRIEVA, T. M., VALEYEV, R. B., and KOZLOV, Y. P. 1992. Sex pheromones of male yellowfin Baikal sculpin (*Cottocomephorus grewingki*): isolation and chemical studies. *J. Chem. Ecol.* 18:2003–2010.

KITAMURA, S., OGATA, H., and TAKASHIMA, F. 1994. Activities of F-type prostaglandins as releaser sex pheromones in cobitide loach, *Misgurnus anguillicaudatus. Comp. Biochem. Physiol. A* 107:161–169.

MACINNIS, A. J., and CORKUM, L. D. 2000. Fecundity and reproductive season of the round goby, *Neogobius melanostomus*, in the upper Detroit River. *Trans. Am. Fish. Soc.* 129:136–144.

MILLER, P. J. 1984. The tokology of Gobiid fishes, pp. 119–153, *in* G. W. Potts and R. W. Wootton (eds.). Fish Reproduction: Strategies and Tactics. Academic Press, New York.

MOISEYEVA, Y. B., and RUDENKO, V. I. 1976. The spawning of the round goby, *Gobius melanostomus*, under aquarium conditions in winter. *J. Ichthyol.* 8:690–692.

MURPHY, C. A. 1998. Olfactory and behavioural responses to putative steroidal pheromones in the round goby, *Neogobius melanostomus*. MSc thesis, University of Alberta, Edmonton, Alberta.

MURPHY, C. A., and STACEY, N. E. 1999. Putative steroidal sex pheromones in the round goby, *Neogobius melanostomus*: Good correlation between sensory and behavioral discrimination, pp. 599–605, *in* R. E. Johnston, D. Muller-Schwarze, and P. W. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Publishers, New York.

MURPHY, C. A., MANIAK, P. J., and SORENSEN, P. W. 2000. Functional and biochemical characterization of a novel sex pheromone in the Eurasian ruffe, *Gymnocephalus cernuus*, p. 211, *in* Proceedings of the 9th International Zebra Mussel and Aquatic Nuisance Species Conference, Duluth, Minnesota, April 16–30, 1999. The Professional Edge, Pembroke, Ontario, Canada.

NEVITT, G. A. 1991. Do fish sniff? A new mechanism of olfactory sampling in pleuronectid flounders. *J. Exp. Biol.* 157:1–18.

PROTASOV, V. P., TZVETKOV, V. I., and RASHCHEPERIN, V. K. 1965. Acoustical signals in the Azov round goby *Neogobius melanostomus* (Pallas). *Zh. Obshch. Biol.* 26:151–159.

RAY, W. J., and CORKUM, L. D. 1997. Predation of zebra mussels by round gobies, *Neogobius melanostomus. Environ. Biol. Fishes* 50:267–273.

ROBISON, R. R., FERNALD, R. D., and STACEY, N. E. 1998. The olfactory system of a cichlid fish responds to steroidal compounds. *J. Fish Biol.* 53:226–229.

SHEPHERD, G. M. 1987. A molecular vocabulary for olfaction. *Ann. N.Y. Acad. Sci.* 510:98–103.

SORENSEN, P. W., and CAPRIO, J. 1998. Chemoreception, pp. 375–405, *in* D. H. Evans (ed.). The Physiology of Fishes, Second Edition. CRC Press, Boca Raton, Florida.

SORENSEN, P. W., and STACEY, N. E. 1999. Evolution and specialization of fish hormonal pheromones, pp. 15–47, *in* R. E. Johnston, D. Muller-Schwarze, and P. W. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Publishers, New York.

SORENSEN, P. W., SCOTT, A. P., STACEY, N. E., and BOWDIN, L. 1995. Sulfated 17α,20β-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. *Gen. Comp. Endocrinol.* 100:128–142.

STACEY, N. E., and CARDWELL, J. R. 1995. Hormones as sex pheromones in fish: widespread distribution among freshwater species, pp. 244–248, *in* F. W. Goetz and P. Thomas (eds.). Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. Fish Symposium 95, Austin, Texas.

STACEY, N. E., and CARDWELL, J. R. 1997. Hormonally-derived pheromones in fish: new approaches to controlled reproduction, pp. 407–454, *in* M. Fingerman, R. Nagabhushanam, and M.-F. Thompson (eds.). Recent Advances in Marine Biotechnology, Volume 1. Oxford-IBH, New Delhi.

STACEY, N. E., ZHENG, W. B., and CARDWELL, J. R. 1994a. Milt production in common carp (*Cyprinus carpio*): Stimulation by a goldfish steroid pheromone. *Aquaculture* 127:265–276.

STACEY, N. E., CARDWELL, J. R., LILEY, N. R., SCOTT, A. P., and SORENSEN, P. W. 1994b. Hormones as sex pheromones in fish, pp. 438–448, *in* K. G. Davey, R. E. Peter, and S. S. Tobe (eds.). Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa.

SVEINSSON, T., and HARA, T. J. 1995. Mature males of Arctic char, *Salvelinus alpinus*, release F-type prostaglandins to attract conspecific mature females and stimulate their spawning behavior. *Environ. Biol. Fishes* 42:253–266.

TAVOLGA, W. N. 1956. Visual, chemical and sound stimuli as cues in the sex discriminatory behavior of the gobiid fish, *Bathygobius soporator. Zoologica* 41:49–65.

VALENTINCIC, T., WEGERT, S., and CAPRIO, J. 1994. Learned olfactory discrimination versus innate taste responses to amino acids in the channel catfish (*Ictalurus punctatus*). *Physiol. Behav.* 55:865–873.

VAN DEN HURK, R., and LAMBERT, J. G. D. 1983. Ovarian steroid glucuronides function as sex pheromones for male zebra fish, *Brachydanio rerio. Can. J. Zool.* 61:2381–2387.

VAN DEN HURK, R., and RESINK, J. W. 1992. Male reproductive system as sex pheromone producer in teleost fish. *J. Exp. Zool.* 261:204–213.

VERMEIRSSEN, E. L. M., and SCOTT, A. P. 1996. Excretion of free and conjugated steroids in rainbow trout (*Oncorhynchus mykiss*): Evidence for branchial excretion of the maturation-inducing steroid 17α,20β-dihydroxy-4-pregnen-3-one. *Gen. Comp. Endocrinol.* 101:180–194.

WARING, C. P., and MOORE, A. 1997. Sublethal effects of a carbamate pesticide on pheromonal mediated endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. *Fish Physiol. Biochem.* 17:203–211.

WILKINSON, L. 1994. Systat 5.05 for Windows. SPSS, Evanson, Illinois.

ZAR, J. H. 1984. Biostatistical Analysis, 2nd ed. Prentice Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

1-OCTEN-3-OL ISOLATED FROM BONT TICKS ATTRACTS *Amblyomma variegatum*

C. McMAHON, P. M. GUERIN,* and Z. SYED

Institute of Zoology
University of Neuchâtel
Rue Emile-Argand 11
2007-Neuchâtel, Switzerland

(Received July 23, 2000; accepted November 12, 2000)

Abstract—Volatiles from various life-stages of the bont ticks *Amblyomma variegatum* and *A. hebraeum* were collected by using solid-phase microfibers and charcoal traps. An octenol isomer was found to be a major constituent of most of the tick material sampled and was identified as 1-octen-3-ol by gas chromatography–mass spectrometry and by using antenna of the tsetse fly *Glossina brevipalpis* in gas chromatography-linked antennogram detection. Release of this compound increased during molt to adulthood and following mechanical disturbance of adult ticks. (*R*)-(−)-1-Octen-3-ol and racemate 1-octen-3-ol both induce an increase in upwind walk to the odor source from *A. variegatum* in an airstream on a servosphere. Volatiles from tick exuviae plus feces and from dead ticks also attracted *A. variegatum*, suggesting that 1-octen-3-ol may contribute to the aggregation response of *Amblyomma* spp. on such substrates. 2,6-Dichloroanisol and 2,5-dimethylpyrazine also were detected in volatiles from the ticks but induced no behavioral responses on the servosphere. The suspected tick pheromone component, 2,6-dichlorophenol, was detected from *A. variegatum* adults cut into pieces but had no effect on the behavior of *A. variegatum* on the servosphere at a range of doses.

Key Words—1-Octen-3-ol, ixodid tick, *Amblyomma variegatum*, tick attractant.

INTRODUCTION

Secretions from dermal glands of both prostriate and metastriate ticks have been widely reported (Lees, 1947; Balashov, 1972, 1983; Yoder et al., 1993a; Walker et al., 1996). Particular attention has been paid to the aggregation–attachment pheromone of some *Amblyomma* spp. (Gladney et al., 1974; Apps et al., 1988;

*To whom correspondence should be addressed. e-mail: Patrick.Guerin@zool.unine.ch

0098-0331/00/0300-0471$19.50/0

Diehl et al., 1991) and the suspected tick pheromone product 2,6-dichlorophenol (De Bruyne and Guerin, 1994). It is known that several metastriate ticks transiently release cuticular droplets in response to physical disturbance, for which a defensive role has been suggested. Yoder et al. (1993a) found that *Dermacentor andersoni*, *D. variabilis*, *A. americanum* and *A. maculatum* release a "pungent odor" associated with the appearance of droplets on the cuticle. Pavis et al. (1994) observed the release of droplets by *A. variegatum* after disturbance, and Walker et al. (1996) reported the release of droplets with a "sour, nut-like smell" from unfed and fed *Rhipicephalus appendiculatus* adults after handling. Behavioral tests with these exudates were carried out by Yoder et al. (1993b) on *D. variabilis* and by Pavis et al. (1994) on *A. variegatum*. Each demonstrated that the exudate gave significant protection to ticks for a day in a Petri dish assay against fire ant predation. It is likely that protection was afforded in these studies by nonvolatile components of the exudates and not by the transient odor associated with them upon release from the cuticle.

Here, we collected odor volatiles released by both resting and physically disturbed *Amblyomma variegatum* (Fabricius) and *A. hebraeum* (Koch) by using solid-phase microfibers and charcoal. The volatiles were analyzed by gas chromatography-linked mass spectrometry (GC-MS) and by gas chromatography-linked electroantennogram detection (GC-EAD). A servosphere apparatus was used to examine the behavioral responses of *A. variegatum* adults to the headspace of ticks, to 1-octen-3-ol and other volatiles identified.

METHODS AND MATERIALS

Ticks. The two tick species examined, *A. variegatum* and *A. hebraeum* (Acari, Ixodidae), have been reared at the Centre de Recherche Santé Animale S.A., Novartis, St. Aubin, Switzerland since 1981. All stages (immature and adult) are fed on the tails of Simmental calves at 22–24°C and kept under constant darkness during molt at 28°C/80–90% relative humidity. For our experiments, engorged females, unfed adults of both *A. variegatum* and *A. hebraeum*, and unfed nymphs of *A. variegatum* were maintained in a 10L : 10D cycle with 2-hr ramps of dawn and dusk at 19°C/90% relative humidity. *A. variegatum* adults of either sex, between 1 and 9-months old, were prepared for behavioral tests on the servosphere as in McMahon and Guerin (2000). Each tick was tested only once.

Odor Sampling with SPME. Two solid-phase microextraction (SPME) stationary phases (Supelco) were used: Carbowax/divinylbenzene (Carbowax/DVB), which can adsorb volatile alcohols, and Carboxen/polydimethylsiloxane (Carboxen/PDMS), which has an affinity for hydrocarbons after several hours with the analyte. The latter phase with its lower affinity for water is more suit-

able for sampling over biological material in humid environments. The fibers were preconditioned for 2 hr at 300°C in a N_2 stream.

Odor Sampling over Undisturbed Ticks. Volatiles from resting or dead ticks were sampled by using the Carboxen/PDMS fiber that was inserted without disturbing the resting ticks into a horizontally held glass tube (12 cm long, 2 cm diam.) containing 200 ticks and sealed at both ends with perforated plastic stoppers. The control consisted of placing a fiber in an empty glass tube within 50 cm of the test tube. The collection fibers were left to stand overnight. In this manner, volatiles from the following tick substrates were sampled: 9-month-old *A. variegatum* nymphs, 1- and 9-month-old *A. variegatum* adults, *A. variegatum* adults that had died in the previous two months, and 12-month-old *A. hebraeum* adults.

Odor Sampling over Manipulated Ticks. The Carbowax/DVB fiber was used to sample volatiles from disturbed unfed *A. variegatum* adults of both sexes by holding five unfed adults in a 2-ml vial and squeezing the ticks individually with forceps. The vial was sealed immediately with Parafilm, and a Carbowax/DVB fiber was inserted through the seal and held over the ticks for 1 hr. The sampling was repeated overnight. The control for this test was to hold the fibers above undisturbed ticks for the same time intervals. The Carbowax/DVB fiber also was used to collect volatiles from four *A. variegatum* females killed by freezing and immediately cut into four pieces.

Odor Sampling with Charcoal. Air was sucked with a membrane pump (200 ml/min) over groups of five gently shaken engorged *A. hebraeum* or *A. variegatum* females (<1 week after drop-off) held in a 500-ml gas-wash bottle. Volatiles from the ticks were trapped on a 5-mg charcoal trap held in a 60-mm long × 4-mm-diam. glass tube in the closed-loop stripping apparatus of Grob and Zürcher (1976). As water condensation can reduce the effectiveness of charcoal as an adsorbant, the charcoal trap was heated to 10°C above room temperature during odor entrainment. After collection of volatiles for 10 min, the flask was gently shaken again to induce the release of more droplets from the dermal glands, and odors were collected onto the charcoal trap for another 10 min. The charcoal trap was extracted with 12 μl dichloromethane, of which 2 μl were analyzed (below).

Volatiles from 9-month-old unfed *A. variegatum* adults were entrained onto a charcoal trap (above) from air (200 ml/min) passing over 200 ticks held in a 50-ml gas-wash bottle and shaken to induce release of volatiles from dermal glands. The odor collected was extracted with dichloromethane (as above).

Gas Chromatography-Linked Mass Spectrometry Analysis. The SPME fibers were withdrawn into the hollow needle of the holding syringe after odor sampling and reexposed for 1 min to desorb the trapped volatiles in a split/splitless injection port (injection temperature, 280°C) of a gas chromatograph (Hewlett-Packard 5890 GC) linked to a HP 5971A mass selective detector

(MSD). The analyte was swept from the injector port onto a DB-Wax high-resolution fused-silica capillary column (30 m long, 0.25 mm ID, 0.25-μm film thickness, J&W Scientific) with a precolumn (1 m deactivated fused silica). The column was connected via a 1-m deactivated fused-silica capillary (0.25 mm ID) to the MSD ionization chamber (temperature 160°C, ionization energy 70 eV). The MSD, in the EI mode, scanned for masses m/z 20–300. Helium was used as carrier gas at 50 kPa head pressure (but was set at a constant flow, 30 cm/sec for on-column injection of the charcoal trap extract, see above). Components of the desorbed material were identified by comparing the mass spectra of unknowns in the analyte with those of standards in a library of the HP Chemstation software and by matching retention times with synthetic equivalents.

Gas Chromatography-Linked Electroantennogram Detection. Tick volatiles were tested for the presence of 1-octen-3-ol by using the antenna of the tsetse fly *Glossina brevipalpis* in GC-linked electroantennogram detection (GC-EAD) (Arn et al., 1975). The antenna of *G. brevipalpis* is sensitive to subnanogram levels of this compound eluting from a GC column. Furthermore, by using the specific response of the antenna of *G. brevipalis* to 1-octen-3-ol (Ujváry et al., 2000), the product can be identified by its retention time and by comparing the response of the antenna to the analyte with that to a similar amount of the naturally occurring pure (*R*)-(−) isomer. This was achieved by comparing the ratio of the EAG to FID peak heights for the extract and natural (*R*-(−)-1-octen-3-ol.

The procedure for electroantennogram detection briefly described is as follows. Volatiles were delivered on-column to a Carlo Erba 5160 GC with a flame ionization detector (FID at 260°C). The extract or headspace was injected in splitless mode onto a 25-m fused-silica SE-54 column (Macherey-Nagel), 0.25 mm ID, 0.35-μm film thickness with H_2 as carrier gas (27 cm/sec). The column effluent was split (glass Y-splitter) so that 60% was directed to the FID and 40% to the electrophysiological preparation. The latter was swept by a conditioned airstream (see below) to the electrophysiological preparation from a heated transfer line (240°C) in the wall of the chromatograph in such a way that the column effluent was simultaneously monitored by the FID and fly antenna. The method of mounting the fly head for EAG recording was as in Guerin and Visser (1980). Briefly, the fly head was excised after momentarily anaesthetizing it with CO_2. A chloridized silver wire was placed in a drawn-out capillary (10-μm tip diameter) filled with 0.1% KCl that was inserted through the occipital opening reaching into the pediculus and served as the reference electrode. This preparation was then mounted under a microscope (WILD Kombistereo M3Z) where the antenna was bathed in a humidified airstream (90–100% relative humidity, 23 ± 2°C) flowing at 1 m/sec via a glass water-jacketed tube (6 mm ID) whose outlet was about 1 cm from the preparation. The unbroken tip of the recording electrode was brought into contact with the funiculus surface upon which the tip

broke. Funiculus surface contact was enough to record the EAG. Micromanipulators (Leitz) permitted accurate positioning of the preparation and recording electrode. The EAG signal was captured via a silver wire in the electrolyte-filled (0.1 M KCl) glass electrode connected to a high-impedance preamplifier and an AC/DC amplifier (UN-03, Syntech) and recorded on the hard disk of a PC with the FID signal via a 16-bit analog–digital IDAC card (Syntech) by using the GC-EAD software package (Syntech), and monitored simultaneously with an oscilloscope (Tektronix 5103).

G. brevipalpis antennae were employed in GC-EAD to test for the presence of 1-octen-3-ol in volatiles collected on charcoal from 9-month-old disturbed *A. variegatum* adults as described above. *G. brevipalpis* EAD responses also were used to monitor directly for the presence of 1-octen-3-ol in the air above tick material. For this, *A. variegatum* exuviae (0.3 g; 5 days after molt) from 150 individuals were placed in a closed 5-ml vial with a Teflon-coated rubber seal. One day later, 2 ml of air was withdrawn from this vial with a gas-tight syringe and injected on to the DB-Wax column in splitless mode (1 min). To facilitate the large-volume injection, the column head pressure was reduced manually for 5 sec during injection and then raised to 65 kPa. Analysis for the presence of 1-octen-3-ol over 10 unmolted *A. variegatum* ticks was carried out in the same manner. The influence of molting on the release of 1-octen-3-ol in the headspace was analyzed by sealing 10 unmolted *A. variegatum* ticks in a 5-ml vial as before. After two animals had molted, 2 ml of this headspace was withdrawn in the gas-tight syringe without disturbing the animals and injected in splitless mode as above. In addition, odor puffs from groups of five undisturbed and just disturbed *A. variegatum* adults were delivered to the antenna of *G. bevipalpis* from the barrels of 5-ml plastic syringes in which the ticks were held to test for electroantennogram responses (Steullet and Guerin, 1992).

Behavior. A locomotion compensator or servosphere (Kramer, 1976) was used to evaluate the behavioral responses of walking ticks to volatiles from tick material borne in an airstream (18 cm/sec, 70% relative humidity, 23–25°C) (McMahon and Guerin, 2000). Only the responses to *A. variegatum* adults were tested. Briefly, an animal is maintained at the apex of a Perspex sphere (50 cm diam.) to which the airstream is directed. Displacements of the sphere caused by the moving animal are monitored by pulse generators allowing the reconstruction of the tracks described by the arthropod (Kramer, 1976). This permits an evaluation of several parameters such as speed, angular velocity, the duration of the walk, and direction. The latter parameters can be used to calculate upwind (attractive) responses to volatiles. Two estimates of the upwind responses elicited by a treatment in the test period (1 min) compared to a control period of equal duration were employed in this study: (1) the percentage time spent walking in a cone 60° either side of due upwind, and (2) the change in "target vector" (Jones, 1977). The target vector provides an estimate of the efficiency with which a par-

ticular direction is followed. It is independent of the arbitrarily chosen upwind cone and is calculated by multiplying the cosine of the mean direction (∅) by the path straightness (circular statistics after Batschelet, 1981). Values for target vector range from −1 (downwind direction) to +1 (upwind direction).

Walks of the ticks also were monitored for a further minute (end-control period) following the withdrawal of test vapors to determine if they executed any off-responses. Such off-responses consist of the tick describing small circles, abruptly turning downwind, or both, within 10 sec of the loss of chemical. These behaviors never occur during control runs (McMahon and Guerin, 2000). Intertreatment comparisons of the proportion of ticks displaying such off-responses were carried out by using the Fischer exact test.

Tick Odors Tested on the Servosphere. Vapors from the following biological substrates were presented to walking *A. variegatum* adults on the servosphere from 500-ml gas-wash bottles (after McMahon and Guerin, 2000): (1) air at 150 ml/min swept over 200 dead *A. variegatum* adults, (2) air at 10 ml/min passed over 0.6 g of exuviae plus feces and of newly molted (<2 weeks) *A. variegatum* adults, (3) air at 75 ml/min over 20 unmolted *A. variegatum* adults, (4) air at 75 ml/min over 10 freshly molted *A. variegatum* adults (<48 hr after emergence), (5) air at 75 ml/min over five engorged *A. hebraeum* females, and (6) air at 75 ml/min over five engorged *A. variegatum* females. During experiments 5 and 6, the bottle containing engorged females was shaken gently after every two tests to provoke the release of more droplets from the dermal glands. The blank controls for the above experiments consisted of passing air through empty gas-wash bottles at the same flow rate as used in the tests.

Synthetic Volatiles Tested on the Servosphere. Vapors of the following compounds were delivered separately to *A. variegatum* adults on the servosphere from 500-ml gas-wash bottles at 150 ml/min (after McMahon and Guerin, 2000): 2,5-dimethyl pyrazine, 2,6-dichloroanisol (Aldrich; source doses 10 ng and 1 μg), 2,6-dichlorophenol (2,6-DCP, Supelco; source doses 100 fg to 10 ng in log steps), (*R*)-(−)-1-octen-3-ol (Robertet; source dose 10 ng); racemate 1-octen-3-ol (Merck; source dose 10 ng). All compounds were >97% pure as indicated by GC and were prepared in dichloromethane (Merck, analytical grade). The racemate was a 50 : 50 mixture of (*R*)-(−)-1-octen-3-ol and *S*)-(+)-1-octen-3-ol (separated on a 0.25-μm film of the chiral phase 6-TBDMS-2,3-diacetyl-β-cyclodextrin 100% in OV-1701 coated on a 30-m × 0.32-mm-ID capillary column with He as carrier gas).

RESULTS

Volatiles identified from A. hebraeum *and* A. variegatum. An octenol isomer matching the mass spectrum and retention time of racemate 1-octen-3-ol

was identified by GC-MSD over engorged *A. variegatum* (6 ng) and *A. hebraeum* females (0.2 ng) collected onto charcoal (Figure 1a, Table 1). This product also was identified in all the headspace samples of either species sampled with the Carboxen/PMDS fiber (Table 1) at levels between 0.2 and 2 ng. Furthermore, GC-MSD analysis of the volatiles over five unfed, undisturbed, and disturbed *A. variegatum* adults with the Carbowax/DVB fiber demonstrated that release of octenol (0.6 ng) was associated with squeezing *A. variegatum* (Figure 1b, Table 1). No octenol was detected after sampling the headspace for a further 13 hr, or in the headspace of the undisturbed control ticks with this SPME fiber.

The octenol isomer was identified as 1-octen-3-ol since it elicited the same response as the natural product from the antenna of *G. brevipalpis* in GC-EAD analysis of the vapors over disturbed unfed *A. variegatum* collected on charcoal (Figure 2). In the other GC-EAD analyses that used *G. brevipalpis* antennae, 1-octen-3-ol was the predominant chemical detected over freshly molted ticks at levels of 1 ng/tick. However, 1-octen-3-ol was not found over unmolted ticks or exuviae, the only tick headspace samples in which this compound was not detected. Direct stimulation of the *G. brevipalpis* antennae with volatiles from undisturbed and disturbed tisks held in syringes corroborated the GC-MSD and GC-EAD finding, i.e., the release of 1-octen-3-ol by ticks is enhanced by physical manipulation. Air passing over undisturbed ticks to the antenna failed to induce an EAG response, whereas the air over disturbed ticks caused a strong depolarization of the antennal receptors.

Other compounds, including short-chain aliphatic aldehydes and alcohols were recovered from both the charcoal trap extract and the SPME fibers in the vapors over engorged *A. variegatum* and *A. hebraeum* females. However, few of these volatiles were recovered in sufficient quantities to permit identification (the MSD has a detection limit for a full mass spectrum at ca. 0.1 ng). Apart from 1-octen-3-ol, only three compounds above the MSD threshold were identified over ticks (Table 1). 2,6-Dichlorophenol was detected only in the headspace of *A. variegatum* females cut into pieces (ca. 1 ng), and 2,6-dichloroanisol and 2,5-dimethyl pyrazine were identified only over 9-month-old or dead *A. variegatum* and *A. hebraeum* adults and, as such, may be degradation products. These three tick products had matching mass spectra and retention times with synthetic equivalents. Retrospective analysis for the presence of the major ion of each of these three compounds in the other tick headspace samples analyzed by GC-MSD failed to indicate the presence of any of these products at their respective retention times.

Behavior. Odors from tick material elicited behavioral responses in *A. variegatum* adults walking on the servosphere. Exuviae plus feces from freshly molted *A. variegatum* adults induced a significant increase in the time spent walking in the upwind cone ($P < 0.05$; Table 2). Volatiles of dead conspecifics attracted *A. variegatum* adults ($P < 0.01$; Table 2) and also induced a significant

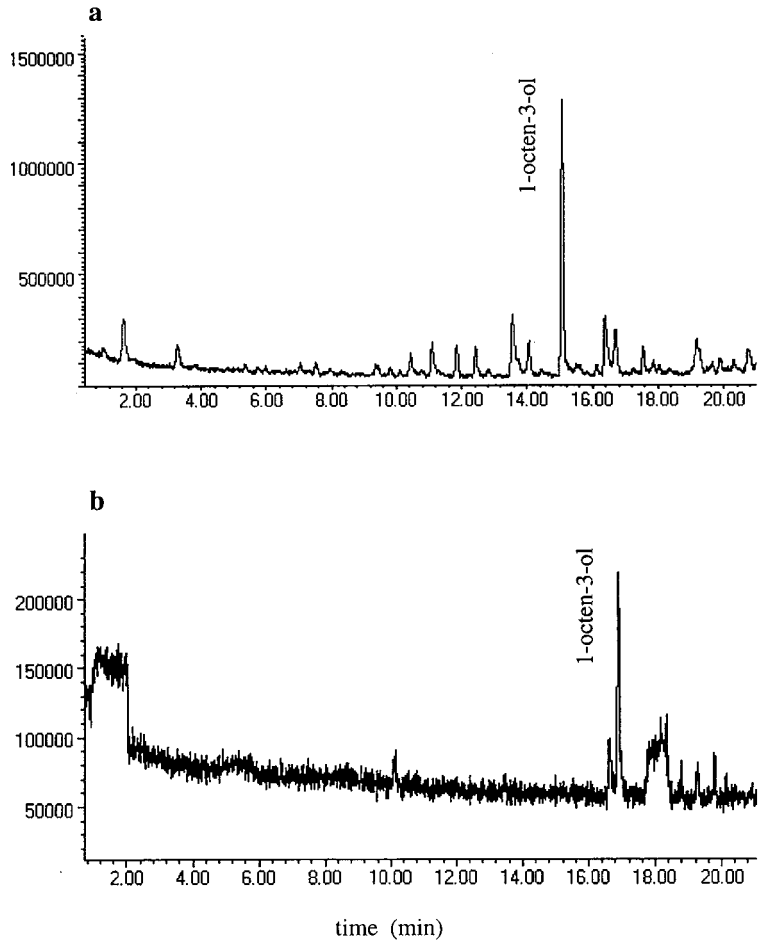


FIG. 1. Total ion chromatograms of volatiles collected over (a) disturbed engorged *Amblyomma variegatum* females by using a charcoal trap and (b) over unfed disturbed *A. variegatum* adults sampled by solid-phase microextraction with a Carbowax/divinylbenzene coated fiber (see text). Each 2-min interval (abscissa on the chromatogram) is equivalent to a 10°C rise in the oven temperature, starting at 40°C after 5 min. 1-Octen-3-ol predominates in both analyses occurring at a level of ca. 6 ng in a and ca. 0.6 ng in b in the 5–135°C elution temperature range presented. It was identified by matching the retention time and mass spectrum with a synthetic standard. The shift in the retention time of 1-octen-3-ol between the two chromatograms is due to a difference in column head pressure employed for the two analyses (on-column for the charcoal trap extract, splitless desorption for SPME fiber).

TABLE 1. COMPOUNDS IDENTIFIED IN HEADSPACE OVER *A. variegatum*

Compound	Identification by GC-MS			Identification by GC-EAD with *Glossina* antenna as detector	
	SPME		Charcoal entrainment	Direct headspace sampling	Charcoal entrainment
	Carboxen/PDMS	Carbowax/DVB			
1-Octen-3-ol	Disturbed adults Engorged females[a] Nymphs Dissected females Dead ticks Exuviae plus feces Undisturbed adults (1 month old)[a] Undisturbed adults (9 months old)[a]	Disturbed adults	Disturbed adults Engorged females[a]	Newly moulted adults	Disturbed adults
2,6-Dichlorophenol	Dissected females				
2,6-Dichloroanisol	Dead ticks Undisturbed adults (9 months old)[a]				
2,5-Dimethyl pyrazine	Dead ticks Undisturbed adults (9 months old)[a]				

[a]Compounds were also detected in the headspace of the same life stage of *A. hebraeum*.

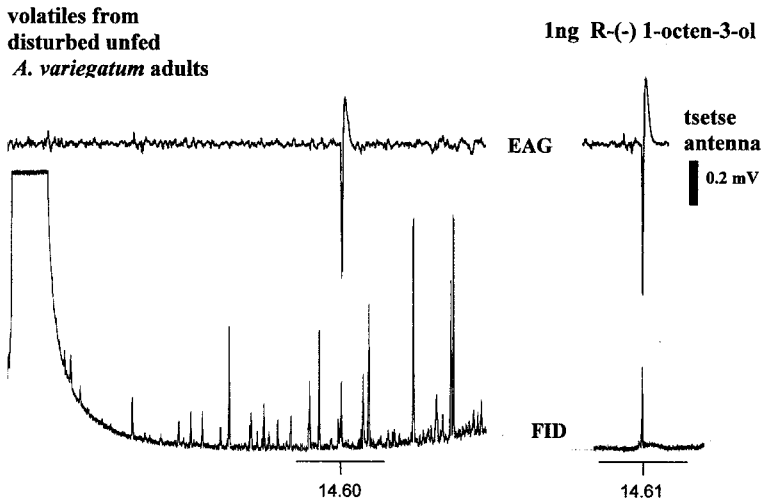


FIG. 2. Analysis of volatiles collected on a charcoal trap from disturbed unfed *Amblyomma variegatum* adults with the antenna of the tsetse fly, *Glossina brevipalpis*, as a biological detector in gas chromatography coupled electroantennogram detection (EAD). 1-Octen-3-ol eluted at 14.60 min. The amplitude of the EAG response (right) to 1 ng of (*R*)-(−)-1-octen-3-ol matched the response of a similar amount of the biologically active product in the extract. Several of the FID peaks are laboratory pollutants present in controls.

decrease in speed ($P < 0.01$). Odors from freshly cut females and unmolted and freshly molted *A. variegatum* adults did not attract conspecifics or influence their walking behavior. Odors from disturbed engorged *A. hebraeum* or *A. variegatum* adult females were unattractive, but *A. variegatum* responded to the odor of disturbed engorged *A. hebraeum* with a significant decrease in speed ($N = 14$; $P < 0.05$) and to disturbed engorged conspecifics with a decrease in angular velocity ($N = 24$; $P < 0.01$). No off-response occurred upon the withdrawal of tick odor.

The response to 1-octen-3-ol on the sphere was typified by an increase upwind walk (Figures 3 and 4) at a reduced speed and increased angular velocity. Both racemate 1-octen-3-ol and (*R*)-(−)-1-octen-3-ol attracted male and female *A. variegatum*. Nonetheless, (*R*)-(−)-1-octen-3-ol was a weaker attractant than any other treatment (Table 2), eliciting an increase in the walk in the upwind cone of just 8% and a moderate but insignificant increase in target vector. Although racemate 1-octen-3-ol was a strong attractant, evoking an increase in the walk in the upwind cone of 47%, no other differences in the behavior of the ticks were detected to these treatments. Furthermore, both 1-octen-3-ol

TABLE 2. UPWIND AND OFF-RESPONSES OF *A. variegatum* ADULTS ELICITED BY TICK ATTRACTANTS ON SERVOSPHERE[a]

Treatment (source dose)	*N*	% Change in time spent walking in upwind cone			Change in target vector			Off-responses	
		Median upwind response (interquartile range[b])	Wilcoxon signed rank test for a treatment (two-tailed *P* values)	Significance of change in duration of walk in upwind cone between treatments $P < 0.05$)	Median upwind response (interquartile range[b])	Wilcoxon signed rank test for a treatment (two-tailed *P* values)	Significance of change in target vector between treatments ($P < 0.05$)	Proportion of ticks showing an off-response	Significance of proportion of ticks showing off responses between treatments (Fisher exact test, two-tailed, $P < 0.05$)
Exuviae plus faeces (0.6 g)	18	11 (−1 to 31)	0.05	a	0.26 (−0.00 to 0.62)	0.01	a	1/18	b
Dead ticks (200)	13	21 (2–41)	0.01	a	0.23 (−0.05 to 0.64)	0.05	a	0/13	b
(*R*)-(−)-1-octen-3-ol (10 ng)	20	8 (−6 to 54)	0.05	ab	0.04 (−0.23 to 0.87)	0.23 (NS)	a	8/20	a
Racemate 1-octen-3-ol (10 ng)	21	47 (22–62)	0.001	b	0.71 (0.42 to 1.06)	0.001	b	13/20	a

[a] The index of the upwind response presented is the percentage difference in time spent walking in an upwind cone 60° either side of the air stream in the test period compared to the preceding control period. Intertreatment comparisons of the upwind responses were carried out by applying the Wilcoxon-Mann-Whitney test (two-tailed) to both the percentage change in the time spent walking in the upwind cone and to the change in target vector (values not shown) elicited by the treatments. Intertreatment comparisons of the proportion of off-responses induced by loss of stimulus were made with the Fisher Exact test (two-tailed). NS: Vapors of (*R*)-(−)-1-octen-3-ol did not elicit a significant change in target vector. Within a behavioral category, responses followed by the same letter are not significantly different.

[b] The interquartile range represents the 25 and 75 percentiles of the upwind response.

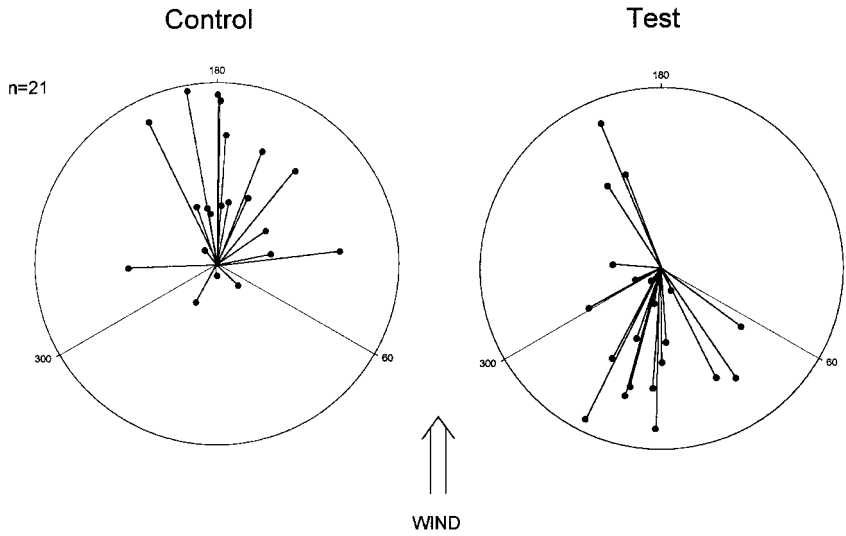


FIG. 3. Polar plot of the distribution of the mean direction (ϕ) of walks of *A. variegatum* adults ($N = 21$) on the servosphere during a 1-min control period followed by a 1-min test period where the same ticks were presented with vapors of a racemic mixture of 1-octen-3-ol (source dose 10 ng). The nearer the vector weighting (r) to the perimeter, the straighter the walk in that direction [circular statistics, after Batschelet (1981)]. Note the shift in mean walking directions in the presence of racemic 1-octen-3-ol.

samples elicited off responses (Table 2 and Figure 4) in 21 of a total of 41 *A. variegatum* adults tested, where the ticks reacted to the loss of the attractant either by describing small circles, abruptly turning downwind, or both in the 10 sec after loss of stimulus. This off-response was unique to 1-octen-3-ol. No such responses were observed for any other chemicals or tick volatiles presented to *A. variegatum* adults (Table 2).

The synthetic equivalents of the other products identified from ticks, i.e., vapors of 2,6-chloroanisol and 2,5-dimethyl pyrazine (source doses of 10 ng and 1 μg) presented separately to *A. variegatum* adults ($N = 15$, $N = 20$, respectively) failed to elicit any behavioral responses from *A. variegatum* on the servosphere, nor did 2,6-DCP have any effect on *A. variegatum* behavior on the servosphere in several tests spanning a source dose range with 10-fold increases from 100 fg to 10 ng ($N > 10$ for each dose).

DISCUSSION

1-Octen-3-ol is present in volatiles over various life-stages of both *A. variegatum* and *A. hebraeum*. Moreover, this compound attracts unfed *A. variegatum*

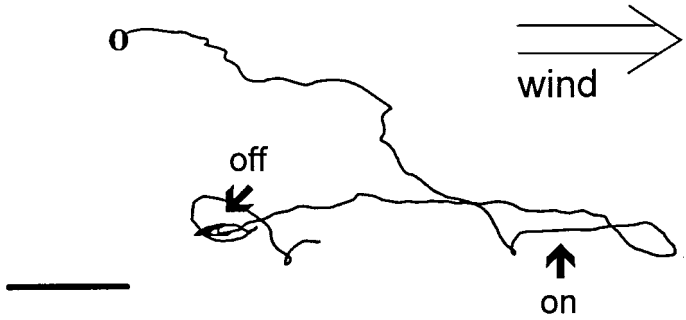


FIG. 4. Track described by an *Amblyomma variegatum* adult on the servosphere in response to vapors from racemate 1-octen-3-ol (source dose 10 ng). The track started (o) with the tick walking downwind (open arrow) in the control period. Note the off-response on loss of the attractant at odor off. The arrows on the track indicate odor on and odor off, and the bar beneath the track represents a displacement of 20 cm. Control, test, and end control periods of 1 min each.

adults on the servosphere. Whereas 1-octen-3-ol is a known semiochemical for hematophagous insects such as tsetse flies (Hall et al., 1984) and mosquitoes (Braverman et al., 1991), to our knowledge, this is the first report of 1-octen-3-ol being attractive to ticks. Although Norval et al. (1987) recorded activation of *A. hebraeum* in the field by odors of cattle and sheep, these authors recorded no such response to an open vial containing neat 1-octen-3-ol in the same study. Osterkamp et al. (1999) demonstrated that this compound enhanced the questing behavior of *Boophilus microplus*, albeit at the source dose of 3 μg compared to the 10-ng source dose used here. The level tested in this study is equivalent to about one twentieth that in bovid breath (Vale and Hall, 1985), and within the range of concentrations found attractive in wind-tunnel tests to the tsetse fly *Glossina morsitans morsitans* (Paynter and Brady, 1993) and the stable fly *Stomoxyes calcitrans* (Schofield and Brady, 1997).

The identity of the 1-octen-3-ol isomer released by the two *Amblyomma* tick species in our study is unknown. (*R*)-(−)-1-octen-3-ol predominates in bovid breath (Hall et al., 1984), probably arising from the enzymatic oxidation of linoleic acid (Tressl et al., 1982), but the racemate can arise from the nonenzymatic degration of this polyunsaturated fatty acid (Porter et al., 1980). In behavior, both 1-octen-3-ol samples tested elicited a similar proportion of off-responses upon withdrawal of the stimulus for *A. variegatum*, responses that were all but absent from the other treatments. Nonetheless, we found racemate 1-octen-3-ol to be a superior attractant to (*R*)-(−)-1-octen-3-ol, suggesting that the (*S*)-(+) isomer in the racemate might exert an independent effect. However, it is probably in the context of other as yet unidentified volatiles from ticks that

1-octen-3-ol exerts its effect. A difference in the efficacy of single compounds as attractants compared to the mixtures in which they are released has already been reported for this tick species (McMahon and Guerin, 2000).

The exact biological role of 1-octen-3-ol in the biology of *A. variegatum* and *A. hebraeum* remains to be determined. The greater amounts of 1-octen-3-ol recovered over disturbed ticks is consistent with reports such as that of Yoder et al. (1993b), wherein even bright light was sufficient to provoke the secretion of droplets by engorged *D. variabilis* females. Nonetheless, odors from tick material releasing relatively high amounts of 1-octen-3-ol in this study (newly molted ticks and engorged female *A. hebraeum* and *A. variegatum*) did not induce either attraction or repellency in *A. variegatum* adults on the servosphere. This suggests that ticks are either insensitive to the strong odor released or that some essential components of the odor did not reach the ticks on the servosphere. In contrast, attraction was recorded to samples emitting lower levels of volatiles, such as from dead ticks and exuviae plus feces. In the latter treatment, fecal volatiles are suggested as the potential source of the attractants, as 1-octen-3-ol was not detected over *A. variegatum* exuviae by using the sensitive electroantennogram detection method. The contribution of fecal attractants to "self-odor" in arthropods has long been recognized, particularly in aggregation. Ammonia has been implicated in the aggregation responses of arthropods, such as kissing bugs (Taneja and Guerin, 1997) and flour mites (Levinson et al., 1991) to their own feces, but fecal volatiles and odors released from the arthropods themselves may interact. Torto et al. (1996) demonstrated that phenols released from nymphal feces of the desert locust *Schistocerca gregaria* combined with several fatty acids and aldehydes released from nymphs themselves synergized the aggregation response. A similar role may exist for the several unidentified aldehydes present over engorged females (a particularly rich source of volatiles) in this study. Moreover, 1-octen-3-ol and several short chain aldehydes also are emitted by the host (Hall et al., 1984; Steullet and Guerin, 1994), and host volatiles attract *A. variegatum* (McMahon, 1999). It appears that ticks may be making parsimonious use of 1-octen-3-ol as a cue for aggregation with conspecifics and for host finding when in search of a blood meal.

Acknowledgments—This research was supported by the Swiss National Science Foundation (grants 31-37420.93 and 31-50658.97 entitled "Studies on the Chemical Ecology, Sensory Physiology and Behavior of Ticks"). We are indebted to the Hasselblad, Roche, and Sandoz Foundations, the Ciba-Geigy-Jubilaeums-Stiftung, and the Swiss Office for Education and Science for funding studies on tick sensory physiology and behavior at Neuchâtel. We thank Messrs P. Bula and J. Jonczy of Novartis Centre de Recherche Santé Animale S.A., Fribourg, Switzerland, for supplying us with ticks, and the International Atomic Energy Agency, Siebersdorf Laboratories, Austria, for supplying us with tsetse flies. We also thank Dr. D. Joulain, Robertet S. A., Grasse, France for the gift of (*R*)-(1)-1-octen-3-ol. The authors are grateful to Mainak Das for help with the behavioral tests. This paper is part of the PhD thesis submitted by C. McMahon at the University of Neuchâtel.

REFERENCES

APPS, P. J., VILJOEN, H. W., and PRETORIUS. V. 1988. Aggregation pheromones of the bont tick *Amblyomma hebraeum*: Identification of candidates for bioassay. *Onderstepoort J. Vet. Res.* 55:135–137.

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in gas chromatographic analysis of insect pheromones. *Z. Naturforsh.* 30:722–725.

BALASHOV, Y. S. 1972. Bloodsucking ticks (Ixodoidea)—Vectors of diseases of man and animals. *Misc. Publ. Entomol. Soc. Am.* 8:161–376.

BALASHOV, Y. S. 1983. An Atlas of Ixodid Tick Ultrastructure. Entomological Society of America Special Publication, 289 pp.

BATSCHELET, E. 1981. Circular Statistics in Biology. Academic Press, London.

BRAVERMAN, Y., KITRON, U., and KILLICK-KENDRICK, R. 1991. Attractiveness of vertebrate hosts to *Culex pipiens* (Diptera: Culicidae) and other mosquitoes in Israel. *J. Med. Entomol.* 28:133–138.

DE BRUYNE, M., and GUERIN, P. M. 1994. Isolation of 2,6-dichlorophenol from the cattle tick *Boophilus microplus*: Receptor cell responses but no evidence for a behavioral response. *J. Insect. Physiol.* 40:143–154.

DIEHL, P. A., GUERIN, P. M., VLIMANT, M., and STEULLET, P. 1991. Biosynthesis, production site, and emission rates of aggregation-attachment pheromone in males of two *Amblyomma* ticks. *J. Chem. Ecol.* 17:833–847.

GLADNEY, W. J., GRABBE, R. R., ERNST, S. E., and OEHLER, D. D. 1974. The gulf coast tick: Evidence of a pheromone produced by males. *J. Med. Entomol.* 11:303–306.

GROB, K., and ZÜRCHER, F. 1976. Stripping of trace organic substances from water, equipment and procedure. *J. Chromatogr.* 117:285–294.

GUERIN, P. M., and VISSER, J. H. 1980. Electroantennogram responses of carrot fly, *Psila rosae*, to plant volatiles. *Physiol. Entomol.* 5:111–119.

HALL, D. R., BEEVOR, P. S., CORK, A., NESBITT, B. F., and VALE, G. A. 1984. 1-Octen-3-ol: A potent olfactory stimulant and attractant for tsetse isolated from cattle odours. *Insect Sci. Appl.* 5:335–339.

JONES, R. E. 1977. Search behaviour: A study of three caterpillar species. *Behaviour* 60:237–259.

KRAMER, E. 1976. The orientation of walking honeybees in odour fields with small concentration gradients. *Physiol. Entomol.* 1:27–37.

LEES, A. D. 1947. Transpiration and the structure of the epicuticle in ticks. *J. Exp. Biol.* 23:379–410.

LEVINSON, H. Z., LEVINSON, A. R., and MÜLLER, K. 1991. Functional adaptation of two nitrogenous waste products in evoking attraction and aggregation of flour mites (*Acarus siro* L.). *Anz. Schaedlingskde Pflanz., Umweltschutz* 64:55–60.

MCMAHON, C. 1999. Behavioural responses of the tropical bond tick, *Amblyomma variegatum* (Fabricius) to odours of hosts and of conspecifics. PhD thesis. University of Neuchâtel, Neuchâtel, Switzerland.

MCMAHON, C., and GUERIN, P. M. 2000. Responses of the tropical bont tick, *Amblyomma variegatum* (Fabricius), to its aggregation-attachment pheromone presented in an air stream on a servosphere. *J. Comp. Physiol. A* 186:95–103.

NORVAL, R. A. I., YUNKER, C. E., and BUTLER, J. F. 1987. Field sampling of unfed adults of *Amblyomma hebraeum* Koch. *Exp. Appl. Acarol.* 3:213–217.

OSTERKAMP, J., WAHL, U., SCHMALFUSS, G., and HAAS, W. 1999. Host-odour recognition in two tick species is coded in a blend of vertebrate volatiles. *J. Comp. Physiol. A* 185:59–67.

PAVIS, C., MAULÉON, H., BARRÉ, N., and MAIBECHE, M. 1994. Dermal gland secretions of tropical bont tick, *Amblyomma variegatum* (Aracrina: Ixodidae): Biological activity on predators and pathogens. *J. Chem. Ecol.* 20:1495–1503.

Paynter, Q., and Brady, J. 1993. Flight responses of tsetse flies (*Glossina*) to octenol and acetone vapour in a wind-tunnel. *Physiol. Entomol.* 18:102–108.

Porter, N. A., Weber, B. A., Weenen, H., and Khan, J. A. 1980. Autooxidation of polyunsaturated lipids. Factors controlling the stereochemistry of product hydroperoxides. *J. Am. Chem. Soc.* 102:5597–5601.

Schofield, S., and Brady, J. 1997. Effects of carbon dioxide, acetone and 1-octen-3-ol on the flight responses of the stable fly, *Stomoxys calcitrans*, in a wind tunnel. *Physiol. Entomol.* 22:380–386.

Steullet, P., and Guerin, P. M. 1992. Perception of breath components by the tropical bont tick, *Amblyomma variegatum* Fabricius (Ixodidae) I. CO_2-excited and CO_2-inhibited receptors. *J. Comp. Physiol. A* 170:665–676.

Steullet, P., and Guerin, P. M. 1994. Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* Fabricius (Ixodidae) I. Receptors within the Haller's organ capsule. *J. Comp. Physiol. A* 174:27–38.

Taneja, J., and Guerin, P. M. 1997. Ammonia attracts the haematophagous bug *Triatoma infestans*: Behavioural and neurophysiological data on nymphs. *J. Comp. Physiol. A* 181:21–34.

Torto, B., Njagi, P. G. N., Hassanali, A., and Amiani, H. 1996. Aggregation pheromone system of nymphal gregarious desert locust, *Schistocerca gregaria* (Forskal). *J. Chem. Ecol.* 22:2273–2281.

Tressl, R., Bahri, D., and Engel, K.-H. 1982. Formation of eight-carbon and ten-carbon components in mushrooms (*Agaricus campestris*). *J. Agric. Food Chem.* 30:89–93.

Ujváry, I., Tóth, M., and Guerin, P. M. 2000. Synthetic analogues of Natural Semiochemicals as Promising Insect Control Agents. In: K.-H. Tan (ed.): Area-wide Control of Fruit Flies and other Insect Pests; Joint Proceedings of the FAO/IAEA International Conference on Area-Wide Control of Insect Pests (28 May–2 June 1998) and the 5th International Symposium on Fruit Flies of Economic Importance (1–5 June 1998), USM Press, Penang, Malaysia, pp. 301–309.

Vale, G. A., and Hall, D. R. 1985. The role of 1-octen-3-ol, acetone and carbon dioxide in the attraction of tsetse flies, *Glossina* spp. (Diptera: Glossinidae) to ox odour. *Bull. Ent. Res.* 75:209–217.

Walker, A. R., Lloyd, C. M., McGuire, K., Harrison, S. J., and Hamilton, J. G. C. 1996. Integumental glands of the tick *Rhicephalus appendiculatus* (Acari: Ixodidae) as potential producers of semiochemicals *J. Med. Entomol.* 33:743–759.

Yoder, J. A., Pollack, R. J., Spielman, A., Sonenshine, D. E., and Johnston, D. E. 1993a. Secretion of squalene by ticks. *J. Insect Physiol.* 39:291–296.

Yoder, J. A., Pollack, R. J., and Speilman, A. 1993b. An ant-diversionary secretion of ticks: First demonstration of an acarine allomone. *J. Insect Physiol.* 39:429–435.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

PREPARATIVE CHIRAL LIQUID CHROMATOGRAPHY FOR ENANTIOMERIC SEPARATION OF PHEROMONES

CHRISTOPHER I. KEELING,* HIEN T. NGO,
KATHRYN D. BENUSIC, and KEITH N. SLESSOR

Department of Chemistry
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

(Received January 18, 2000; accepted November 12, 2000)

Abstract—Cellulose triacetate was investigated as a chiral stationary phase for preparatively separating the enantiomers of lineatin, frontalin, *exo*-brevicomin, *endo*-brevicomin, verbenone, (*E*)-conophthorin, and grandisol. Tens of milligrams of both enantiomers were efficiently prepared in high percentage enantiomeric excess from one injection of each compound except grandisol. We prepared grandisyl acetate, benzoate, and 4-bromobenzoate to determine if derivatization of the free alcohol might improve separation. Of these, grandisyl 4-bromobenzoate provided the best separation but was still not very well resolved. Preparative separation of enantiomers on cellulose triacetate is a viable alternative to stereoselective synthesis when semiochemicals of very high enantiomeric purity are required for biological testing.

Key Words—Cellulose triacetate, preparative chiral liquid chromatography, lineatin, frontalin, *exo*-brevicomin, *endo*-brevicomin, verbenone, (*E*)-conophthorin, grandisol.

INTRODUCTION

When a pheromone component is chiral, the emitting organism often produces only one enantiomer or a specific blend of enantiomers and the receiving organism responds differently to each enantiomer. There are 10 categories for classifying the stereochemistry–bioactivity relationship, all with examples in nature (Mori, 1998). For instance, examples are known where either only one enantiomer is biologically active (Hoover et al., 2000), a specific blend of enantiomers is most active (Miller et al., 1996), or one enantiomer inhibits the other

* To whom correspondence should be addressed.

0098-0331/00/0300-0487$19.50/0

(Camacho et al., 1994). In some instances, closely related or sympatric species use the same pheromone components but vary the enantiomeric composition to distinguish each other (Leal, 1996). To fully understand these interactions, chemical ecologists need to investigate which enantiomer(s) the organism is producing and what behaviors the two enantiomers elicit in the receiving organism.

When chiral pheromones are first identified, the racemic form is usually synthesized (or purchased) to compare physical, chemical, and behavioral properties with the isolated pheromone. The next step is to investigate the stereochemistry of the pheromone component in the extract. Chirality can be assessed using chromatography on chiral stationary phases (Beesley and Scott, 1998) and other means, but to establish the absolute configuration, stereoselective synthesis is often necessary. To test the biological activity of each enantiomer, however, they both must be available in high enantiomeric purity in quantities suitable for bioassay or field testing that might involve two, often lengthy and difficult, chiral syntheses. Since the racemic form has already been made, and synthesis of the racemic form is usually much easier, the preparative separation of the enantiomers from the racemate can be a time- and labor-saving option.

We report here our investigations on the preparative chiral resolution of enantiomers of several pheromones that are difficult to obtain enantiomerically pure. We were initially only interested in separating the enantiomers of lineatin for field-testing, but this separation was so successful (Hoover et al., 2000; Lindgren et al., 2000) that we investigated other pheromones as well. We selected cellulose triacetate (CTA I) as the chiral stationary phase (CSP) for liquid chromatography for the following reasons: (1) it is available inexpensively in bulk; (2) it has high loading capacities because it is a pure chiral polymer, unlike most CSPs, which have a chiral compound bound to silica gel; (3) it has high chiral recognition; and (4) it is the most often used CSP for preparative separations, including a γ-lactone pheromone (Francotte and Lohmann, 1987) and a spiroacetal pheromone component with similarities to (*E*)-conophthorin (Isaksson et al., 1984). It is clearly the first CSP to try when attempting a preparative separation of enantiomers. The main drawback of this CSP is the slow kinetics of the adsorption–desorption process, which can broaden peaks and require very slow flow rates. A useful review is available of the various CSPs for preparative separation of enantiomers (Francotte and Junker-Buchheit, 1992). Free alcohols are often poorly resolved on CTA I, but esterification can improve resolution, depending on the acyl group (Francotte and Wolf, 1990). We prepared and tested grandisyl acetate, benzoate, and 4-bromobenzoate to possibly improve resolution.

METHODS AND MATERIALS

Racemic frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane), verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one), the brevicomins (*exo*- and *endo*-

7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane), and lineatin (3,3,7-trimethyl-2,9-dioxatricyclo[3.3.1.$0^{4,7}$]nonane) were obtained from PheroTech Inc. (Delta, British Columbia, Canada). We obtained (±)-grandisol [*cis*-2-(1-methylethenyl)-1-methylcyclobutaneethanol] from Bedoukian Research Inc. (Danbury, Connecticut), and (±)-(*E*)-conophthorin [(*E*)-7-methyl-1,6-dioxaspiro[4.5]decane] was kindly provided by H. D. Pierce, Jr. (Simon Fraser University). Grandisyl acetate, benzoate, and 4-bromobenzoate were prepared by conventional esterification conditions with acetic anhydride or the corresponding acyl chloride in pyridine. The spectroscopic data for these esters were consistent with their expected structure.

A 2.5- × 100-cm water-jacketed glass column (Altex Scientific Inc., Berkeley, California) was slurry packed with approx. 200 g (dry wt) of cellulose triacetate (25–40 μm, EM Science, Gibbstown, New Jersey) previously swollen for 30 min in refluxing 10% water in ethanol. Both ends of the column had fixed bed supports each containing a porous Teflon filter (30–60 μm) backed by a woven Teflon defusing mesh. The column was connected to a Varian (Palo Alto, California) model 5000 liquid chromatograph with a 500-μl sample loop, and a Waters (Milford, Massachusetts) fraction collector. Injections of 50 mg of each analyte were made in ethanol unless stated otherwise. Freshly distilled anhydrous ethanol and purified water [Millipore (Bedford, Massachusetts) Alpha-Q Reagent Grade Water System] were used for the mobile phase, 10% water in ethanol unless stated otherwise. Shrinkage or swelling of the packed bed occurred when changing to different solvent systems that required the stationary phase to be added or removed as necessary. Previous work (Rimböck et al., 1985; Rizzi, 1989c; Isaksson et al., 1990; Bevan and Mutton, 1992) has shown that resolution improves dramatically at very low flow rates and that higher temperatures can increase column efficiency but often at the expense of resolution as retention and separation decrease. Therefore, we set the flow rate to 0.2 ml/min and ran separations at various column temperatures. Initial separations of lineatin at higher flow rates gave very broad peaks. Column back-pressure at 0.2 ml/min was less than 100 kPa.

Lacking a refractive index detector, an in-line polarimeter, or good chromophores for UV detection in most of the compounds, we followed each separation by gas chromatography. Fractions were collected every 30 min or 1 hr and analyzed to follow the elution of each enantiomer as shown in Figure 1. The critical fractions were usually located quickly by scent. Fractions and extracted peaks were analyzed by gas chromatography (Varian 3400 GC with Varian 8100 Autosampler, split, FID) with a J&W Scientific (Folsom, California) chiral capillary column (Cyclodex-B, 30 m × 0.25 mm ID × 0.25 μm, He carrier gas, injector and detector at 220°C, optimized pressures and column temperatures as noted in Table 1). Fractions were extracted with hexane before gas chromatography if the mobile phase contained more than 10% water. Otherwise, 1-μl samples of

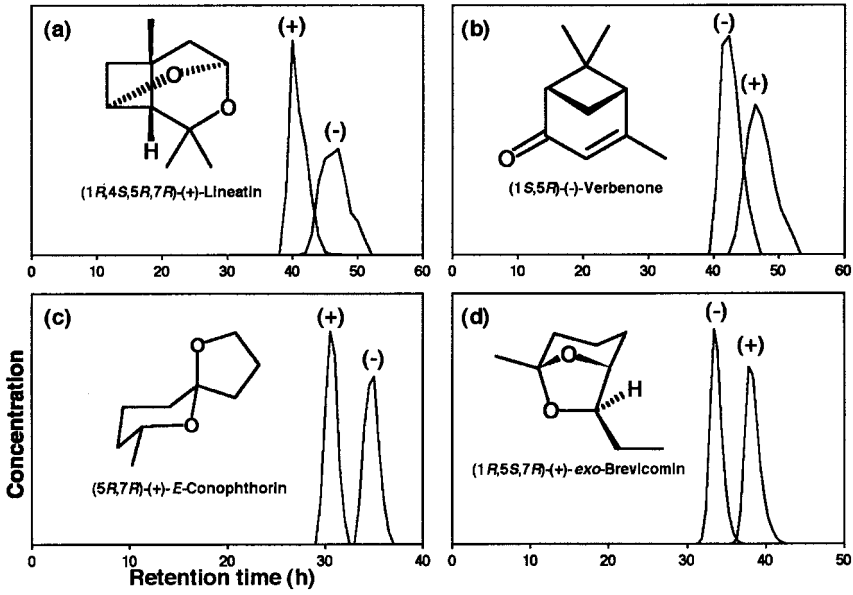


FIG. 1. Preparative resolutions of racemic pheromones on CTA I at specified temperatures. Mobile phase 10% water in ethanol and 50 mg of racemate injected unless otherwise specified: (a) 117 mg of (±)-lineatin, 50°C, absolute configuration from Kandil and Slessor (1985); (b) (±)-verbenone, 15°C, absolute configuration from Mori (1989); (c) (±)-(*E*)-conophthorin, 30°C, absolute configuration from Pierce et al. (1995); (d) (±)-*exo*-brevicomin, 15°C, absolute configuration from Mori (1989); (e) (±)-*endo*-brevicomin, 15°C, absolute configuration from Mori and Seu (1985); (f) (±)-frontalin, 30°C, absolute configuration from Mori (1975); (g) 100 mg of (±)-grandisol, mobile phase 40% water in ethanol, 30°C, absolute configuration from Mori (1989); (h) (±)-grandisyl 4-bromobenzoate, 10°C, sign of optical rotation refers to grandisol from the hydrolyzed ester.

each critical fraction were directly injected onto the chiral GC column. The enantiomers of grandisyl 4-bromobenzoate could not be resolved on the Cyclodex-B GC column, so fractions containing grandisyl 4-bromobenzoate were hydrolyzed for 1 hr at room temperature in 1 M ethanolic KOH, extracted into hexane, and then dried over anhydrous Na_2SO_4 before chiral-GC analysis of grandisol was completed.

To recover the enantiomerically enriched products, we combined fractions with similar enantiomeric purity and then diluted them with 3% aqueous $NaHCO_3$ so that two layers would form when extracted with 4 × 100 ml pentane (glass distilled). The combined pentane extract was dried over anhydrous Na_2SO_4, filtered, and rotary-evaporated. A Perkin Elmer (Wellesley, Massachusetts) Polarimeter 341 was used to determine the sign of optical rota-

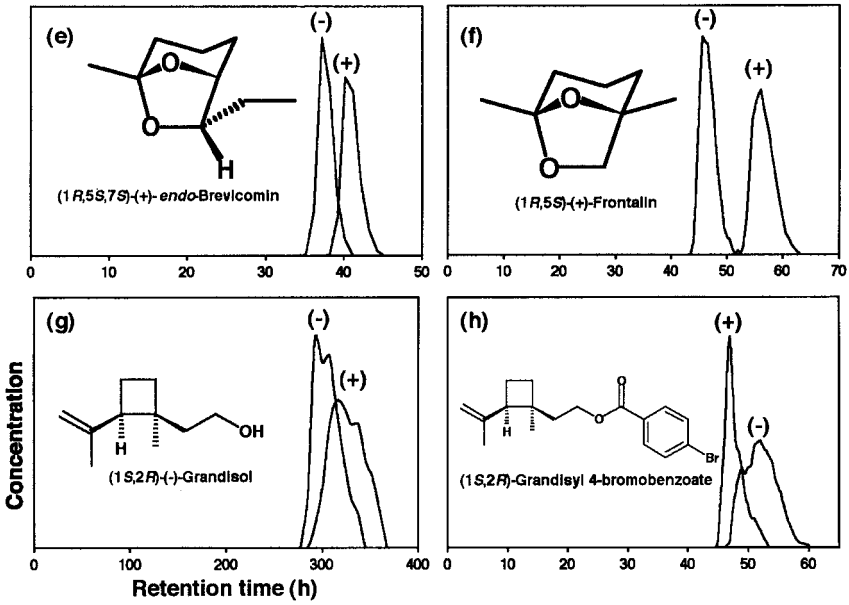


FIG. 1. Continued.

tion of the extracted peaks in pentane at 20°C and 589 nm (sodium D line). We checked the enantiomeric purity of the recovered enantiomers by chiral GC. The chemical purities of the extracted peaks were determined on a Hewlett-Packard

TABLE 1. SEPARATION DATA ON CHIRAL CYCLODEX-B GAS CHROMATOGRAPH COLUMN

Compound	Temp (°C)	Head pressure (kPa He)	Retention times (min)[a]
Lineatin	100	135	(+)13.73, (−)14.09
Verbenone	100	135	(−)28.79, (+)29.45
(*E*)-Conophthorin	85	85	(+)13.64, (−)13.93
exo-Brevicomin	100	135	(+)5.69, (−)6.25
endo-Brevicomin	100	85	(−)11.46, (+)11.73
Frontalin	70	135	(+)12.37, (−)12.75
Grandisol	115	135	(+)14.81, (−)15.95
Grandisyl acetate	110	135	17.48, 17.84
Grandisyl benzoate	[b]	135	68.11, 68.44

[a] (+) and (−) refer to the sign of optical rotation of peak extracted from liquid chromatography.
[b] Chiral GC column was temperature programmed as follows: 130°C for 1 min, then 0.5°C/min to 180°C. These peaks were broad and unresolved.

(Palo Alto, California) 5890 GC with a J&W DB-1 column (30 m × 0.25 mm ID × 0.25 μm, split, FID, head pressure 135 kPa He, injector and detector at 260°C, and column programmed 60°C for 1 min then 10°C/min to 260°C).

RESULTS

Most compounds were sufficiently resolved by preparative chiral liquid chromatography on CTA I to allow efficient preparation of both enantiomers in very high enantiomeric purity (Table 2 and Figure 1). Resolution approaching baseline separation was achieved for (*E*)-conophthorin, *exo*-brevicomin, and frontalin (Figures 1c, d, and f). The enantiomeric purity of both extracted peaks for these compounds exceeded 99.7% e.e. without discarding many overlapping, intermediate fractions. Each enantiomer was recovered in 50–80% yield, with the lowest yields for the enantiomers of (*E*)-conophthorin, which were easily lost during rotary evaporation. The enantiomers of lineatin, verbenone, and *endo*-brevicomin were not as well resolved (Figures 1a, b, and e) but recovery of enantiomers with high enantiomeric purity was still possible. As reported previously by Hoover et al. (2000), lineatin peaks were divided to yield two extracts of different % e.e. for each enantiomer. (+)-Lineatin was recovered with >99.98% e.e. in 34% yield and with 97.6% e.e. in 38% yield. (−)-Lineatin was recovered with 99.7% e.e. in 17% yield and 97.4% e.e. in 47% yield. For verbenone, we split the fractions where % e.e. = 0 and recovered the two peaks in 86 and 70% yield, both with 76% e.e. With some peak shaving, both peaks of *endo*-brevicomin were recovered in 60% yield, with >99.9 and 93% e.e. for the first and second peaks, respectively. However, with more severe peak shaving, higher enantiomeric purity is possible in reasonable yields.

The separation of grandisol was particularly difficult. The free alcohol could not be separated in 10% water in ethanol. Increasing the proportion of water in the mobile phase improved separation somewhat but dramatically increased retention (Figure 1g). By splitting the peaks where % e.e. = 0, these enantiomers were recovered with 43 and 42% e.e. in 81 and 89% yield for the first and second eluting peaks, respectively. Grandisyl acetate and grandisyl benzoate showed no signs of separating (Table 2). However, grandisyl 4-bromobenzoate run at 50°C and 10% water in ethanol showed a slight separation that improved when the column temperature was lowered to 10°C (Figure 1h). By splitting the peaks where % e.e. = 0, these enantiomers were recovered (after hydrolysis) with 54 and 61% e.e. for the first and second eluting peaks of grandisyl 4-bromobenzoate, respectively. Higher enantiomeric purity is possible by peak shaving, but this would decrease the yield. Enantiomeric purity can also be improved by rechromatographing an extracted peak with high, but not sufficient, enantiomeric purity. Unfortunately, less than 50% of the injected mass of this ester is grandisol,

TABLE 2. SEPARATION DATA ON CHIRAL CELLULOSE TRIACETATE LIQUID CHROMATOGRAPH COLUMN

Compound	Temp (°C)	Eluant H_2O : ethanol	Retention time (hr)[a]	R^b	α^c
Lineatin	51	10 : 90	(+)40, (−)46	0.70	1.15
	30	10 : 90	56, 68	0.83	1.22
Verbenone	50	10 : 90	31, 32	n.d.	1.03
	30	10 : 90	37, 40	n.d.	1.08
	15	10 : 90	(−)42, (+)46	0.62	1.10
(*E*)-Conophthorin	30	10 : 90	(+)31, (−)35	1.44	1.13
exo-Brevicomin	51	10 : 90	28, 30	0.80	1.07
	30	10 : 90	31, 34	1.18	1.10
	15	10 : 90	(−)33, (+)38	1.47	1.13
endo-Brevicomin	51	10 : 90	28, 29	n.d.	1.04
	30	10 : 90	33, 36	0.61	1.07
	15	10 : 90	(−)37, (+)41	0.90	1.09
Frontalin	51	10 : 90	37, 41	1.34	1.11
	30	10 : 90	(−)46, (+)56	1.52	1.22
Grandisol	51	10 : 90	28, 28	0	1.00
	30	60 : 40	(−)310, (+)325	0.41	1.05
	10	10 : 90	36, 36	0	1.00
Grandisyl acetate	51	5 : 95	24, 24	0	1.00
	35	30 : 70	42, 42	0	1.00
Grandisyl benzoate	51	10 : 90	28, 28	0	1.00
Grandisyl	50	10 : 90	30, 31	n.d.	1.02
4-Bromobenzoate	10	10 : 90	(+)[d]47, (−)52	0.60	1.11

[a] (+) and (−) refer to the sign of optical rotation of peak extracted from liquid chromatography.
[b] Resolution (R) = separation between peaks/(1.7 × average peak width at half height).
[c] Separation factor (α) = retention time_2/retention time_1.
[d] Sign of optical rotation refers to grandisol from the hydrolyzed ester.

and this method requires hydrolysis to recover the free grandisol. A more efficient separation is necessary to provide reasonable quantities of grandisol with higher enantiomeric purity. Grandisal, the corresponding aldehyde of grandisol, might resolve better on CTA I but is probably too unstable for such treatment (Webster et al., 1987).

The separation and extraction process did not introduce additional impurities, so the chemical purity of the extracted peaks was similar to the starting materials. Both commercial *exo*- and *endo*-brevicomin contained 5–10% of the other as an impurity. These compounds were partially resolved on CTA I (see Figures 1d and e), which resulted in some of the enantiomers becoming free from these impurities and thus chemical purity actually improved during enantiomeric separation.

We saw no change in the order of elution of enantiomers when changing

temperature and, for grandisol and its derivatives, mobile phase composition. The order of elution of enantiomers during chiral liquid chromatography did not correlate with elution during chiral gas chromatography, suggesting that the chiral selectivities of CTA I and Cyclodex-B were different.

Separation, retention, and peak widths increased with decreasing column temperature. However, the increase in separation compensated for increased peak widths as resolution also improved at lower column temperatures for all compounds tested. We preparatively separated lineatin at 50°C at the expense of some loss in resolution because retention times were faster and the second enantiomer tailed less than at lower temperatures, allowing faster throughput and resulting in smaller volumes to extract.

DISCUSSION

CTA I exhibits unique chromatographic properties. Separations are strongly temperature and flow-rate dependent, suggesting slow mass transfer. Many studies have shown that very slow flow rates provide the best resolution (Rimböck et al., 1985; Isaksson et al., 1990; Bevan and Mutton, 1992; Jacobson et al., 1993). Although 5–10% water in ethanol is the most common mobile phase, a range of mobile phases from water to cyclohexane has been used with CTA I. This CSP can be operated in either normal or reverse phase to allow separation of both polar and nonpolar racemates (Rizzi, 1989b,c; Francotte and Junker-Buchheit, 1992). The unusual and not completely understood separation mechanisms of CTA I makes prediction of success for particular racemates difficult (Rizzi, 1989a; Francotte and Wolf, 1990; Isaksson et al., 1990; Francotte and Junker-Buchheit, 1992). Most successful separations are with compounds that contain a ring close to the chiral center. Free alcohols often do not separate well on CTA I. However, simple esterification can improve the separation dramatically. Benzoates are particularly useful, with substitution in the *para* position having a strong influence on both retention and separation (Francotte and Wolf, 1990). With the large number of preparative separations already reported, CTA I appears to have broad applicability.

Although we did not investigate the loss of resolution at sample loads higher than the lineatin injection of 117 mg (Figure 1a), examples of much greater sample loads do appear in the literature (Francotte and Junker-Buchheit, 1992). For example, 2.47 g of (±)-γ-ethyl-γ-butyrolactone, the sex attractant pheromone of *Trogoderma* species, was separated on a 5- × 100-cm column (Francotte and Lohmann, 1987). Higher throughput can also be realized by injecting again before the previous injection has eluted. When resolution is not complete, yield versus the enantiomeric purity desired requires optimizing. By peak shaving, both enantiomers of all compounds tested here could be obtained in essentially

100% e.e., although yields for some compounds would be low. However, the intermediate fractions can be extracted and reinjected if the starting racemate is valuable.

The excellent separation achieved with spiroacetals, reported both here and by Isaksson et al. (1984), suggests that other spiroacetals of biological interest (Perron and Albizati, 1989; Moore et al., 1994; Weston et al., 1997) might be similarly resolved.

As was shown by Francotte and Wolf (1990), this method also can yield chiral building blocks or precursors to aid chiral synthesis if the final product is not resolvable. For example, derivatized chiral epoxy alcohols, which can be used to synthesize long chain epoxide-containing pheromones, are well separated on CTA I (E. Plettner, personal communication). However, milligram quantities of several epoxydiene pheromone components have also been separated directly on other CSPs (Qin et al., 1997). The separation of verbenone completed here also could provide, via reduction of the ketone, both enantiomers of the pheromones *cis*- and *trans*-verbenol in high enantiomeric purity. An acyclic pheromone, if it does not separate directly, could be prepared via a cyclic precursor. For example, a lactone that separates well on CTA I (Francotte and Lohmann, 1987) can be hydrolyzed to provide both enantiomers of an acyclic acid for further synthesis.

CONCLUSIONS

Using a readily achieved separation, we prepared excellent yields of both enantiomers of several pheromones in very high enantiomeric purity from the racemates significantly faster and less expensively than could be prepared by stereoselective synthesis. Under good conditions, hundreds of milligrams of racemic material can be completely separated into its enantiomers. Preparative chiral liquid chromatography with cellulose triacetate is a powerful alternative to stereoselective synthesis and should be considered when biological testing requires both enantiomers in very high enantiomeric purity.

Acknowledgments—We thank Dr. A. J. Bennet for the use of the fraction collector and polarimeter. This research was supported by a Frank A. Linville Graduate Scholarship in Olfaction and a Marshall Noble Memorial Graduate Bursary in Chemical Ecology to CIK and the Natural Sciences and Engineering Research Council of Canada to KNS.

REFERENCES

BEESLEY, T. E., and SCOTT, R. P. W. 1998. Chiral Chromatography. John Wiley & Sons, Toronto.

BEVAN, C. D., and MUTTON, I. M. 1992. Ultra-low flow preparative chiral high-performance liquid chromatography on cellulose triacetate. *Anal. Proc.* 29:240–242.

CAMACHO, A. D., PIERCE, H. D., JR., and BORDEN, J. H. 1994. Aggregation pheromones in *Dry-*

ocoetes affaber (Mann.) (Coleoptera: Scolytidae): Stereoisomerism and species specificity. *J. Chem. Ecol.* 20:111–124.

FRANCOTTE, E., and JUNKER-BUCHHEIT, A. 1992. Preparative chromatographic separation of enantiomers. *J. Chromatogr.* 576:1–45.

FRANCOTTE, E., and LOHMANN, D. 1987. Analytic and preparative resolution of racemic γ- and δ-lactones by chromatography on cellulose triacetate. Relationship between elution order and absolute configuration. *Helv. Chim. Acta* 70:1569–1582.

FRANCOTTE, E., and WOLF, R. M. 1990. Preparation of chiral building blocks and auxiliaries by chromatography on cellulose triacetate (CTA I): Indications for the presence of multiple interaction sites in CTA I. *Chirality* 2:16–31.

HOOVER, S. E. R., LINDGREN, B. S., KEELING, C. I., and SLESSOR, K. N. 2000. Enantiomer preference of *Trypodendron lineatum* and effect of pheromone dose and trap length on response to lineatin-baited traps in interior British Columbia. *J. Chem. Ecol.* 26:667–677.

ISAKSSON, R., LILJEFORS, T., and REINHOLDSSON, P. 1984. Preparative separation of the enantiomers of *trans*,*trans* and *cis*,*trans*-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, main pheromone components of *Adrena* bees, by liquid chromatography on triacetylcellulose. *J. Chem. Soc. Chem. Commun.* 1984:137–138.

ISAKSSON, R., ERLANDSSON, P., HANSSON, L., HOLMBERG, A., and BERNER, S. 1990. Triacetylcellulose as a chiral stationary phase for high-performance liquid chromatography. *J. Chromatogr.* 498:257–280.

JACOBSON, S. C., SEIDEL-MORGENSTERN, A., and GUIOCHON, G. 1993. Study of band broadening in enantioselective separations using microcrystalline cellulose triacetate. I The linear case. *J. Chromatogr.* 637:13–18.

KANDIL, A. A., and SLESSOR, K. N. 1985. A chiral synthesis of (+)-lineatin, the aggregation pheromone of *Trypodendron lineatum* (Olivier), from D-ribonolactone. *J. Org. Chem.* 50:5649–5655.

LEAL, W. S. 1996. Chemical communication in scarab beetles: Reciprocal behavioral agonist-antagonist activities of chiral pheromones. *Proc. Natl. Acad. Sci. U.S.A.* 93:12112–12115.

LINDGREN, B. S., HOOVER, S. E. R., MACISAAC, A. M., KEELING, C. I., and SLESSOR, K. N. 2000. Lineatin enantiomer preference, flight periods, and effect of pheromone concentration and trap length on three sympatric species of *Trypodendron* (Coleoptera: Scolytidae). *Canad. Entomologist* 132:877–887 (2000).

MILLER, D. R., BORDEN, J. H., and SLESSOR, K. N. 1996. Enantiospecific pheromone production and response profiles for populations of pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae), in British Columbia. *J. Chem. Ecol.* 22:2157–2172.

MOORE, C. J., HÜBENER, A., TU, Y. Q., KITCHING, W., ALDRICH, J. R., WAITE, G. K., SCHULZ, S., and FRANCKE, W. 1994. A new spiroketal type from the insect kingdom. *J. Org. Chem.* 59:6136–6138.

MORI, K. 1975. Synthesis of optically active forms of frontalin, the pheromone of *Dendroctonus* bark beetles. *Tetrahedron* 31:1381–1384.

MORI, K. 1989. Synthesis of optically active pheromones. *Tetrahedron* 5:3233–3298.

MORI, K. 1998. Semiochemicals—Synthesis, stereochemistry, and bioactivity. *Eur. J. Org. Chem.*: 1479–1489.

MORI, K., and SEU, Y.-B. 1985. Synthesis of both enantiomers of *endo*-brevicomin, the aggregation pheromone of *Dryocoetes autographus. Tetrahedron* 41:3429–3431.

PERRON, F., and ALBIZATI, K. F. 1989. Chemistry of spiroketals. *Chem. Rev.* 89:1617–1661.

PIERCE, H. D. JR., DE GROOT, P., BORDEN, J. H., RAMASWAMY, S., and OEHLSHLAGER, A. C. 1995. Pheromones in the red pine cone beetle, *Conophthorus resinosae* Hopkins, and its synonym, *C. banksianae* McPherson (Coleoptera: Scolytidae). *J. Chem. Ecol.* 21:169–185.

QIN, X.-R., ANDO, T., YAMAMOTO, M., YAMASHITA, M., KUSANO, K., and ABE, H. 1997. Resolution

of pheromonal epoxydienes by chiral HPLC, stereochemistry of separated enantiomers, and their field evaluation. *J. Chem. Ecol.* 23:1403–1417.

RIMBÖCK, K.-H., KASTNER, F., and MANNSCHRECK, A. 1985. Liquid chromatography on triacetylcellulose: Preparative separation of enantiomers on an axially compressed column. *J. Chromatogr.* 329:307–310.

RIZZI, A. M. 1989a. Band broadening in high-performance liquid chromatographic separations of enantiomers with swollen microcrystalline cellulose triacetate packings. I. Influence of capacity factor, analyte structure, flow velocity and column loading. *J. Chromatogr.* 478:71–86.

RIZZI, A. M. 1989b. Band broadening in high-performance liquid chromatographic separations of enantiomers with swollen microcrystalline cellulose triacetate packings. II. Influence of eluent composition, temperature and pressure. *J. Chromatogr.* 478:87–99.

RIZZI, A. M. 1989c. Evaluation of the optimization potential in high-performance liquid chromatographic separations of optical isomers with swollen microcrystalline cellulose triacetate. *J. Chromatogr.* 478:101–119.

WEBSTER, F. X., ZENG, X.-N., and SILVERSTEIN, R. M. 1987. Extreme sensitivity of grandisal to acids. *J. Chem. Ecol.* 13:1725–1738.

WESTON, R. J., WOOLHOUSE, A. D., SPURR, E. B., HARRIS, R. J., and SUCKLING, D. M. 1997. Spiroacetals and other venom constituents as potential wasp attractants. *J. Chem. Ecol.* 23:553–568.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

ANALYSIS OF VOLATILES INDUCED BY OVIPOSITION OF ELM LEAF BEETLE *Xanthogaleruca luteola* ON *Ulmus minor*

ROBERT WEGENER,[1] STEFAN SCHULZ,[1,*] TORSTEN MEINERS,[2] KATJA HADWICH,[2] and MONIKA HILKER[2]

[1]*Institute of Organic Chemistry*
Technische Universität Braunschweig
Hagenring 30, 38106 Braunschweig, Germany
[2]*Institute of Biology*
Freie Universität Berlin
Haderslebener Str. 9, 12163 Berlin, Germany

(Received July 26, 2000; accepted November 8, 2000)

Abstract—Egg deposition of the elm leaf beetle *Xanthogaleruca luteola* causes the emission of volatiles from its food plant, *Ulmus minor*. These volatiles are exploited by the egg parasitoid, *Oomyzus gallerucae*, to locate its host. In contrast to other tritrophic systems, the release of volatiles is not induced by feeding but by egg deposition. Previous investigations showed that the release is systemic and can be triggered by jasmonic acid. Comparison of headspace analysis revealed similarities in the blend of volatiles emitted following egg deposition and feeding. The mixture consists of more than 40 compounds; most of the substances are terpenoids. Leaves next to those carrying eggs emit fewer compounds. When treated with jasmonic acid, leaves emit a blend that consists almost exclusively of terpenoids. Dichloromethane extracts of leaves treated with jasmonic acid were also investigated. After separation of extracts of jasmonate induced elm leaves on silica, we obtained a fraction of terpenoid hydrocarbons that was attractive to the parasitoids. This indicates that jasmonic acid stimulates the production of terpenoid hydrocarbons that convey information of egg deposition to the parasitoid.

Key Words—Egg parasitoid, synomones, plant defense, systemic induction, jasmonic acid, *Ulmus minor*, elm leaf beetle, *Oomyzus gallerucae*, sesquiterpenes.

*To whom correspondence should be addressed.

0098-0331/00/0300-0499$19.50/0

INTRODUCTION

Plants respond to herbivory with the emission of volatiles that facilitate the ability of natural enemies of the herbivores to distinguish between infested and noninfested host plants (Dicke, 1994). Predators and parasitoids exploit compounds of different origin to locate their prey or their host (Dicke et al., 1990; Paré and Tumlinson, 1999; Mattiacci et al., 1995; Turlings et al., 1990). While some parasitoids are guided by green leaf volatiles (Du et al., 1998), others respond mainly to terpenoids (Turlings et al., 1993). They also have to cope with variations in the synomone blends. The total amount and composition of herbivore induced volatile emission depends on abiotic factors, such as light and water stress, and biotic factors, such as herbivore species and age, the plant species, the cultivar, and the age of leaves that have been fed on (Takabayashi et al., 1994). Nevertheless, parasitoids are able to distinguish between herbivory damage caused by a suitable host and nonhost insects. Generalists learn differences in odor composition and associate volatiles detected on the plant with host kairomones and visual cues (Vinson, 1985). The dependence of specialist parasitoids on specific herbivore-induced volatiles is more rigid. Foraging specialist parasitoids must express the highest degree of specificity in host recognition because a mistake in the final step of the host-location process would necessarily cause a loss of fitness. Release of herbivore-induced volatiles is also time dependent. Although some green leaf volatiles are emitted directly after feeding of herbivores, due to damage of leaf tissue, the major part of the components is synthesized de novo and emitted following a cascade of biochemical transformations (Paré and Tumlinson, 1997; Turlings et al., 1998).

Jasmonic acid is well known to act as a mediator of physiological changes in plants in response to herbivory. Exogenous addition of jasmonic acid or methyl jasmonate leads to an increase of defensive compounds (see, e.g., Baldwin et al., 1994; Baldwin and Preston, 1999; Farmer and Ryan, 1992; Miksch and Boland, 1996). Furthermore, it acts as a growth regulator and can promote senescence (Creelman and Mullet, 1997; Sembdner and Parthier, 1993). In the last decade, jasmonic acid was also shown to be involved in triggering the emission of plant volatiles in a wide variety of plants (Boland et al., 1995). Plant volatiles induced by herbivory were proved to attract predators and parasitoids of the herbivores in numerous systems (Dicke and Vet, 1999). Even though the substance pattern induced by jasmonic acid resembles the blend emitted after herbivory to a large degree in a studied case (Gols et al., 1999), predators also were shown to be able to differentiate between feeding and jasmonate-induced plants (Dicke et al., 1999). In the lima bean, the role of jasmonic acid in transforming the information of herbivory into the emission of sesquiterpene volatiles has been proved (Koch et al., 1999).

The elm leaf beetle, *Xanthogaleruca luteola* (Coleoptera, Chrysomelidae),

that feeds on the field elm (*Ulmus minor*) is attacked by the egg parasitoid *Oomyzus gallerucae* (Hymenoptera, Eulophidae). Prior to egg deposition, gravid elm leaf beetles gnaw a groove into the under-surface of a leaf. Into this groove they glue an egg mass of about 10–30 eggs by means of their oviduct secretion. After an induction period, the leaf emits volatiles that attract *Oomyzus gallerucae*. In contrast to the tritrophic systems mentioned above, emission of attractive volatiles from the elm is not caused by feeding but by egg deposition of the host (Meiners and Hilker, 1997). Previous investigations showed that the emission of volatiles is systemic (Meiners and Hilker, 1997, 2000). Plant parts located near leaves carrying eggs emit volatiles that attract parasitoids. It was further demonstrated that jasmonic acid renders elm leaves attractive to wasps (Meiners and Hilker, 2000).

In this work, we investigated the chemical composition of the odor emitted by differently treated elm leaves by using headspace techniques. Odor production induced by egg deposition, by jasmonic acid treatment, and by feeding damage was compared. We also analyzed the emission of volatiles of intact leaves next to leaves damaged by feeding or adjacent to leaves carrying eggs. In addition, extracts of jasmonic acid-treated leaves were fractionated in an effort to identify compounds responsible for parasitoid attraction.

METHODS AND MATERIALS

Plants and Insects. Elm twigs (*Ulmus minor*) from the lower part of a tree were cut from June to September in the botanic garden of the Freie Universität Berlin for headspace analysis or at Lake Ölper in Braunschweig for extracts. Twigs of the latter tree were also subject to headspace analysis after treatment with jasmonic acid. Adults and eggs of *X. luteola* were collected near Montpellier and Perpignan (southern France). All stages of *X. luteola* were kept at 20°C and 16L : 8D. Adults and larvae were fed elm leaves of *U. minor*. *O. gallerucae* emerging from elm leaf beetle eggs on *U. minor* were held at 10°C and 16L : 8D and fed diluted honey. Due to the availability of field elm leaves, only three generations of beetles and parasitoids were produced in the laboratory. Several days before testing they were transferred to 20°C conditions. Only experienced females with prior contact with host eggs were tested. These females encountered eggs two days prior to the experiment for a period of 24 hr (Meiners and Hilker, 1997).

Leaf Extracts. Freshly cut elm twigs with 10–20 leaves were placed in tap water containing 1 μmol/ml jasmonic acid and Tween 20 (Fluka, 0.05%). After 72 hr, elm leaves were ground under liquid nitrogen, and 100 ml of dichloromethane was added. The mixture was stirred overnight in a closed vial before the plant material was filtered off by using a chromatography column

filled with 10 g silica (Merck, mesh 70-230 ASTM). The filtrate was concentrated by distillation to a small volume. For control, freshly cut elm twigs with 10–20 leaves were kept in tap water and Tween 20 without jasmonic acid for 72 hr. The extraction of these so-called intact (control) leaves was conducted as described for the jasmonate-treated leaves. For fractionation, the extract was loaded onto a silica column (10.7 g, Merck, mesh 70-230 ASTM). After elution with 18 ml pentane, 9 ml of a solution containing sesquiterpenoids was obtained.

Headspace Samples. Headspace samples were taken 72 hr after starting the induction by closed-loop analysis. Samples were taken from: (1) an untreated elm twig kept in tap water for 72 hr, (2) an elm twig with 10–20 leaves without eggs that elm leaf beetles had fed on, (3) an elm twig with 10–20 leaves that carried 15–20 egg masses (i.e., about 1 egg mass per leaf), and (4) an elm twig with 10–20 leaves that was treated by jasmonic acid as described for the leaf samples.

Samples of systemically induced elm leaves were attained by placing an elm twig in a Plexiglas cylinder and dividing its lower part (15–20 leaves) from the upper part (15–20 leaves) by use of a partition plate (Meiners and Hilker, 2000). The lower part was offered to male elm leaf beetles or 15–20 egg masses were transferred onto its leaves. The twig was cut into two pieces, and the upper part was used for headspace sampling.

Sampling duration was 6 hr (Boland et al., 1984). The charcoal filters with 1 mg charcoal used were extracted with approximately 20 μl dichloromethane.

Chemicals. Jasmonic acid was synthesized by alkaline hydrolytic cleavage of methyl jasmonate (Baldwin et al., 1994). Its purity was checked by silylation with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), followed by GC analysis. (*E*,*E*)-2,6-Dimethyl-3,5,7-octatrien-2-ol was prepared by reduction and rearrangement of ocimenone (van Dort et al., 1993). (*E*)-2,3-Epoxy-2,6-dimethyl-6,8-nonadiene was synthesized by oxidation of (*E*)-4,8-dimethyl-1,3,7-nonatriene with *m*-chlorperbenzoeic acid in dichloromethane (Kaiser, 1991).

Analysis. Samples were analyzed by GC-MS with a Hewlett Packard GC 6890 MSD 5973 with a split/splitless inlet, equipped with a 30-m HP5MS capillary column (0.25 mm ID, $d_f = 0.25$ μm). Some samples were analyzed by using a Gerstel KAS4 programmed temperature vaporization (PTV) inlet. Its heater was programmed from 50°C (0.1 min hold) to 280°C at 120°C/min. The oven temperature was programmed from 50°C (5 min hold) to 300°C at 5°C/min. Helium flow was adjusted to 1 ml/min in constant flow mode. The mass spectrometer was operated in 70 eV EI ionization mode. Compounds were identified by comparing mass spectra and retention indices with those of reference compounds or critical evaluated literature data (Joulain and König, 1998).

Bioassays. The effect of headspace samples and leaf extracts on the parasitoids was studied in a four-arm airflow olfactometer (for details see Vet et al., 1983; Meiners and Hilker, 1997, 2000). Odorless humidified air was presented

in three control fields to a female parasitoid, while the fourth field contained air that had passed the sample under investigation. The duration of stay of the parasitoids was observed over a period of 600 sec. The data were recorded with the help of the Noldus Observer program, version 3.0 (Wageningen, the Netherlands). The number of closed-loop samples tested was four each for the extracts of jasmonate treated and untreated leaves. The number of parasitoids was 20.

Statistical Analysis. Data were statistically evaluated by the Friedman test. Durations of stay in each of the four olfactometer fields were compared to each other by the Wilcox-Wilcoxon test (Bortz, 1993).

RESULTS

Analysis of Closed-Loop Extracts. Intact leaves emit only small amounts of volatiles consisting of terpenes (seven monoterpenes, a homomonoterpene, and seven sesquiterpenes). In one sample, no compound was detected at all.

Closed-loop extracts of elm leaves on which eggs were deposited and of leaves damaged by feeding contained more compounds. The emitted blends consisted mainly of terpenes, with (*E*,*E*)-α-farnesene, β-caryophyllene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) as major compounds (Table 1, Figure 1). Aromatic compounds were emitted also from feeding-damaged and egg-laden leaves, but the monoterpenes identified in untreated leaves were detected only in minute amounts. Repeated samples showed a great variability in their quantitative composition. Terpenoids such as β-caryophyllene, (*E*,*E*)-α-farnesene, and (*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene (see Figure 2 for structures of various terpenoids identified), an oxidation product of DMNT previously only identified in the flower scent of *Selenicereus hamatus* (Kaiser, 1991), especially vary in their concentration. Considerable changes in the concentration of *cis*-jasmone and aromatic compounds such as methyl salicylate and esters of benzoic acid also were noted. No significant differences could be established between the two different treatments due to the variability of the samples.

(*Z*)-3-Hexenol and (*Z*)-3-hexenyl acetate are the main components emitted by leaves that were systemically induced by feeding on other leaves of the same twig. Leaves systemically induced by egg deposition produced terpenes with β-caryophyllene and (*E*)-4,8-dimethyl-1,3,7-nonatriene as main compounds (Figure 3). The headspace of the systemically oviposition-induced leaves contained several sesquiterpenes that were not detected in the two feeding induced samples.

The headspace of elm leaves induced by jasmonic acid consisted almost exclusively of terpenes (Figure 4). Again, the concentration of (*E*,*E*)-α-farnesene was quite variable in repeated samples. The homomonoterpenoid (*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene was identified in all samples examined.

TABLE 1. VOLATILES COLLECTED FROM LEAVES OF *U. minor* FOLLOWING DIFFERENT TREATMENTS[a]

Compound	Relative amount						
	I	F	E	SF	SE	J1	J2
Aromatic compounds							
Phenylacetonitrile	0,0,0	4,1,1,0,3,4,1	3,0,5,5,4,3,3,1	0,0	0,0	0,0	2,3,0
Methyl salicylate	0,0,0	3,0,1,0,3,3,1	1,1,0,0,1,3,3,1	0,6	0,0	0,0	3,5,6
Indole	0,0,0	4,3,0,1,5,2,3	5,4,1,4,5,4,4,3	0,0	0,0	0,1	2,1,0
Esters							
(*Z*)-3-Hexenyl acetate	0,0,0	3,1,5,3,4,5,1	1,1,5,4,1,3,1,1	6,1	0,6	0,0	0,1,6
(*E*)-2-Hexenyl acetate	0,0,0	2,1,5,3,3,3,1	1,0,5,4,1,3,1,1	0,1	0,0	0,1	0,1,4
(*E*)-2-Hexenyl propanoate	0,0,0	1,0,3,0,0,0,0	0,1,1,0,0,0,0,0	0,0	0,0	0,0	0,0,0
(*Z*)-3-Hexenyl butanoate	0,0,0	3,1,4,3,4,3,1	3,2,4,1,1,1,3,1	0,0	0,4	0,0	0,3,4
Hexyl butanoate	0,0,0	0,0,4,4,3,0,1	0,0,4,1,1,0,1,0	0,0	0,0	0,1	0,1,0
(*E*)-2-Hexenyl butanoate	0,0,0	4,2,5,4,4,6,3	4,4,5,4,4,4,3,3	0,0	0,0	0,2	0,1,0
(*Z*)-3-Hexenyl 2-methylbutanoate	0,0,0	4,1,4,4,5,3,3	3,4,4,3,3,4,3,3	0,0	0,0	4,1	1,4,4
(*E*)-2-Hexenyl 2-methylbutanoate	0,0,0	4,4,5,5,5,6,4	4,4,5,5,5,5,4,4	0,0	0,0	4,2	0,0,0
(*Z*)-3-Hexenyl hexanoate	0,0,0	2,1,0,1,4,1,3	3,3,3,4,3,1,1,1	0,0	0,0	0,0	0,1,2
(*E*)-2-Hexenyl hexanoate	0,0,0	2,0,1,3,4,3,3	3,3,4,4,3,3,1,1	0,0	0,0	0,0	0,1,0
(*Z*)-3-Hexenyl benzoate	0,0,0	4,1,0,0,3,1,3	4,4,0,0,1,3,1,1	0,0	0,0	0,0	4,4,5
(*E*)-2-Hexenyl benzoate	0,0,0	4,4,0,0,1,0,0	3,4,0,0,1,1,1,0	0,0	0,0	0,0	0,0,0
Ketones							
(*Z*)-Jasmone	0,0,0	4,3,0,0,3,0,0	4,4,0,0,1,3,1,1	0,0	0,0	0,0	0,0,0
Hydrocarbons							
Hexadecane	0,0,0	4,0,0,0,0,0,0	0,0,3,0,0,0,1,0	0,0	0,0	0,0	0,0,0
2,6,10,14-Tetramethylpentadecane	0,0,0	4,1,1,0,0,0,0	4,5,0,0,0,0,0,0	0,0	5,5	0,0	0,0,0
2,6,10,14-Tetramethylhexadecane	0,0,0	4,1,1,0,0,0,0	4,5,0,0,0,0,0,0	0,0	5,5	0,0	0,0,0
Alcohols							
(*Z*)-3-Hexen-1-ol	0,6,0	0,0,6,0,0,0,0	0,0,4,1,0,0,0,0	6,0	0,0	0,0	0,1,6
1-Hexanol	0,5,0	0,0,6,4,0,0,0	0,0,1,2,0,0,0,0	0,0	0,0	0,0	0,0,6
(*E*)-2-Hexen-1-ol	0,0,0	0,0,6,0,0,0,0	0,0,5,0,0,0,0,0	0,0	0,0	0,0	0,0,0

Terpenes							
α-Pinene	0,2,6	0,0,0,0,0,0,0	0,0,1,0,0,0,0,0	0,0	0,0	2,0	0,0,0
Camphene	0,4,4	0,0,0,0,0,0,0	0,0,1,0,0,0,0,0	0,0	0,0	2,0	0,0,0
β-Pinene	0,1,6	0,0,0,0,0,0,0	0,0,1,0,0,0,0,0	0,0	0,0	4,0	0,0,0
Myrcene	0,0,0	0,0,1,0,0,0,0	0,0,1,0,0,0,0,0	0,0	0,5	0,0	0,0,0
Δ^3-Carene	0,3,0	0,0,0,0,0,0,0	0,0,0,0,0,0,0,0	0,0	0,5	0,0	0,0,0
Limonene	0,2,0	1,3,1,1,1,0,0	2,0,4,1,0,0,1,1	0,0	5,6	1,0	0,1,0
(*Z*)-β-Ocimene	0,0,0	0,0,0,0,0,0,0	0,0,1,0,0,0,0,0	0,0	0,0	0,0	0,0,0
(*E*)-β-Ocimene	0,0,0	0,0,0,0,1,0,1	0,0,5,0,0,0,0,1	0,0	0,0	0,3	0,0,3
(*Z*)-4,8-Dimethyl-1,3,7-nonatriene	0,0,0	1,0,0,0,0,0,0	0,0,1,0,0,0,0,0	0,0	0,0	0,0	0,0,0
Linalool	0,6,0	4,2,0,0,2,0,1	4,3,4,0,1,1,1,1	0,0	0,0	2,1	0,0,3
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	0,4,5	6,5,5,5,5,5,0	4,4,6,5,4,5,6,5	6,0	5,6	5,3	1,4,5
Borneol	0,3,0	0,1,1,0,0,0,0	0,0,1,0,0,0,0,0	5,0	4,5	0,0	0,0,0
(*E,E*)-2,6-Dimethyl-3,5,7-octatrien-2-ol	0,0,0	4,0,1,3,3,3,3	0,3,4,4,3,4,3,3	0,0	0,0	4,2	1,4,4
(*E*)-2,3-Epoxy-2,6-dimethyl-6,8-nonadiene	0,0,0	4,3,1,0,1,1,1	0,0,5,0,3,2,3,1	0,0	0,0	4,3	1,1,3
β-Elemene	0,0,0	1,3,1,0,0,0,0	0,0,3,0,0,0,0,0	0,0	0,0	0,0	0,0,0
β-Caryophyllene	0,2,0	6,4,4,2,5,3,5	4,4,6,4,4,4,5,2	4,0	5,6	5,3	0,4,0
β-Copaene	0,0,0	1,1,0,0,0,0,0	1,1,3,0,0,0,0,0	0,0	0,0	0,0	1,3,0
α-Humulene	0,1,0	4,1,3,1,3,1,3	3,3,4,3,1,1,1,1	0,0	5,4	4,0	0,0,0
α-Amorphene	0,1,0	2,0,0,0,0,0,0	4,0,1,0,0,0,0,0	0,0	0,0	0,0	0,0,0
Germacrene D	0,1,0	3,2,1,1,4,1,3	0,0,5,4,0,3,1,3	0,0	0,0	5,3	0,5,1
(*Z,E*)-α-Farnesene	0,0,0	1,1,1,1,0,0,1	0,0,3,0,0,0,1,0	0,0	0,0	0,1	4,4,3
(*E,E*)-α-Farnesene	0,0,5	5,6,5,6,6,3,6	2,3,6,6,5,4,0,6	0,0	5,0	0,3	6,6,6
γ-Cadinene	0,1,0	2,0,1,0,0,0,0	4,2,1,0,0,0,0,0	0,0	0,4	3,0	2,0,0
δ-Cadinene	0,1,0	3,3,2,3,3,1,3	2,2,3,1,3,1,1,1	0,0	0,4	3,2	2,0,0
(*E*)-Nerolidol	0,0,0	4,0,0,1,1,1,0	4,4,1,1,3,1,1,1	0,0	0,0	0,0	2,0,3
Unknown, $b = 83$	0,0,0	2,0,1,0,1,1,0	3,2,4,0,3,3,2,3	0,0	0,0	0,0	0,0,0
Unknown, $b = 159$	0,0,0	5,0,4,1,4,4,1	5,0,4,3,5,4,6,5	0,0	0,0	2,0	5,4,4
Unknown, $M = 220$, $b = 162$	0,0,0	6,6,5,6,6,6,4	4,6,5,6,6,5,6,6	0,0	0,6	5,0	6,6,6

[a] I, intact leaves, 3 samples; F, fed on by *X. luteola*, 7 samples; E, carrying eggs, 8 samples; SF, systemically induced by feeding, 2 samples; SE, systemically induced by egg deposition, 2 samples; J1, after treatment with jasmonic acid, 2 samples; J2, leaves from a different elm tree individual after treatment with jasmonic acid, 3 samples; M, molecular mass; b, base peak. Columns show results of single analyses. Relative proportions are indicated by numbers: 0, not detected; 1 < 0.1%; 2 < 0.2%; 3 < 0.5%; 4 < 2%; 5 < 5%; 6 > 5%.

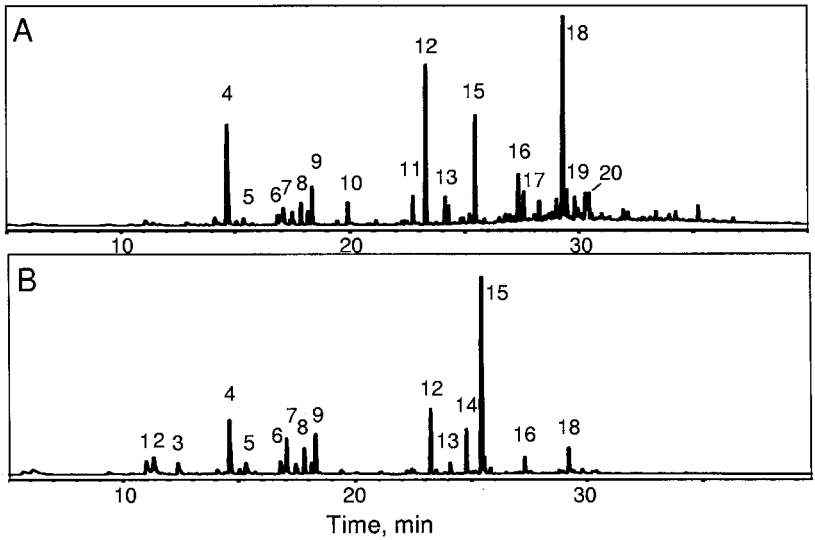


FIG. 1. Headspace analysis. Gas chromatograms of volatiles emitted from elm leaves after feeding (A) and egg deposition (B) of the elm leaf beetle. 1, (*Z*)-3-hexenyl acetate; 2, (*E*)-2-hexenyl acetate; 3, (*E*)-β-ocimene; 4, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 5, phenylacetonitrile; 6, (*Z*)-3-hexenyl butanoate; 7, (*E*)-2-hexenyl butanoate; 8, (*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene; 9, (*E*)-2-hexenyl 2-methylbutanoate; 10, indole; 11, (*Z*)-jasmone; 12, β-caryophyllene; 13, α-humulene; 14, germacrene D; 15, (*E*,*E*)-α-farnesene; 16, oxygenated sesquiterpene, b = 159; 17, hexadecane; 18, oxygenated sesquiterpene, *M* = 220; 19, solvent impurity; 20, 2,6,10,14-tetramethylhexadecane; *b* = base peak; *M* = molecular mass.

The composition of volatiles induced with jasmonic acid also differs when comparing elm trees from different regions. The leaves of one elm tree investigated in Braunschweig produced large amounts of methyl salicylate after treatment with jasmonic acid; an elm tree in another location in Berlin lacked this ability, but emitted β-caryophyllene.

Analysis of Extracts. The composition of the extracts of jasmonate-induced and noninduced components showed some similarities. Again, the major group of compounds was terpenoids. Eight additional sesquiterpenes occurred in jasmonate-treated leaves compared to the extract of intact leaves. Chromatography on silica of the extract of jasmonate-induced leaves yielded a mixture of terpenoid hydrocarbons that was active in bioassays: (*E*,*E*)-α-farnesene, germacrene D, squalene, and other terpenoid hydrocarbons (Figure 5 and Table 2).

Attractiveness of Odor Blends from Closed-Loop Extracts. The closed-loop extracts of untreated leaves were not attractive (Friedman ANOVA $\chi^2 = 0.950$,

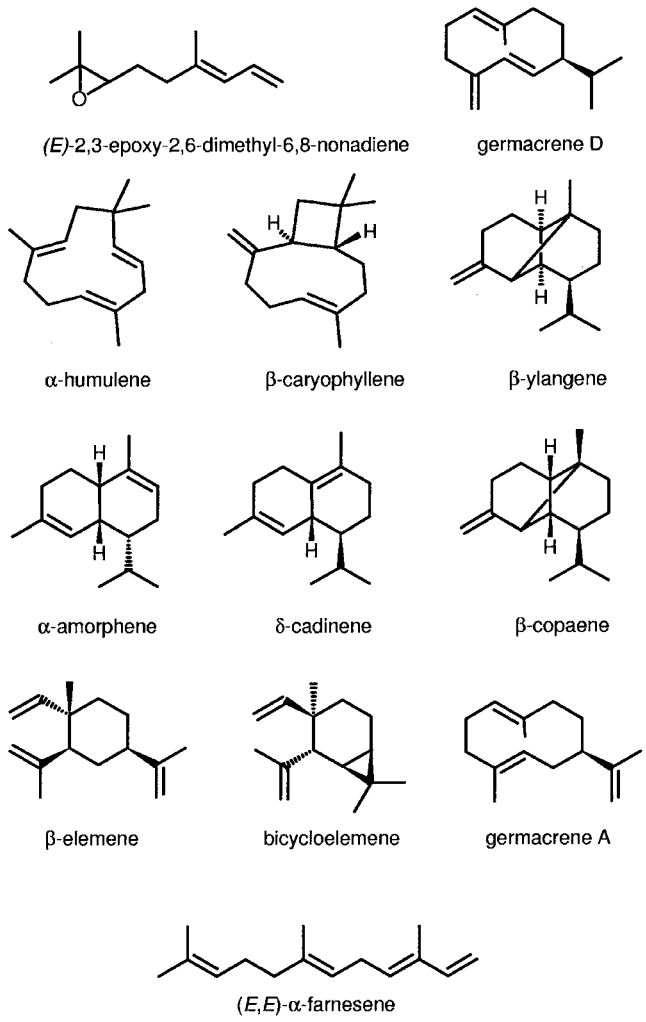


FIG. 2. Terpenoids identified or mentioned in text. Formulas show relative configurations only.

df = 3, ns; Figure 6a). The closed-loop extracts of jasmonic acid-treated leaves attracted the parasitoids in a four-arm olfactometer (Friedman ANOVA χ^2 = 18.588, *df* = 3, $P < 0.001$; Figure 6b). The odor of a fraction obtained by silica chromatography of an extract of jasmonate induced elm leaves also attracted the parasitoids (Friedman ANOVA χ^2 = 12.528, *df* = 3, $P < 0.01$. Figure 6c).

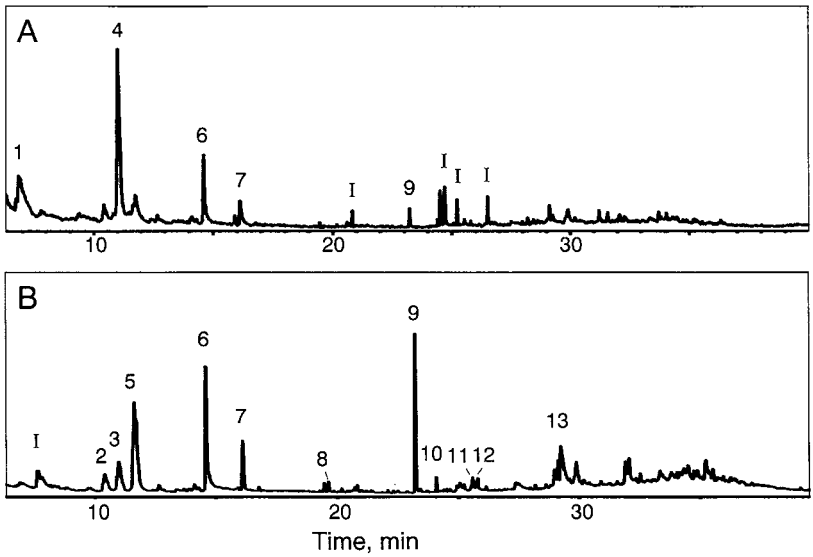


FIG. 3. Headspace analysis. Gas chromatograms of volatiles from leaves systemically induced by feeding (A) and by egg deposition (B) of the elm leaf beetle. Peaks: 1, (*Z*)-3-hexenol; 2, myrcene; 3, Δ^3-carene; 4, (*Z*)-3-hexenyl acetate; 5, limonene; 6, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 7, borneol; 8, borneyl acetate; 9, β-caryophyllene; 10, α-humulene; 11, γ-cadinene; 12, δ-cadinene; 13, oxygenated sesquiterpene; M = 220; I, impurity; M = molecular mass.

DISCUSSION

Egg parasitoids can distinguish volatile profiles of elm leaves induced by egg deposition of the host from those elicited by feeding of the host. The way egg deposition evokes a plant response resembles processes following host feeding in other systems. The process is initiated by an elicitor and cannot be mimicked by simply wounding or feeding the leaves. The gnawing of a female elm leaf beetle prior to oviposition affects only the leaf surface and differs substantially from feeding (Meiners and Hilker, 2000). It cannot be excluded that the female also applies to the egg-associated elicitor an elicitor through the mouthparts when scratching the grooves for her eggs. The mechanical damage by this gnawing is minute, however, enabling no or only small amounts of saliva to contact the leaf. The mimicking of the gnawing by removing leaf surface tissue with a scalpel did not induce the emission of attractive volatiles (Meiners and Hilker, 2000) or of a substantial amount of green leaf volatiles (Meiners and Hilker, unpublished).

The elicitor was present in the oviduct secretion of *X. luteola*, which

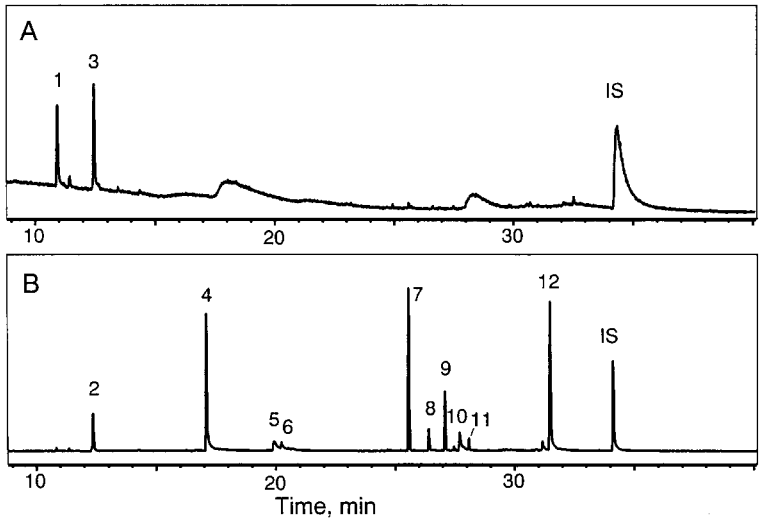


FIG. 4. Headspace analysis. Gas chromatograms of volatiles from jasmonic acid treated elm leaves (B) compared to untreated elm leaves (A). Peaks: 1, α-pinene; (2), (*E*)-β-ocimene; 3, β-pinene; 4, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 5, (*E*,*E*)-2,6-dimethyl-3,5,7-octatrien-2-ol; 6, (*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene; 7, β-caryophyllene; 8, α-humulene; 9, germacrene D; 10, γ-cadinene; 11, δ-cadinene; 12, oxygenated sesquiterpene, M = 220; M = molecular mass; PTV inlet; oven programming was 40°C (5 min hold) to 300°C (5°C/min). Added reference compound was octadecane (10 μl/ml, IS).

attaches the eggs to the leaves (Meiners and Hilker, 2000). Elm leaves emit a complex mixture of compounds when eggs are deposited on them. Some of the components are derived from the lipoxygenase pathway (green leaf volatiles),

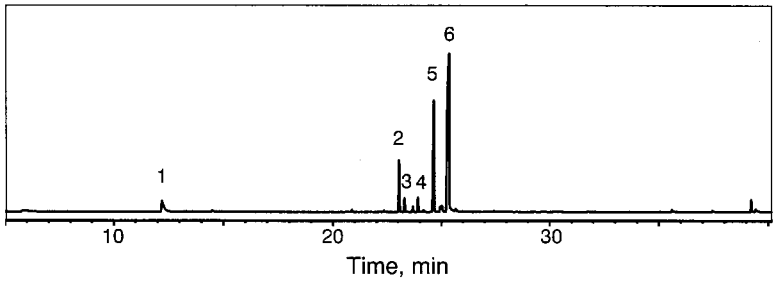


FIG. 5. Gas chromatogram of a fraction from the extract of jasmonic acid treated elm leaves that was used for a bioassay. Peaks: 1, (*E*)-β-ocimene; 2, β-ylangene; 3, β-copaene; 4, α-humulene; 5, germacrene D; 6, (*E*,*E*)-α-farnesene.

TABLE 2. LEAF EXTRACTS[a]

Compound	Relative amount I	J	A	Compound	Relative amount I	J	A
Aldehydes				Ketones			
Hexanal	4	3,0	0	2-Heptacosanone	1	2,2	0
(*E*)-2-Hexenal	4	4,0	0	Hydrocarbons			
Nonanal	2	1,1	0	Heptacosane	4	4,4	0
Pentadecanal	5	5,5	0	Octacosane	3	2,3	0
Heptadecanal	2	3,2	0	Nonacosane	6	6,6	0
Docosanal	1	2,3	0	Hentriacontane	4	4,4	0
Tricosanal	1	1,2	0	Terpenoids			
Tetracosanal	3	3,4	0	Limonene	1	0,1	0
Pentacosanal	2	2,3	0	(*E*)-β-Ocimene	0	2,1	5
Triacontanal	4	3,3	0	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	1	0,1	3
Aromatic compounds				Bicycloelemene	0	0,0	3
Benzaldehyde	4	3,2	0	β-Elemene	0	1,1	2
Benzylalcohol	1	2,1	0	β-Caryophyllene	0	1,1	0
Phenylacetaldehyde	4	2,1	0	β-Ylangene	0	0,0	6
Methyl benzoate	0	1,0	0	β-Copaene	0	1,1	4
Phenylacetonitrile	0	2,1	0	α-Humulene	0	1,0	4
Methyl salicylate	4	4,4	0	Germacrene D	2	3,3	6
Indole	0	1,1	0	β-Ionone	0	1,1	0
(*Z*)-3-Hexenyl benzoate	0	4,1	0	(*Z,E*)-α-Farnesene	0	1,2	3
Benzyl benzoate	0	5,1	0	(*E,E*)-α-Farnesene	4	6,6	6
Phenylethyl benzoate	0	1,1	0	δ-Cadiene	0	1,1	3
Eugenol	0	0,1	0	(*E*)-Nerolidol	0	1,0	0
Benzyl salicylate	2	1,1	0	Phytol	4	5,4	0
Benzyl hexadecanoate	0	1,1	0	Squalene	6	6,6	6
2-Phenylethyl hexadecanoate	0	1,1	0	γ-Tocopherol	6	5,5	0
Benzyl octadecatrienoate	0	1,0	0	Friedelan-3-one like spectrum	4	4,4	0
Esters				Unknown, $M = 428$	5	5,5	0
Hexyl hexadecanoate	3	3,3	0	Friedelan-3-one	6	6,6	0
Tetracosyl acetate	1	0,2	0				
Octacosyl acetate	1	1,1	0				

[a] Compounds identified in extracts from differently treated leaves extracts. I, intact leaves, 1 sample; J, leaves treated with jasmonic acid, 2 samples; A, attractive fraction containing sesquiterpenes, 1 sample; Columns show results of single analyses. Relative proportions are indicated by numbers: 0, not detected; 1 < 0.1%; 2 < 0.2%; 3 < 0.5%; 4 < 2%; 5 < 5%; 6 > 5%.

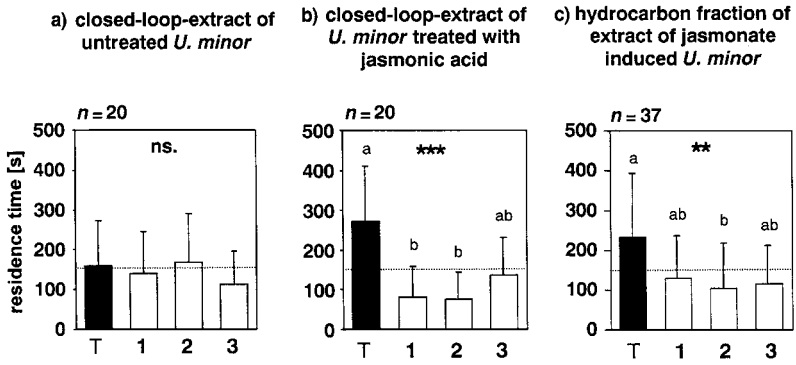


FIG. 6. Responses of females of the egg parasitoid *Oomyzus gallerucae* to volatiles from extracts of elm leaves. Mean values and standard deviations of residence times from females of *O. gallerucae* in test and control fields of a four-arm olfactometer. The observation period per female parasitoid was 600 sec, T = field with test odors; 1, 2, 3 = three field with control air. Treatments: (a) closed-loop extract of intact elm leaves; (b) closed-loop extract of intact elm leaves treated with jasmonic acid at a concentration of 1 μmol/ml; (c) hydrocarbon fraction of an extract of elm leaves treated with jasmonic acid at a concentration of 1 μmol/ml. Friedman test: $^{**}P < 0.01$. $^{***}P < 0.001$. Wilcox-Wilcoxon test: different letters indicate significant ($P < 0.05$) differences.

while most others are of terpenoid origin. A great number of components is found both in the volatile blend of leaves induced by egg deposition and by feeding. The main components are (*E*,*E*)-α-farnesene, β-caryophyllene, and germacrene D. The concentration of certain terpenoids, e.g., (*E*,*E*)-α-farnesene varies in leaves induced by egg deposition and by feeding. Additionally, there is variation in the concentration of these compounds in repeated experiments. Acetates, butyrates, and 2-methylbutyrates of the green leaf alcohols (*Z*)-3-hexenol and (*E*)-2-hexenol also contribute to the elm leaf odor. The overlap of compounds in the blend following egg deposition and herbivory can be attributed to the lack of necessity of plants that are under attack by a variety of herbivores to release highly specific synomones. As long as specialized parasitoids are among the species attracted, it is not necessarily beneficial for the plant to maximize the emission of volatiles in response to a certain herbivore (Turlings et al., 1995). Due to the variation in the composition of the emitted blends, we were not able to detect significant differences between bouquets induced by feeding or by egg deposition. Nevertheless, the herbivore might select only a few significant components of the whole mixture, or other compounds that eluded our analytical methods.

The induction of volatiles by egg deposition in the elm leaf is systemic, as described for herbivory (Meiners and Hilker, 2000; Dicke et al., 1992). Compar-

ing the volatiles emitted by leaves carrying eggs and adjacent egg-free leaves, the number of compounds emitted by the latter is lower. Green leaf esters and aromatic compounds are not emitted. This could be due to a dilution effect, because the signal that mediates egg deposition may be spread throughout the plant. Mechanical damage facilitates the liberation of compounds from the leaf tissue of plants (Paré and Tumlinson, 1998). With the exception of (*Z*)-3-hexenyl acetate, esters of green leaf alcohols are found only in small quantities in the blend of elm leaves systemically induced by feeding, but they are major components in the blend of damaged leaves.

Jasmonic acid renders intact elm leaves attractive to parasitoids (Meiners and Hilker, 2000). The odor composition caused by jasmonic acid treatment differs from the blend emitted from elm leaves with deposited eggs in regard to the absence of esters. It seems that compounds such as the green leaf volatiles and compounds derived from the shikimic acid pathway are not vital for parasitoids to detect the host plant.

The finding that induction with jasmonic acid leads to the formation of attractants for predators or parasitoids agrees with other studies (Gols et al., 1999). The effect of jasmonic acid on the production of terpenoids also could be demonstrated by comparing the dichloromethane extracts of noninduced and induced leaves, in which the number of sesquiterpenes is higher.

By separating apolar terpenoid hydrocarbons from more polar compounds with column chromatography on silica, we demonstrated that induction with jasmonic acid leads to the production of terpenoid hydrocarbons attracting the specialist parasitoid *Oomyzus gallerucae*. Removal of polar compounds, however, does not render the leaf extract unattractive. Both closed-loop extracts and the sesquiterpene fraction of the extract of jasmonic acid treated leaves attract parasitic wasps, although the concentration of the main components is different.

The importance of different terpenoids for parasitoid host location can be estimated by comparison of their occurrence in closed-loop extracts of leaves carrying eggs (Table 1, column E), jasmonate-induced leaves (Table 1, column J1), and the active fraction of jasmonate induced leaves (Table 2, column A). The least complex blend is found in the active fraction A, containing 12 compounds. The two sesquiterpenes, β-ylangene and bicycloelemene, were not detected in the eight analyses of the oviposition-induced leaves (Table 1, column E), while (*E*)-β-ocimene, β-elemene, and (*Z*,*E*)-α-farnesene were detected only once or twice. Thus, they might be less important for host location. The other volatile terpenoids, (*E*)-4,8-dimethyl-1,3,7-nonatriene, α-humulene, germacrene D, (*E*,*E*)-α-farnesene, and δ-cadinene might be involved, while β-copaene shows no clear trend. Some terpenoids, such as limonene, β-caryophyllene or α-amorphene are absent in the attractive fraction (Table 2, column A) and, therefore, seem to be inactive. In addition, the known instability of some sesquiterpenes, especially germacrenes, complicates the situation. Germacrene A, not detected

in any extract, is an unstable compound known to undergo Cope rearrangement to β-elemene at temperatures over 165°C, as normally encountered in the injection port of a GC (Teisseire, 1994). The same is true for bicycloelemene (rearrangement from bicyclogermacrene) (Garnero and Tabacchi, 1987). Germacrene D can rearrange to various compounds, preferably to δ-cadinene under treatment with silica, which was employed as medium in the fractionation of the leaf extract. Light can induce rearrangement to β-copaene, while heat above 190°C leads to its diastereomer β-ylangene as well as bicycloelemene (Garnero and Tabacchi, 1987; Bülow and König, 2000). In contrast, major rearrangement products such as the bourbonenes were not present. By use of a PTV injector, rearrangement processes are reduced (Garnero and Tabacchi, 1987). Comparison analyses performed with a PTV injector did not show significant differences compared to splitless injection. The rearrangements also can take place during biosynthesis of the sesquiterpenes, thus making it difficult to decide whether some of them are true naturally occurring components or artifacts. Many of the identified sesquiterpenes are biosynthetically related, for example, β-caryophyllene and α-humulene share the same precursor cation (Teisseire, 1994). In addition, germacrene A, or other sesquiterpenes even more susceptible to heat may be involved in the attraction. Furthermore, the specialist herbivore may be able to cope with changes in volatile composition.

Despite the similarity of the terpenoid blend elicited by jasmonate treatment and by egg deposition, it remains unclear whether the signal pathway proceeds via jasmonic acid. On the other hand, the absence of a variety of compounds may indicate that other mediators are involved. In the future, blocking experiments could confirm the role of jasmonic acid in the process of signal transaction.

Further insight into which terpenes are used by the parasitoid for host location will be obtained from electrophysiological studies. The activity of extracts obtained from jasmonic acid-induced leaves will provide access to larger amounts of volatiles relative to those from headspace techniques. Preparative chromatography will be performed to elucidate the function of single components in the behavior of *Oomyzus gallerucae*.

REFERENCES

BALDWIN, I. T., and PRESTON, C. A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137–145.

BALDWIN, I. T., SCHMELZ, E. A., and OHNMEISS, T. E. 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* Spegazzini and Comes. *J. Chem. Ecol.* 20:2139–2157.

BOLAND, W., NEY, P., JÄNICKE, L., and GASSMANN, G. 1984. A "Closed-loop-stripping" technique as a versatile tool for metabolic studies of volatiles, pp. 371–380, in P. Schreier (ed.). Analysis of Volatiles: Method, Applications, de Gruyter. Berlin.

BOLAND, W., HOPKE, J., DONATH, J., NÜSKE, J., and BUBLITZ, F. 1995. Jasmonic acid and coronatine induce odor production in plants. *Angew. Chem. Int. Ed. Engl.* 34:1600–1602.

BORTZ, J. 1993. Statistik für Sozialwissenschaftler, 4th ed. Springer. Berlin.

BÜLOW, N., and KÖNIG, W. A. 2000. The role of germacrene D as a precursor in sesquiterpene biosynthesis: Investigations of acid catalyzed, photochemically and thermally induced rearrangements. *Phytochemistry* 55:141–168.

CREELMAN, R. A., and MULLET, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:355–381.

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant–carnivore mutualism. *J. Plant Physiol.* 143:465–472.

DICKE, M., and VET, L. 1999. Plant–carnivore interactions: Evolutionary and ecological consequences for plant, herbivore and carnivore, pp. 483–520, *in* H. Olff, V. K. Brown, and R. H. Drent (eds.). Herbivores: Between Plants and Predators. Blackwell Science, Oxford.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A. 1990. Isolation and identification of volatile kairomone that effects predator–prey interactions. Involvement in host plant in its prdocution. *J. Chem. Ecol.* 16:381–396.

DICKE, M., VAN BAARLEN, P., WESSELS, R., and DIJKMAN, H. 1992. Herbivory induces systemic production of plant volatiles that attract predators of the herbivore: Extraction of endogenous elicitor. *J. Chem. Ecol.* 19:581–599.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25:1907–1922.

DU, Y., POPPY, G. M., POWELL, W., PICKET, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1998. Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidus ervi. J. Chem. Ecol.* 24:1355–1368.

FARMER, E. E., and RYAN, C. A. 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4:129–134.

GARNERO, J., and TABACCHI, R. 1987. Examples of artefact formation by chromatographic techniques, pp. 359–367, *in* P. Sandra, and C. Bicchi (eds.). Capillary Gas Chromatography in Essential Oil Analysis. Dr. Alfred Huethig Verlag, Heidelberg.

GOLS, R., POSTHUMUS, M. A., and DICKE, M. 1999. Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis. Entomol. Exp. Appl.* 93:77–86.

JOULAIN, D., and KÖNIG, W. A. 1998. The Atlas of Spectral Data of Sesquiterpene Hydrocarbons. EB-Verlag, Hamburg.

KAISER, R. 1991. Trapping, investigation and reconstitution of flower scents, pp. 213–250, *in* P. M. Müller and D. Lamprarsky (eds.). Perfumes, Art, Science and Technology. Elsevier Applied Science, London.

KOCH, T., KRUMM, T., JUNG, V. ENGELBERTH, J., and BOLAND W. 1999. Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiol.* 121:153–162.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1995. β-Glucosidase: An elicitor of herbivore induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92:2036–2040.

MEINERS, T., and HILKER, M. 1997. Host location in *Oomyzus gallerucae* (Hymenoptera: Eulophidae), an egg parasitoid of the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae). *Oecologia* 112:87–93.

MEINERS, T., and HILKER, M. 2000. Induction of plant synomones by oviposition of a phytophagous insect. *J. Chem. Ecol.* 26:221–232.

MIKSCH, M., and BOLAND, W. 1996. Airborne methyl jasmonate stimulates the biosynthesis of furanocoumarins in the leaves of celery plants (*Apium graveolescens*). *Experientia* 52:739–743.

PARÉ, P. W., and TUMLINSON, J. H. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114:1161–1167.

PARÉ, P. W., and TUMLINSON, J. H. 1998. Cotton volatiles synthesized and released distal to the site of insect damage. *Phytochemistry* 47:521–526.

PARÉ, P. W., and TUMLINSON, J. H. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121:325–331.

SEMBDNER, G., and PARTHIER, B. 1993. The biochemistry and the physiological and molecular actions of jasmonates. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:569–589.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1994. Volatile herbivore-induced terpenoids in plant–mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol.* 20:1329–1354.

TEISSEIRE, P. J. 1994. Chemistry of Fragrant Substances. VCH publishers, New York, pp. 200–218.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., MCCALL, P. J., ALBORN, H., and TUMLINSON, J. H. 1993. An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps. *J. Chem. Ecol.* 19:412–425.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92:4169–4174.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L., and WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta* 207:146–152.

VAN DORT, H. M., JÄGERS, P. P., TER HEIDE, R., and VAN DER WEERDT, J. A. 1993. *Narcissus trevithian* and *Narcissus geranium*: Analysis and synthesis of compounds. *J. Agric. Food Chem.* 41:2063–2075.

VET, L. E. M., VAN LENTEREN, J. C., HEYMANNS, M., and MEELIS, E. 1983. An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. *Physiol. Entomol.* 8:97–106.

VINSON, S. B. 1985. The behavior of parasitoids, pp. 417–469, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Press, New York.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

ECOLOGICALLY ACTIVE 2-OCTANOYLCYCLOHEXANE-1,3-DIONE FROM *Philodendron guttiferum*

HESHAM R. EL-SEEDI,[1,2] LARS BOHLIN,[1] FELIPE GHIA,[3] and KURT B. G. TORSSELL[4,*]

[1] *Department of Pharmacy, Biomedical Centre*
Uppsala University
Box 579, S-57123 Uppsala, Sweden

[3] *Facultad de Quimica Ingeneria, EPN*
Quito, Ecuador

[4] *Department of Organic Chemistry, Chemical Institute*
University of Aarhus
8000 Aarhus C, Denmark

(Received March 1, 2000; accepted November 6, 2000)

Abstract—The novel naturally occurring 2-octanoylcyclohexane-1,3-dione **1** was isolated in its enol form from *P. guttiferum* (Araceae). Its chemical ecological relevance is discussed. There was mass spectral evidence for the presence of small amounts of the homologous 2-decanoyl and 2-dodecanoyl derivatives.

Key Words—Philodendron guttiferum, Araceae, 2-acylcyclohexane-1,3-diones, kairomone, ecological relevance.

INTRODUCTION

There are approximately 300 species of the genus *Philodendron* that are native to the tropical Americas and the West Indies. Several of these have been used in folk medicine for treatment of various ailments such as skin infections, boils, stomach disorders, and snake bites (Schultes and Raffauf, 1990). They have also been used as a contraceptive and a fish poison (Schultes and Raffauf, 1990). In particu-

*To whom correspondence should be addressed at current address: Finn Malmgrens Väg 3A, S-12138 Johanneshov, Sweden.
[2] On leave from the Department of Organic Chemistry, Faculty of Science, El Menoufia University, Shebin El-Kom, El-Menoufia, Egypt.

0098-0331/00/0300-0517$19.50/0

lar, *Philodendron guttiferum* Kunth, (Araceae) is a "tree-loving" epiphytic species, common in the primary forest, that exudes a latex. It has been used in Ecuadorian traditional medicine as an antimicrobial agent. Our preliminary pharmacological tests of the crude extracts indicated moderate antiinflammatory, antiviral, and toxic properties. This, coupled with our observations of some interesting features in the ^{1}H NMR spectrum, prompted further investigation. The aim of our study was to isolate and characterize the biologically active compounds.

METHODS AND MATERIAL

Plant Material. P. guttiferum was collected in 1990 in the Provincia del Napo, Ecuador. A voucher specimen, F. G. 390, is kept in the Herbario Economico, Escuela Politecnica National, Quito.

General Procedures. The NMR spectra were recorded with a 270-MHz Jeol JNM EX-270/4000 instrument. Heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments were performed using a Varian VXR-400 NMR instrument. EI-MS and positive-ion FAB-MS (with glycerol as matrix) spectra were recorded with a Jeol JMS SX/SX102A instrument. Analytical TLC was performed with silica gel 60 F_{254} on precoated aluminum sheets. Preparative TLC was carried out on silica gel 60 $PF_{254+360}$-coated glass plates (20 × 20 × 0.25 and 0.50 mm; Merck, Darmstadt, Germany). UV light at 245 and 366 nm, and iodine chamber or spraying with vanillin–sulfuric acid were used to visualize compounds. MPLC was performed using Separo AB, MPLC equipment (Baeckström Separo AB, Lidingö, Sweden).

Extraction. The whole plant was dried at 40°C in the dark, in a ventilated hood, and ground. The material, 0.54 kg, was extracted at room temperature three times with light petroleum (40–60°C) with occasional stirring and filtered, followed by three extractions with methanol for seven days each time. The extracts were evaporated in vacuo to give 7.7 and 42.5 g of a gelatinous and oily material, respectively. The methanol extract was partitioned between ethyl acetate and water to give 13 g of an ethyl acetate soluble fraction. An insoluble residue of 1.5 g was discarded. The water phase was freeze-dried to give 27.4 g of a crude material, which mainly consisted of carbohydrates. It was not further investigated.

Isolation and Purification. The ethyl acetate fraction, 12.5 g, was subjected to Separo column chromatography on silica gel 60 (30 g) with gradient elution using hexane–CH_2Cl_2 and EtOAc–MeOH. From the nonpolar fractions, straight-chain hydrocarbons, fatty acids, and fatty acid methyl esters were isolated. At low solvent polarity 5 mg stigmasterol and 4 mg β-sitosterol were eluted and purified by preparative TLC using CH_2Cl_2 and $CHCl_3$–MeOH as eluents, respectively. They were identified by their ^{1}H NMR spectra and by comparison with authentic specimens.

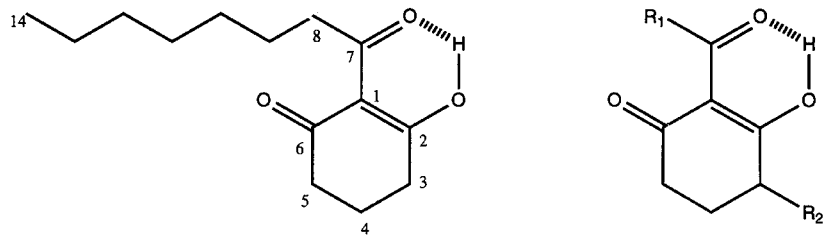
The novel naturally occurring compound **1** was eluted as an oil at a slightly higher eluent polarity and was purified by repeated MPLC and preparative TLC by using CH_2Cl_2 as eluent, 39 mg. ^{1}H NMR ($CDCl_3$), δ ppm: 0.89 (3H-14, t), 1.29 (8H-10-13, m), 1.59 (2H-9, quint), 1.98 (2H-4, quint), 2.47 (2H-3, t), 2.64 (2H-5, t), 2.97 (2H-8, t). ^{13}C NMR ($CDCl_3$), δ ppm: 14.09 (C-14), 19.07 (C-4), 22.60 (C-13), 24.60 (C-9), 29.09 (C-10), 29.34 (C-11), 31.72 (C-12), 33.21 (C-5), 38.69 (C-3), 40.50 (C-8), 112.85 (C-1), 194.68 (C-2), 198.36 (C-6), 206.09 (C-7). FAB-MS (positive mode) m/z (rel. int.): 239, $(M+1)^+$ ion, (98), 139 (18), 125 (13), 80 (28), 67 (35), 41 (55), corresponding to a molecular composition of $C_{14}H_{22}O_3$.

The mass spectra of the main chromatographic fractions containing **1** showed also small peaks at m/z 267 and m/z 295, $(M+1)^+$ ions and fragmentation peaks indicating the presence of the homologous compounds $C_{16}H_{26}O_3$ and $C_{18}H_{30}O_3$, **2**, $R_1 = (CH_2)_8CH_3$, $R_2 = H$ and $R_1 = (CH_2)_{10}CH_3$, $R_2 = H$, respectively. Inspection of the ^{1}H NMR spectrum of the crude petroleum ether extract showed that considerable amounts of metabolites of type **1** were present.

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of **1** showed only absorptions in the saturated aliphatic region, δ 2.97–0.89. The ^{13}C NMR spectrum was revealing in that it showed three C═O groups, C-2,6,7 but only one olfenic C-1 atom at δ 112.8, indicating an enolic fragment. This information, together with further HMBC experiments, led to the simple structure **1** for the component (Figure 1). The three strongly electron withdrawing acyl groups increase the acidity of the central H-1 atom sufficiently to transform the compound into its enol form, and **1** is consequently a chelating agent. Compound **1** has not been isolated previously from natural sources.

A literature search showed, however, that **1** was previously prepared by



1 **2**

FIG. 1. 2-Octanoylcyclohexane-1,3-dione and its dodecanoyl derivative. $R_1 = (CH_2)_{10}CH3$; $R_2 = H_X$.

chemical synthesis (Mudd, 1981) for the purpose of preparing model compounds for kairomones identified in the Mediterranean flour moth *Ephestia kuehniella* Zeller (Mudd, 1978, 1981, 1983) and other pyralid species (Corbet, 1971, 1973). Our ^{13}C assignments are in agreement with the literature (Mudd, 1981). The dodecanoyl derivatives **2**, $R_1 = (CH_2)_{10}CH_3$, $R_2 = H$ (Figure 1), has also been prepared by chemical synthesis (Oliver and Lusby, 1988).

Several insects secrete 2-acylcyclohexane-1,3-diones, **2**. R_1 is usually a saturated or unsaturated long chained alkyl group and R_2 is H or OH. Metabolites of this type are biologically active as insecticides and fungicides and affect the egg laying behavior and crowding in insects, hence controlling the population density (Corbet, 1971, 1973; Mudd et al., 1984). They act both as kairomones, attracting their predators, and allomones, of advantage to the host by regulating overcrowding. It is therefore conceivable that **1** in *P. guttiferum* acts as a protecting agent associated with the regulation of the number of feeding insects. It is not known whether these cyclic triketones are synthesized by the larva or are sequestered from the food. A compound related to **2**, $R_1 = (CH_2)_{10}Ph$, $R_2 = OH$, was isolated from Brazilian *Virola* and *Horsfeldia* species (Kato et al., 1992, and references therein). The possibility that the kairomones are sequestered from the food can not be excluded. A comprehensive review of the occurrence, biological effects, biogenesis, etc., of 2-acylcycloalkane-1,3-diones has recently been published (Rubinov et al., 1995).

Compounds **1** and **2** are biosynthetically closely related to the 2-acylresorcinols, which also have been found in the larval secretions, *Stephanitis* species (Oliver et al., 1985, 1990). These derivatives are reported to strongly inhibit PGH synthetase (Jurenka et al., 1989). A slightly different (folding of the polyketide chains in the biosynthesis leads to the 5-alkyl-1,3-resorcinols by decarboxylation. 5-Heptadecatri-8(*Z*),11(*Z*)-enylresorcinol was in fact isolated from *Philodendron scandens* (Reffstrup et al., 1982). Resorcinols were not observed in our extracts.

Since the ^1H NMR spectra of **1** and **2** are not particularly different from those of many fatty acids or hydrocarbons, always present in large quantities in plant extracts, the presence of minor amounts of this type compounds may occasionally have escaped detection in plants.

Acknowledgments—We are indebted to the Swedish Institute and the Egyptian government for fellowships to H.E. This work was supported by funds from the Swedish Council for Forestry and Agricultural Science and the Danish Natural Science Research Council. We wish to thank Dr. H. Balslev, Department of Botany, University of Aarhus, for identifying our plant material.

REFERENCES

CORBET, S. A. 1971. Mandibular gland secretion of flour moth *Anagasta kueniella* contains an epideictic pheromone and elicits oviposition movements in a hymenopteran parasite. *Nature* 232:481–484.

CORBET, S. A. 1973. Oviposition pheromone in larval mandibular glands of *Ephestia. Nature* 243:537–538.

JURENKA, R. A., NEAL, J. W., JR., HOWARD, R. W., OLIVER, J. E., and BLOMQUIST, G. E. 1989. In vitro inhibition of prostaglandin H synthase by compounds from the exocrine secretions of lace bugs. *Comp. Biochem. Physiol.* 93C:253.

KATO, M. J., YOSHIDA, M., and GOTTLIEB, O. R. 1992. Flavons and lignans in flowers, fruits and seedlings of *Virola venosa. Phytochemistry* 31:283–287.

MUDD, A. 1978. Novel β-triketones from Lepidoptera. *J. Chem. Soc. Chem. Commun.* 1978:1075–1076.

MUDD, A. 1981. Novel 2-acylcyclohexanes-1,3-diones in the mandibular glands of lepidopteran larvae. Kairomones of *Ephestia kueniella. J. Chem. Soc. Perkin I* 1981:2357–2362.

MUDD, A. 1983. Further novel 2-acylcyclohexane-1,3-diones in the mandibular glands of lepidopteran larvae. *J. Chem. Soc. Perkin I* 1983:2161–2164.

MUDD, A., WALTERS, J. H. H., and CORBET, S. A. 1984. Relative kairomonal activities of 2-acylcyclohexane-1,3-diones in eliciting oviposition behavior from parasite *Nemeretis canescens. J. Chem. Ecol.* 10:1597–1601.

OLIVER, J. E., and LUSBY, W. R. 1988. Synthesis of 2-acyl-3,6-dihydroxy-2-cyclohexen-1-ones. *Tetrahedron* 44:1591–1598.

OLIVER, J. E., NEAL, J. W., JR., LUSBY, W. R., ALDRICH, J. R., and KOCHANSKY, J. P. 1985. *J. Chem. Ecol.* 11:1223–1227.

OLIVER, J. E., LUSBY, W. R., and NEAL, J. W., JR., 1990. Exocrine secretions of the Andromeda lace bug *Stephanites takeyai. J. Chem. Ecol.* 16:2243–2252.

REFFSTRUP, T., HAMMERSHÖJ, O., BOLL, P. M., and SCHMIDT, H. 1982. *Philodendron scandens* Koch et Sello subsp. *oxycardium* (Schott) Bunting: A new source of allergenic alkylresorcinols. *Acta Chem. Scand. B* 32:291–294.

RUBINOV, D. B., RUBINOV, I. L., and AKHREM, A. A., 1995. 2-Acylcycloalkane-1,3-diones. Occurrence in nature, biological activity, biogenesis, chemical synthesis. *Khim. Prir. Soedin.* 635–663 (Engl. transl. *Chem. Nat. Comp.* 31:537–559, 1995.

SCHULTES, R. E., and RAFFAUF, F. F. 1990. *The Healing Forest.* Dioscorides Press. Portland, Oregon, p. 87.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

NOOTKATONE IS A REPELLENT FOR FORMOSAN SUBTERRANEAN TERMITE (*Coptotermes formosanus*)

BETTY C. R. ZHU,[1] GREGG HENDERSON,[2,*], FENG CHEN,[2] LARA MAISTRELLO,[2] and ROGER A. LAINE[1]

[1] *Department of Biological Sciences*
[2] *Department of Entomology*
Louisiana State University Agricultural Center
Louisiana Agricultural Experiment Station
Louisiana State University
Baton Rouge, Louisiana 70803

(Received July 31, 2000; accepted November 14, 2000)

Abstract—We examined the behavior of Formosan subterranean termites toward one of the components of vetiver grass oil, the roots of which manufacture insect repellents. We found nootkatone, a sesquiterpene ketone, isolated from vetiver oil is a strong repellent and toxicant to Formosan subterranean termites. The lowest effective concentration tested was 10 μg/g substrate. This is the first report of nootkatone being a repellent to insects.

Key Words—*Vetiveria zizanioides*, vetiver oil, sesquiterpenes, ketones.

INTRODUCTION

The Formosan subterranean termite is the most destructive termite species wherever it occurs in the world. These termites search for and find cellulose largely using chemical cues from the wood itself (Amburgey and Smythe, 1977; Reinhard et al., 1997), and they exhibit similar chemoresponses to a variety of chemicals. For example, trail-following behavior has been shown to naphthalene, a well-known insect toxicant (Chen et al., 1998b,c), 2-naphthalenemethanol (Henderson et al., 1996), and 2-phenoxyethanol, a component of some ballpoint inks (Chen et al., 1998a). Formosan subterranean termites live in a miasma of chemicals present in their nests, many of which appear to be plant derived (Chen et al., 1998b; Henderson et al., 1999). Our recent investigations have focused

*To whom correspondence should be addressed.

0098-0331/00/0300-0523$19.50/0

on natural plant extracts that may disrupt this insect's ability to recruit food sources.

Vetiver grass (*Vetiveria zizanioides*, Linn Nash), a fast-growing native plant of India, is a distant relative of maize, sorghum, sugarcane and lemongrass (National Research Council, 1993). The plant has been used in India for weaving mats, baskets, fans, sachets, and ornaments that have insect repellent properties. Clothes moths, head lice, and bedbugs are thought to be repelled by vetiver grass so fashioned (National Research Council, 1993). The Formosan subterranean termite, *Coptotermes formosanus* Shiraki, readily consumes sugarcane, causing agricultural damage to the crop in some countries (Chen and Henderson, 1996; National Research Council, 1993). Therefore, it was not readily apparent that termites would be repelled by components of vetiver grass. The compounds in vetiver oil that repel cockroaches and flies are believed to include α- and β-vetivone, khusimone, khusistone, zizanal, and epizizanal (Jain et al., 1982).

Preliminary studies using dried vetiver root in a sand substrate (1 : 10 ratio) indicated that Formosan subterranean termites were effectively repelled from tunneling to a food source (Henderson et al., unpublished data). Gas chromatographic and mass spectrometric analysis of vetiver oil separated on silica columns, along with behavioral bioassays, allowed us to identify nootkatone as a repellent to Formosan subterranean termites.

METHODS AND MATERIALS

Extraction of Vetiver Oil from Roots

We examined the extracts from the roots to determine which components of the oil were repellent. The roots of fresh Louisiana-grown vetiver grass *Vetiveria zizanioides* (provided by the Donald O. Heumann Greenhouse and Laboratory, Poydras, Louisiana) were cleaned, dried at room temperature, and ground with a blender. Vetiver oil was obtained by petroleum ether extraction of the dried roots. The components of vetiver oil were isolated by silica column (2.5 × 20 cm) and eluted with hexane and increasing amounts of methylene chloride. Five fractions were obtained by eluting with hexane–CH_2Cl_2: 80 : 20 for fraction 1, 70 : 30 for fraction 2, 60 : 40 for fraction 3; 40 : 60 for fraction 4, and 20 : 80 for fraction 5. Examination of sesquiterpenic ketones were visualized for their intrinsic fluorescence under UV light (Andersen, 1970) since they are potential insect repellents. The fractions detected by UV were further isolated by preparative TLC (Analtech, Newark, Delaware) using $CHCl_3$ as a mobile phase. The bands were visualized by charring the plate at 120°C after spraying with 50% sulfuric acid.

Gas chromatography–mass spectrometry (GC-MS) was performed on a Finnigan GCQ (Trace GC 2000 coupled with Polaris MSD). A silica capillary

MS column, DB-5MS (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, California), was operated at 60°C for 1 min, increased to 150°C at 2.5°C/min held for 15 min at this temperature and increased to 260°C at 5°C/min, where is was finally held for 10 min. The injector port was operated in splitless mode at 250°C, and helium was used as carrier gas at 0.8 μ/min. The mass spectral detector (MSD) was set on full-scan mode (m/z 41–400). The authentic standard of nootkatone (crystalline, 97%) was purchased from Lancaster Synthesis Inc. (Windham, New Hampshire). Both α- and β-vetivone were kindly provided by Professor Ekkhard Winterfeldt (Institut für Organische Chemie, Technische Universität, Berlin).

Termite Bioassay

Experiment 1. A three-compartment plastic container (18 × 18 × 4 cm; Pioneer Packaging Co., North Dixon, Kentucky) was used for the termite bioassays. A small hole (0.5 cm diameter) was melted at the bottom of each of two inner walls, connecting the bioassay chamber compartments. For testing, 50 mg of nootkatone was dissolved in 10 ml ethanol as a stock solution. Five concentrations of nootkatone were placed into a sand substrate in the middle compartment of the biossay chamber: 0 (control), 10, 20, 100, and 200 μg/g sand. Four replicates were conducted for each concentration. For each concentration, 500 g of blasting sand was mixed with a series of dilutions of the stock solution (except for 0 μg/g) in 25 ml of ethanol in a glass pan and dried in a hood for 2 hr. The following day, 115 g of untreated blasting sand was added to one end of each compartment (home compartment), and 115 g of treated sand at one of five concentrations was added to the middle compartment. The 0-μg concentration was treated only with 25 ml of ethanol. Filter paper (Whatman No. 1, 2.3 cm diameter) was dried at 70°C for 3 hr and cooled to room temperature for 30 min before weighing. A weighed filter paper with 200 μl distilled water added was placed in the third compartment farthest from the introduction end. Ten milliliters of distilled water was added to the treated and untreated sand in the other compartments just prior to the introduction of termites. Fifty workers and five soldiers of Formosan subterranean termites from a large colony collected in Algiers, Louisiana, on November 18, 1997, were placed in the home compartment. The containers were covered with lids and kept in a dark incubator at 29°C. On day 16, each apparatus was dismantled, living termites were counted, and filter papers were cleaned, dried at 70°C for 3 hr, cooled for 30 min, and weighed. Consumption was calculated as the difference between the weight of filter paper before and after the 16-day incubation. The tunnels termites constructed in the sand were copied using a scanner for measurement of total tunnel lengths.

Experiment 2. The materials and methods were the same as described for

experiment 1, except: (a) the concentrations of nootkatone used to treat the sand in the middle compartment were slightly different; (b) observations of tunneling were recorded several times during the experiment; and (c) 50 g sand was placed in the third compartment under the filter paper disk and was moistened every two days with 0.3 ml of distilled water. For this experiment the concentrations of nootkatone were 0 (control), 5, 10, 20, 40, and 100 μg/g sand. Four replicates were performed for each concentration. Every two days all containers were checked for tunneling activity and the evaluating criterion was: 0 = termites on the surface (no tunneling); 1 = tunneling in the first chamber; 2 = tunneling in the first and in the middle (treated) chamber; 3 = tunneling in all chambers.

Statistical Analysis

For experiment 1, the differences among treatments in filter paper consumption, tunnel length, and percentage mortality were analyzed by ANOVA. For experiment 2, we used a nonparametric analysis of variance (Kruskal-Wallis ANOVA) in order to analyze trends in tunneling differences between days of observation. At the end of the experiment, differences among treatments were analyzed as in experiment 1. For both experiments, differences between treatments were analyzed by Tukey's studentized range test (HSD). Although percentage values were transformed to arc-sin of the square root for data analysis, nontransformed means are reported.

RESULTS

Identification of Nootkatone as Active Fraction from Vetiver Oil

Three fractions (3–5) contained autofluorescing bands detected by UV. Each fraction was further separated by preparative TCL with $CHCl_3$ as a mobile phase. In fraction 4, bands 1 and 2 autofluoresced, and were examined by GC-MS. Nootkatone was a major constituent of band 2 (Figure 1A). The chromatograph of authentic nootkatone is shown in Figure 1B. The nootkatone identification was verified by comparing the mass spectrum of its GC peak (Figure 2A) with authentic nootkatone (Figure 2B).

Termite Bioactivity of Nootkatone

Experiment 1. The mean consumption of filter paper decreased significantly in the presence of nootkatone; the decrease in consumption was concentration dependent (Table 1). Even at the lowest concentration tested (10 μg/g sand), nootkatone significantly decreased feeding as compared with the control. Little consumption of filter paper was found when concentrations of nootkatone were higher than 20 μg/g. Termite mortality increased as nootkatone concentrations

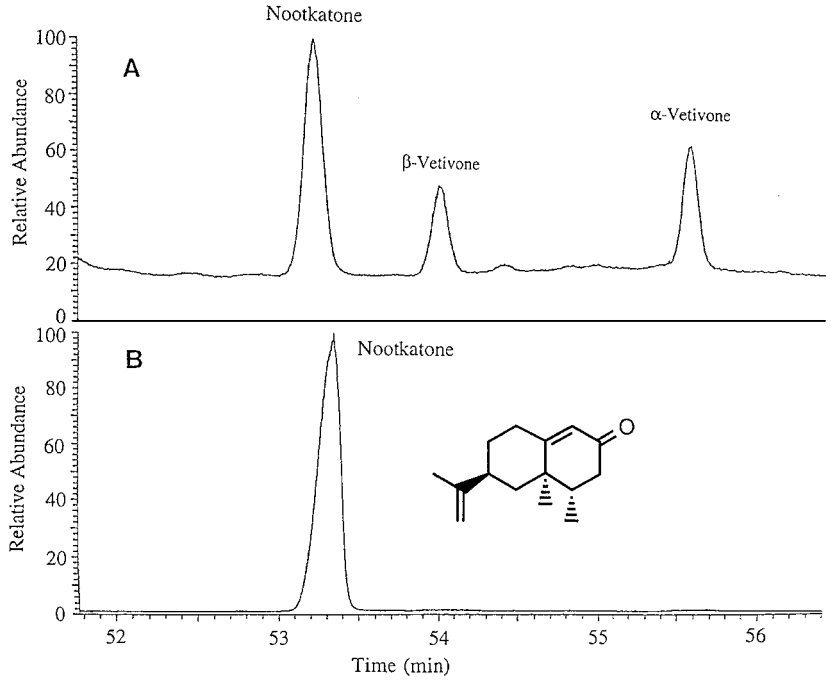


FIG. 1. Gas chromatography of band 2 (A) and the standard of nootkatone (B). The retention time of nootkatone in band 2 is 53.22 min (first peak) and in the standard is 53.35 min.

increased (Table 1). All concentrations of nootkatone had a significant impact on mortality when compared with the control. Ninety percent mortality or greater was observed in all chambers when nootkatone concentrations were ≥100 μg/g sand. The presence of nootkatone substantially decreased the tunneling activity even at the lowest concentration of 10 μg/g sand (Table 1). At higher concentrations of nootkatone (20 μg/g and higher), no tunneling was visible in the middle chamber.

Experiment 2. Units treated with different concentration of nootkatone showed significantly different levels of tunneling activity. On day two, significant differences were detected among treatments ($H = 20.47$, $df = 5$, $P = 0.001$, Kruskal-Wallis ANOVA); almost all groups treated with lowest concentrations were able to tunnel in the first and even in the middle (treated) chamber, while termites faced with the highest concentration remained on the surface. The same trend is confirmed on day 6 ($H = 17.39$, $df = 5$, $P = 0.0038$), where some termite groups (control and concentration 3) tunneled into the feeding chamber,

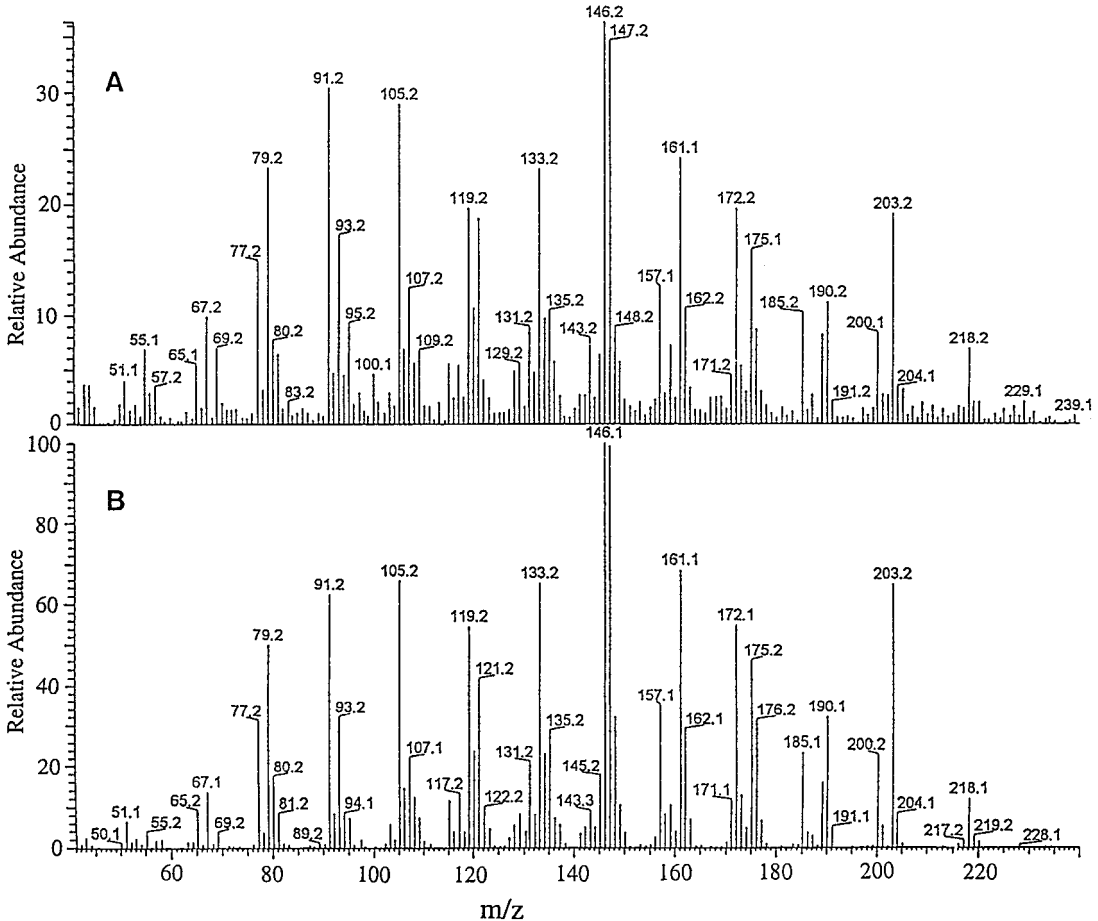


FIG. 2. Mass spectrum of the nootkatone peak in band 2 (A) and authentic nootkatone (B).

TABLE 1. MEAN (±SD) OF PAPER CONSUMPTION, PERCENT MORTALITY, AND TUNNELING LENGTH OF FORMOSAN SUBTERRANEAN TERMITE AFTER 16-DAY EXPOSURE (EXPERIMENT 1)[a]

Concentration of nootkatone (μg/g sand)	Weight loss of filter paper (mg)	Termite mortality (%)	Tunneling length (cm)
0	10.500 ± 2.867A	58.50 ± 8.72A	27.000 ± 6.072A
10	1.900 ± 0.641B	88.50 ± 10.12B	7.225 ± 2.155B
20	0.525 ± 0.709B	87.00 ± 18.22B	0.000 ± 0.000B
100	0.025 ± 0.050B	90.50 ± 11.72B	0.000 ± 0.000B
200	0.275 ± 0.050B	95.00 ± 8.718B	0.000 ± 0.000B

[a] Four replicates were included for each treatment. Means within a column followed by the same letter are not significantly different (ANOVA: $F = 9.39$, $df = 4$ and $P < 0.0005$ for paper consumption; $F = 4.22$, $df = 4$, $P < 0.0174$ for percentage mortality and $F = 43.1$, $df = 4$, $P < 0.0001$ for tunneling length.)

and on day 11 ($H = 14.52$, $df = 5$, $P = 0.0126$) (Table 2). As in experiment 1, significant differences were detected in filter paper consumption, where only the controls and groups treated with 20 and 40 μg/g sand of nootkatone could eat some filter paper. Differences were also detected in tunnel length: only the controls and units treated with the lowest concentration of nootkatone showed tunneling behavior. Due to high variability between units belonging to the same treatment group, no significant differences were detected on termite mortality.

TABLE 2. MEAN (±SD) OF PAPER CONSUMPTION, PERCENT MORTALITY, AND TUNNELING LENGTH OF FORMOSAN SUBTERRANEAN TERMITES AFTER 16-DAY EXPOSURE (EXPERIMENT 2)[a]

Concentration of nootkatone (μg/g sand)	Weight loss of filter paper (mg)	% termite mortality	Tunneling length (cm)
0	3.675 ± 3.500 A	27.727 ± 10.111 A	29.450 ± 8.065 A
5	0.150 ± 0.300 B	36.364 ± 7.120 A	24.975 ± 14.558 A
10	0.650 ± 1.300 AB	52.273 ± 45.281 A	11.000 ± 16.818 AB
20	0.300 ± 0.600 AB	48.182 ± 36.980 A	2.250 ± 2.872 B
40	0 ± 0B	39.091 ± 32.710 A	0 ± 0 B
100	0 ± 0B	58.636 ± 33.008 A	0 ± 0 B

[a] Four replicates were included for each treatment. Means within a column followed by the same letter are not significantly different (ANOVA: $F = 3.416$, $df = 5$, $P = 0.024$ for paper consumption; $F = 0.580$, $df = 5$, $P = 0.715$ for percentage mortality and $F = 7.215$, $df = 5$, $P = 0.0007$ for tunneling length.)

DISCUSSION

Vetiver oil is a complex essential oil containing several hundred individual compounds. Of them, 92 compounds have been characterized and can be divided into 33 esters, 36 sesquiterpenic hydrocarbons, 5 bi- and tricyclic sesquiterpenic alcohols, 9 ketones, 3 aldehydes, and 6 acids (Cazaussus et al., 1989). Nootkatone, 4,4a,5,6,7,8-hexahydro-6-isopropenyl-4,4a-dimethyl-2(3H)-naphthalone, is a mildly pungent sesquiterpene ketone found in the oil of Alaska yellow cedar, *Chamaecyparis nootkatensis* (Lamb) Spach (Erdtman and Hirose, 1962) and in a great number of citrus oils, especially oil from grapefruit, *Citrus pavadisi* Calli (MacLeod and Buigues, 1964). Nootkatone is widely used in the perfume and flavor industries, being essentially nontoxic to humans (National Research Council, 1993).

Our studies determined that nootkatone has potential as a termite repellent barrier. Considering tunneling behavior as a measurement of termite activity, these results confirmed that nootkatone effects termite vigor. Termites from colonies treated with lower concentrations of nootkatone were able to dig tunnels in the home and in the treated chamber, and this ability was negatively correlated with the concentration of nootkatone. Termites treated with the highest concentrations of nootkatone were not able to dig tunnels, even in the home chamber, possibly because of the nootkatone vapors acting as an inhibitor of termite mobility. We believe that a concentration of nootkatone bewteen 10 and 1000 μg/g, preferably between 10 and 200 μg/g, may be useful in repelling and killing termites. We are also evaluating nootkatone as a chemical that could be of value against termites in structural wood treatments or cellulose mulch applications (Henderson et al., unpublished data).

A barrier created by plants that manufacture a termite repellent could potentially provide long-lasting repellency. Vetiver grass is a fast growing plant with a huge spongy mass of roots. The roots grow to depths of 3 m, and with a little attention the plant could live for 50–60 years (National Research Council, 1993). Since the oil occurs primarily in the roots, a low-cost way to prevent invasion by subterranean termites and other insidious underground insects may be to plant a solid band of vetiver grass around a house. Recently, we found that some of the insect repellents found in the roots also occur in the soil surrounding the plant (Henderson et al., in preparation).

Vetiver oil is one of the most complex essential oils (Cazaussus et al., 1989). In addition to nootkatone, we also have found that vetiver oil itself and its major components α- and β-vetivone were powerful repellents and toxicants against Formosan subterranean termite (Zhu et al., in preparation). Jain et al. (1982) reported six substances in vetiver oil that have potent topical irritant activity on cockroaches and flies.

Acknowledgments—This project was approved for publication by the Louisiana State University Agricultural Center and Louisiana Agricultural Experiment Station as manuscript 00-17-0304. Partial funding was provided through a specific cooperative agreement with the USDA/ARS-New Orleans (58-6435-8-084).

REFERENCES

AMBURGEY, T. L., and SMYTHE, R. V. 1977. Shelter tube construction and orientation by *Reticulitermes flavipes* in response to stimuli produced by brown rotted wood. *Sociobiology* 3:27–34.

ANDERSEN, N. H. 1970. Biogenetic implications of the antipodal sesquiterpenes of Vetiver oil. *Phytochemistry* 9:145–151.

CAZAUSSUS, A., ROZE, P. Y., and SELLIER, N. 1989. Positive and negative chemical ionization GC/MS and GC/MS/MS for chromatographic studies of vetiveryl acetate. Use of MS/MS for the differentiation of isomeric sesquiterpenes. *Chromatographia* 28:579–584.

CHEN, J., and HENDERSON, G. 1996. Determination of feeding preference of Formosan subterranean termite (*Coptotermes formosanus* Shiraki) for some amino acid additives. *J. Chem. Ecol.* 22:2359–2369.

CHEN, J., HENDERSON, G., and LAINE, R. A. 1998a. Isolation and identification of 2-phenoxyethanol from a ballpoint pen ink as a trail-following substance of *Coptotermes formosanus* Shiraki and *Reticulitermes* sp. *J. Entomol. Sci.* 33:97–105.

CHEN, J., HENDERSON, G., GRIMM, C. A., LLOYD, S. W., and LAINE, R. A. 1998b. Termites fumigate their nests with naphthalene. *Nature* 392:558–559.

CHEN, J., HENDERSON, G., GRIMM, C. A., LLOYD, S. W., and LAINE, R. A. 1998c. Naphthalene in Formosan subterranean termite carton nests. *J. Agric. Food Chem.* 46:2337–2339.

ERDTMAN, H., and HIROSE, Y. 1962. The chemistry of the natural order Cupressales: 46. The structure of nootkatone. *Acta Chem. Scand.* 16:1311–1314.

HENDERSON, G., STOWELL, J. C., GATTI, S., and GERALDS, R. S. 1996. Trail-following behavior by Formosan termite workers and soldiers on artificial trails. *Proc. Natl. Conf. Urban Entomol.* p. 32.

HENDERSON, G., CHEN, J., and LAINE, R. A. 1999. Compositions and methods for detecting and killing termites. United States Patent Number 5,874,097.

JAIN, S. C., NOWICKI, S., EISNER, T., and MEINWALD, J. 1982. Insect repellents from ventiver oil: 1. Zizanal and epizizanal. *Tetrahedron Lett.* 23:4629–4642.

MACLEOD, JR., W., and BUIGUES, N. M. 1964. Sesquiterpenes; 1. Nootkatone, a new grapefruit flavor constituent. *J. Food Sci.* 29:565–568.

NATIONAL RESEARCH COUNCIL. 1993. Vetiver grass: A thin green line against erosion. (N. D. Vietmeyer & F. R. Ruskin, Eds.) Board on Science and Technology for International Development. National Academy Press Washington, D.C. ISBN 0-309-04269-0.

REINHARD, J., HERTEL, H., and KAIB, M. 1997. Systematic search for food in the subterranean termite *Reticulitermes santonensis* De Feytaud (Isoptera: Rhinotermitidae). *Insectes Soc.* 44:147–158.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

CHEMOSENSORY ASSESSMENT OF PREDATION RISK BY SLIMY SCULPINS (*Cottus cognatus*): RESPONSES TO ALARM, DISTURBANCE, AND PREDATOR CUES

PAMELA J. BRYER,[1] REEHAN S. MIRZA,[1] and DOUGLAS P. CHIVERS[2,*]

[1] *Department of Biological Sciences*
University of Maine
5751 Murray Hall
Orono, ME 04469-5751

[2] *Department of Biology*
University of Saskatchewan
112 Science Place
Saskatoon, Saskatchewan S7N 5E2, Canada

(Received April 26, 2000; accepted November 4, 2000)

Abstract—Slimy sculpins (*Cottus cognatus*) are small benthic fish that commonly co-occur with predatory fishes throughout most of their range in North America. In two laboratory experiments we examined the nature and extent of responses of slimy sculpins to chemosensory cues from predatory brook trout (*Salvelinus fontinalis*) and chemical alarm cues released by conspecific sculpins. Sculpins responded to three different classes of chemical stimuli: (1) cues of predatory brook trout, (2) damage-released alarm cues from conspecific sculpins, and (3) disturbance cues from conspecific sculpins. Sculpins did not distinguish between trout fed different diets; however, the presence of damage-released alarm cues did enhance the intensity of response to the predator chemicals. A histological analysis of the skin of sculpins revealed the presence of large sacciform cells that may act as the source of chemical alarm signals.

Key Words—Predation, chemical cues, alarm signals, disturbance cues, anti-predator behavior, sacciform cells.

*To whom correspondences should be addressed. e-mail: Doug.Chivers@usask.ca

0098-0331/00/0300-0533$19.50/0

INTRODUCTION

Predation is one of the most important selective forces shaping the behavior, morphology, and life-history characteristics of prey animals (Sih, 1987; Lima and Dill, 1990; Chivers and Smith, 1998). A fundamental way in which aquatic organisms assess predation risk is by using chemosensory information (Chivers and Smith, 1998; Kats and Dill, 1998). Under many circumstances chemical cues may be the best source of information available for prey to assess their risk. For example, chemical cues are probably of prime importance when high habitat complexity, low light levels, or high turbidity limit visual cues.

Prey animals assess predation risk using chemical cues from two sources. The first source, generally termed "predator chemicals" or alternatively "predator kairomones," is cues released directly from the predator (reviewed by Kats and Dill, 1998). The second source of chemicals is termed "alarm pheromones" or more generally "chemical alarm signals" (reviewed by Chivers and Smith, 1998). Alarm pheromones are chemicals released by conspecific prey animals when they detect or are captured by a predator. This class of chemicals is further categorized based on the point in the predation sequence upon which the chemical signal is released. Signals released by prey animals that are disturbed, but not injured, are referred to as disturbance signals. Those signals emitted by prey animals only following their capture by a predator are referred to as damage-released alarm signals. The classic Schreckstoff alarm system, characteristic of minnows and other ostariophysan fishes, is probably the best-known example of a damage-released alarm pheromone system. In this system, the pheromone is contained in specialized cells in the epidermis of the fishes (Smith, 1992; Chivers and Smith, 1998).

Damage-released chemical alarm systems are widespread in aquatic organisms. For example, there are estimated to be about 7000 species that possess the Schreckstoff alarm system (Smith, 1992). In addition, there are alarm systems in other groups of fishes (reviewed by Chivers and Smith, 1998), as well as in larval amphibians (Hews and Blaustein, 1985; Hews, 1988), gastropods (Appleton and Palmer, 1988), echinoderms (Lawrence, 1991), sea anemones (Howe and Sheike, 1975) insect larvae (Chivers et al., 1996; Huryn and Chivers, 1999) and crustaceans (Wudkevich et al., 1997; Hazlett, 1994).

Relatively little is known regarding the existence of disturbance signals in aquatic organisms. Disturbance signals are known in one species of crayfish (Hazlett, 1985, 1989), one species of hermit crab (Hazlett, 1990a), one species of larval frog (Kiesecker et al., 1999) and two species of fishes (Wisenden et al., 1995; Jordão and Volpato, 2000).

Antipredator responses to predator chemicals are common among a diversity of aquatic organisms, including fishes (reviewed by Kats and Dill, 1998). Several studies have documented that the presence and/or intensity of an

antipredator response of prey to predator chemicals is dependent upon the diet of the predator. In some cases, the prey animals will respond to the predator only when it is fed conspecifics of the prey (e.g., Mathis and Smith, 1993; Chivers et al., 1996). In other cases, the prey will respond to the predator regardless of the predator's diet, but the intensity of the response is stronger when the predator is fed conspecifics of the prey (Wilson and Lefcort, 1993).

We conducted a series of laboratory studies to examine chemosensory assessment of predation risk by slimy sculpins (*Cottus cognatus*). Slimy sculpins are small benthic fish that inhabit streams and lakes throughout much of northern North America. The sculpins used in our study were collected from streams in central Maine, where they co-occurred with predatory brook trout (*Salvelinus fontinalis*).

In our experiments we tested whether sculpins exhibited an antipredator response to either chemical cues from predatory brook trout, damage-released alarm cues from injured conspecifics, or cues of disturbed conspecifics. Additional experiments examined whether the diet of the brook trout influenced the intensity of the antipredator response of the sculpins and whether chemical alarm signals, presented simultaneously with cues of the predator, enhanced the intensity of the antipredator response. We also completed a histological examination of the skin of sculpins. The source of damage-released alarm pheromones of Ostariophysan fishes is large club cells in the epidermis (Smith, 1992). Similarly, the source of damage-released alarm cues of sculpins may be large sacciform cells in the epidermis (Hugie et al., 1991).

METHODS AND MATERIALS

Fish Collection and Maintenance

In the summer of 1998, we collected slimy sculpins from two adjoining streams in central Maine (Spruce Mountain Brook and White Brook, 69°12′W 45°29′N. Both streams contain brook trout. The fish were caught by backpack electrofishing and were transferred to our laboratory at the University of Maine. The fish were maintained in a 350-liter stream tank at 12–13°C on a 14L : 10D photoperiod. The stream tank was supplied with fresh well water at a rate of 1 liter/min. The fish were fed twice daily ad libitum with frozen brine shrimp (*Artemia salina*).

Brook trout were supplied by the Enfield State Fish Hatchery (Maine Department of Inland Fisheries and Wildlife). They were maintained in a 888-liter circular tank with the same temperature and photoperiod as the sculpins. As a standard diet, the trout were fed previously frozen brine shrimp and commercial trout (Corey Feed Mills). Specific experiments required us to change the diet of some trout (see experiment 2).

Skin Histology

Over the past two years we have made histological examinations of 19 sculpins. Immediately after they were killed, the tissue from the anterior dorsal region was excised and fixed in glutaraldehyde and osmium tetroxide using the microwave enhanced fixation method (Giberson and Demaree, 1995). The tissue was embedded in Epon-Aldarite and 1.5-μm-thick sections were made. We identified the sacciform cells by negative staining with periodic-Schiff's reagent and counterstaining with hemotoxylin.

Experiment 1: Responses of Sculpins to Alarm, Disturbance, and Predator Cues

Behavioural Assay. All experiments took place in a gravitational flow-through test apparatus (modified from Petranka et al., 1987). The testing apparatus consisted of two tiers of tanks. The lower test chambers were transparent plastic storage containers (35 × 50 × 17 cm), each of which had a thin layer of light-colored gravel that matched the substrate color of the stream where the fish were collected. The testing chambers each contained a centrally located shelter object consisting of one third of an overturned ceramic flower pot. Above each test tank there were two header tanks into which the stimuli were placed. The header tanks were nontransparent plastic storage containers (31 × 42 × 15 cm). Throughout the acclimation and testing periods, well water flowed from one of the stimulus tanks into the test chamber at a rate of approximately 1.5 liters/min.

Test fish were introduced to the test tank approximately 22 hr before they were tested. Each morning approximately 6 hr before the observations began, the test fish were each fed 1.5 ml of brine shrimp. The tests were conducted between 13:00 and 16:00 hr. Immediately before all trials, 20 ml of food-odor stimulus was added to the prestimulus tank. This was prepared by taking 1 ml of previously frozen brine shrimp and adding it to 75 ml of well water. After settling, the top layer was decanted and became the food-odor stimulus. Decanting ensured that no small pieces of food were present in the food-odor stimulus. We added 20 ml of food-odor stimulus because this was sufficient to encourage fish to move and explore the tank in search of food. All trials began 1 min after addition of the food-odor stimulus. For each trial we observed the behavior of sculpins for 10 min prior to the introduction of cues to the test tank. Cues were introduced by moving the inlet hose from one header tank to the other header tank that contained the appropriate stimulus. One minute after moving the inlet hose, we resumed recording the behavior of the sculpins for an additional 10-min poststimulus period. Moving the hose between the header tanks did not interrupt the water flow to the test tanks. Dye trials indicated that it took 45 sec for the stimulus water to reach the test tank after moving the inlet hose. The dye was evenly dispersed in the test tank within 1.5 min.

During the 10-min prestimulus and 10-min poststimulus periods, the following behaviors were recorded at 15-sec intervals: (1) number of short moves, (2) number of long moves and (3) shelter use. Short moves included all movements that were less than two body lengths. These are the most common types of sculpin movements. The fish made rapid "hops" along the substrate that ended in a rigid alert posture. Long moves included all movements that were greater than two body lengths. They typically occurred when the fish swam into the water column and glided back to the substrate.

Experimental Treatments and Preparation of Test Stimuli. In this experiment we exposed individual sculpin to chemical cues from: (1) predatory brook trout, (2) injured sculpins, (3) disturbed sculpins (chased with a model fish predator), with controls of (4) injured swordtails (*Xiphophorous helleri*), (5) undisturbed sculpins, and (6) a model fish predator. There were 20 replicates of each treatment. The order of treatments was randomized.

To prepare the brook trout stimulus we randomly removed a single brook trout from our holding tank of approximately 50 trout and placed it into the stimulus container 3 hr prior to the test. A sample of 14 stimulus trout had a mean (±SD) standard length of 129 ± 9 mm.

We used 1.5 cm^2 of sculpin skin to prepare the damage-released alarm cue for every two trials. This amount of skin is similar to that used in several other studies (reviewed by Chivers and Smith, 1998). A sculpin was humanely killed by pithing and decapitation. The skin was removed and separated as much as possible from the adhering muscle tissue and was homogenized in distilled water. The volume of the homogenate was brought up to 100 ml, strained through a fine mesh net, and divided into halves. This solution was added to the stimulus tank just before the beginning of the trial. This allowed the stimulus to disperse evenly in the stimulus tank before the trial.

In the disturbed sculpin treatment, five randomly selected sculpin were placed into the stimulus collecting tanks at 08:00 hr after being fed. All fish were chosen from a pool of previously tested sculpin. At the end of the prestimulus recording period, the sculpin were chased around the tank with a model fish predator. Care was taken to make sure that the sculpin were not accidentally hit during this procedure. They were chased for the entire 1-min interval between the pre- and poststimulus periods. The predatory fish model was a reinforced 6-inch soft fishing lure. We used five sender fish to produce the disturbance cue because sculpins frequently occur at high densities in the stream where they were collected.

In the undisturbed sculpin control treatment, five randomly selected sculpin were placed into the stimulus tanks at 08:00 hr after being fed. All fish were chosen from the same pool of fish used in the disturbed sculpin treatment. In this treatment, the sculpin were not chased around the tank with a model fish predator after the prestimulus period. Consequently, the only chemical cues were from undisturbed conspecifics.

To determine whether the fish model itself had any effect on sculpin behavior, we included a model-only control. Here the model was moved about the tank in the same manner as it was during the disturbed conspecific treatment. However, there were no sculpin present in the stimulus tank.

Stimuli from injured swordtails was prepared in the same manner as the stimuli from injured sculpins. We tested sculpins for a response to swordtails in order to control for a generalized response to injured fish skin. Swordtails were chosen because they are not closely related to sculpins nor do they co-occur with them. The swordtails were bought from a local commercial dealer as adults and maintained on Tetramin flake food.

Statistical Analyses. For each trial we calculated the difference in number of short moves and number of observations under the shelter between the pre- and post-stimulus periods. Long movements were rare and hence were dropped from the analysis. Positive values indicated increased movement and/or shelter use following addition of the stimulus; negative values indicate decreased activity and/or shelter use. Medians of each treatment were compared to the undisturbed sculpin control treatment using one-tailed Wilcoxon Mann-Whitney tests (Siegel and Castellan, 1988). The family-wise error rate was assessed and controlled using the modified Bonferroni test following Keppel (1982). The modified Bonferroni test specifies that corrections to the family-wise error rate be introduced only when the number of comparisons exceeds $k - 1$, where k is the number of treatments (Keppel, 1982). In this experiment, there was a total of six treatments. Since the analysis was restricted to five preplanned comparisons that were based on specific a priori predictions, the rejection probability (P) was set at 0.05 for each comparison (Keppel, 1982).

Experiment 2: Effects of Alarm Cues on Intensity of Sculpin Antipredator Responses

This experiment tested whether alarm cues in the diet of the trout had any influence on the antipredator responses of sculpin and whether chemical alarm signals presented simultaneously with cues of the predator enhanced the intensity of the anti-predator response. The sculpins used in this experiment were collected in November 1998 and were maintained as in experiment 1. The mean (±SD) standard length of sculpins used was 49.2 ± 5.1 mm. The trout used in this experiment were taken from those being held at our laboratory.

Four trout were housed in each of three 105-liter plastic aquaria, each of which had an air stone and was supplied with well water at a rate of 0.5 liters/min. Trout in the three containers were fed ad libitum every other day with brine shrimp, commercial trout pellets (a component of which is fishmeal), or pieces of freshly killed sculpin. The mean (±SD) standard length for the trout was 141 ± 7 mm, 143 ± 8 mm, and 146 ± 9 mm in the brine shrimp, trout, and

sculpin diet treatments, respectively. The fish were fed for at least seven days prior to being used as stimulus donors.

In this experiment, individual sculpins were exposed to one of the following five chemical treatments: (1) trout that had been fed sculpin, (2) trout that had been fed commercial trout pellets, (3) trout that had been fed brine shrimp, (4) trout fed on brine shrimp pluse 50 ml of injured sculpin cue (prepared as in experiment 1) or (5) 50 ml of distilled water. There were 20 replicates of each treatment. The order of the treatments was randomized.

The experimental protocol was nearly identical to experiment 1 except that observations were completed between 17:00 and 18:00 hr. All relative times remained constant between all experiments. As in the first experiment, we recorded the number of short moves and shelter use at 15-second intervals. However, we also recorded data on two additional response variables. In this experiment we recorded short moves only if they occurred out from under the shelter. During the first experiment, we observed that sculpins that moved to shelter often spent considerable time moving under the shelter. Movements under the shelter are probably not as risky as movements out of the shelter. We also recorded an index of total area of the tank used by the sculpins. We divided the total area of the tank (50×30 cm) into nine sections and summed the number of squares that the sculpins used before and after addition of the stimuli.

We employed the same generalized statistical approach as in experiment 1 except that we completed five preplanned comparisons. Because this experiment had five treatments, our rejection probability (P) was set at 0.04 for each comparison (Keppel, 1982).

RESULTS

Histology. We observed large sacciform cells in all 19 sculpins that we examined (Figure 1).

Experiment 1: Responses of Sculpins to Alarm, Disturbance, and Predator Cues. We observed that sculpins exhibited an antipredator response to chemical cues of brook trout, injured conspecifics, and disturbed conspecifics (Figure 2). However, there were some differences in the form of the response between treatments. In comparison to the undisturbed control treatment, sculpins responded to trout cues by decreasing their number of short moves ($U = 61.5$, $P < 0.001$), but they did not increase shelter use ($U = 175.5$, $P = 0.231$). Similarly, sculpins in the disturbed conspecific treatment decreased the number of short moves ($U = 137.5$, $P = 0.046$), but did not increase shelter use ($U = 193$, $P = 0.581$) when compared to the undisturbed controls. In response to cues of injured conspecifics, sculpins increased their shelter use ($U = 138.5$, $P = 0.034$) but did not decrease number of short moves ($U = 151$, $P = 0.092$) when compared to the undisturbed controls.

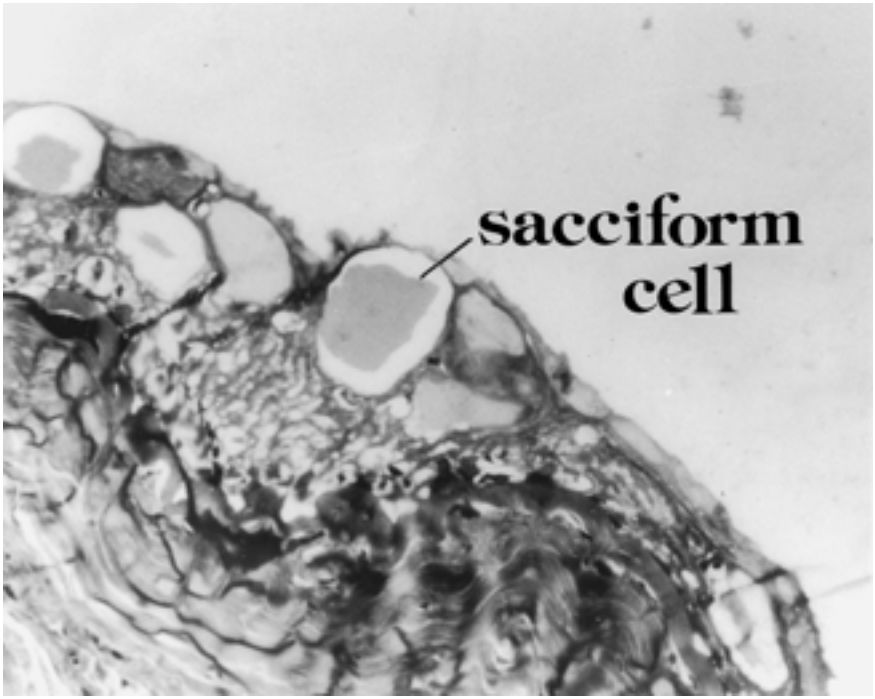


FIG. 1. Micrographs showing a sacciform cell of a slimy sculpin.

In relation to the undisturbed control treatment, sculpins did not increase shelter use or decrease the number of short movements in response to either the swordtail cues (shelter: $U = 159.5$, $P = 0.115$; movement: $U = 180.5$, $P = 0.299$) or the model (shelter: $U = 186.5$, $P = 0.652$; movement: $U = 186.5$, $P = 0.358$).

Experiment 2: Effects of Alarm Cues on Intensity of Sculpin Antipredator Responses. We observed that sculpins responded to chemical cues of brook trout regardless of the diet of the trout (Figure 3). In relation to the distilled water control, sculpins exposed to trout fed sculpins decreased movement ($U = 113.5$, $P = 0.007$), decreased area use ($U = 111$, $P = 0.004$), and increased shelter use ($U = 105$, $P = 0.004$). Similarly, sculpins exposed to trout fed commercial trout pellets decreased movement ($U = 108$, $P = 0.005$), decreased area use ($U = 107$, $P = 0.006$), and increased shelter use ($U = 127$, $P = 0.019$) in relation to the distilled water control.

Sculpins exposed to trout fed brine shrimp decreased movements ($U = 123$, $P = 0.014$), decreased area use ($U = 125.5$, $P = 0.013$) and increased shelter use ($U = 125.5$, $P = 0.016$) in relation to the distilled water control. In response to a combination of cues of trout fed brine shrimp plus cues of injured sculpins,

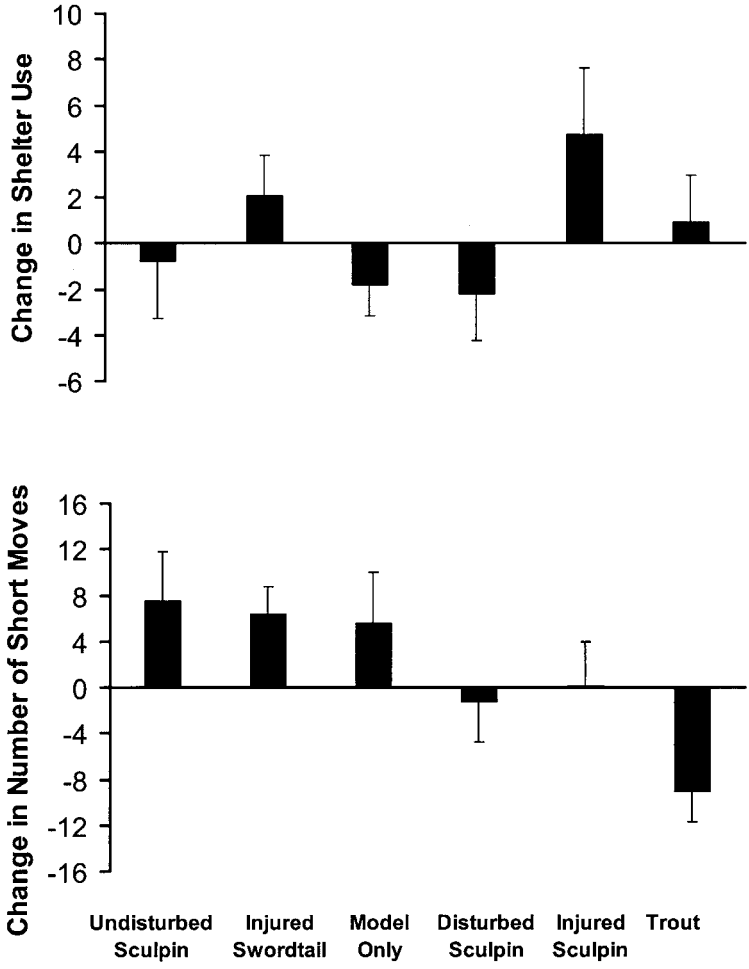


FIG. 2. Mean (±SE) change in shelter use and number of short moves by slimy sculpins following exposure to chemical cues. Treatments include: predatory brook trout, injured sculpins, and disturbed sculpins. Controls include: undisturbed sculpins, injured swordtails and a model fish predator.

sculpins decreased movements ($U = 93.5$, $P = 0.002$), decreased area use ($U = 78.5$, $P < 0.001$), and increased shelter use ($U = 100.5$, $P = 0.003$) in relation to the distilled water control. Even though sculpins responded to both cues of trout fed brine shrimp and cues of trout fed brine shrimp plus cues of injured sculpin, there was a difference in the intensity of response. Sculpins exposed to

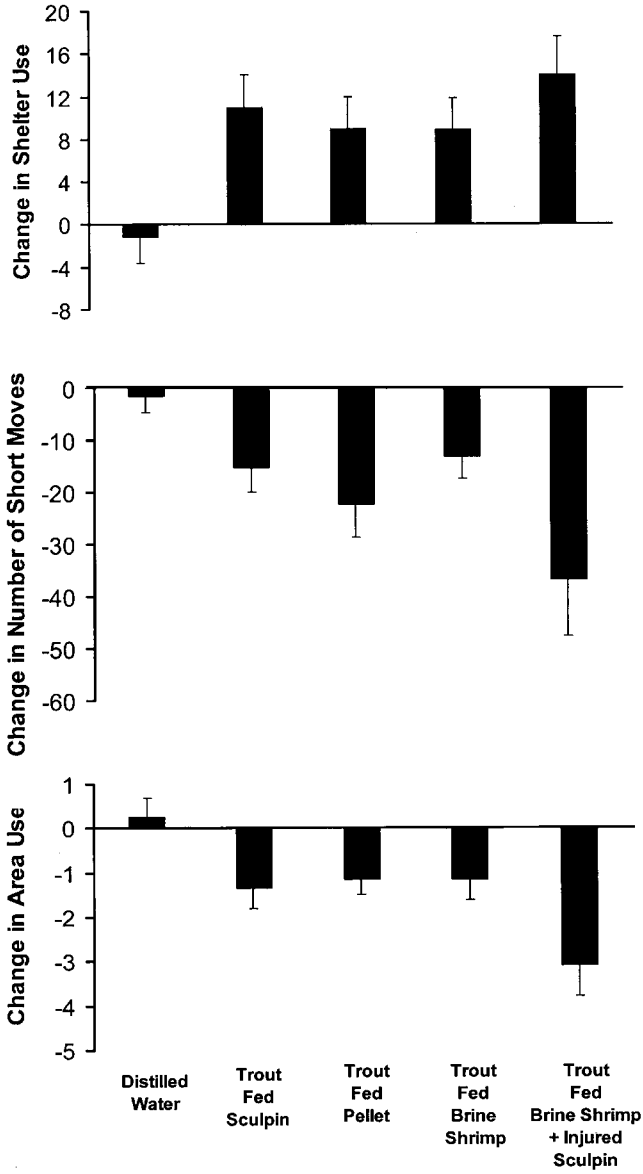


FIG. 3. Mean (±SE) change in shelter use, area use and number of short moves by slimy sculpins following exposure to chemical cues. Treatments include: brook trout fed sculpin, brook trout fed commercial pellets, brook trout fed brine shrimp, and brook trout fed brine shrimp + cues of injured sculpin. The control was distilled water.

cues of trout feeding on brine shrimp and alarm cues responded more strongly in terms of reducing area use than those exposed to cues of trout feeding on brine shrimp ($U = 132$, $P = 0.026$). There was no significant difference in the intensity of response when considering movement ($U = 150.5$, $P = 0.08$) or shelter use ($U = 167.5$, $P = 0.173$).

DISCUSSION

Our data provide evidence that slimy sculpins can assess predation risk by using a diversity of chemosensory information. In our original experiment, we documented that sculpins responded to three different classes of chemical stimuli: (1) predator chemicals, (2) damage-released alarm cues, and (3) cues of disturbed conspecifics. Additionally, we found that damage-released alarm cues enhanced the intensity of responses to predator chemicals.

The antipredator responses that we observed included reductions in activity and movement to shelter. Each of these responses would likely make the sculpins less visible to visually hunting predators such as brook trout.

Our data provide insights into the taxonomic distribution of damage-released alarm signals among members of the sculpin family (Family Cottidae). To date, five of approximately 300 species have been tested for responses to damage-released alarm cues. Neither *Cottus gobio* (Schutz, 1956) nor *Myxocephalus scorpius* (Pfeiffer, 1960) appear to respond to conspecific alarm pheromones, but *Oligocottus maculosus* (Hugie et al., 1991; Houtman and Dill, 1994), *Cottus perplexus* (Chivers et al., 2000), and *Cottus cognatus* (this study) do show responses. Additional studies are needed to elucidate taxonomic patterns of responses in this group. Specifically, we suggest that additional species in the genus *Cottus* be tested. Moreover, we suggest that *Cottus gobio* be re-examined.

The source of the damage-released signals of many fishes is thought to be specialized cells within the epidermis (reviewed by Smith, 1992). Club cells of ostariophysan fishes contain the Schreckstoff alarm signal. During the breeding season male minnows lose their club cells and simultaneously lose their ability to elicit a fright reaction in conspecifics (Smith, 1976). In other species of fishes that have been tested for an alarm response, many have been found to have similar large elonage vacuolated cells in their epidermis. However, there has been no extensive testing of most taxonomic groups for either behavioral responses or histology. In particular, the link between specialized epidermal cells and the fright reaction for fishes other than ostariophysans needs to be examined. Certain fishes outside the Superorder Ostariophysi have sacciform cells that are thought to be analogous to ostariophysan club cells. Darters have been shown to have both a fright reaction to damage-released pheromones and possess sacciform cells (Smith, 1979, 1982). Similarly, Hugie et al. (1991) showed that tidepool sculpin possess both sacciform cells and a fright reaction to cues of injured conspecifics. Our results concur with

the generalized patterns observed in these other fishes. We observed that slimy sculpins possessed both large sacciform cells and a response to damage-released alarm signals. We speculate that damage-released alarm cues are contained in the large sacciform cells; however, additional studies are needed.

Experiments testing for the presence of disturbance signals among fishes have only recently began. Wisenden et al. (1995) showed that Iowa darters (*Etheostoma exile*) exposed to a predator model released disturbance chemicals that caused an increase in vigilance in nearby conspecifics that could not see the predator model. Jordão and Volpato (2000) described similar results for the pacus (*Piaractus mesopotamicus*). Our results demonstrate that slimy sculpins exhibit an antipredator response to chemical stimuli from disturbed conspecifics. Several authors have speculated that the source of disturbance signals may be ammonia released from urine or across the gills during periods of increased activity (Hazlett, 1989, 1990b; Kiesecker et al., 1999). Kiesecker et al. (1999) showed that red-legged frog tadpoles release ammonia upon being disturbed by a predator. Conspecific tadpoles respond with antipredator behavior upon detecting a pulse of ammonia. Similar results are known for crayfish (Hazlett, 1990b). Additional studies are needed to determine whether ammonia is a component of disturbance signals in fishes.

Behavioral responses of prey animals to chemical stimuli from predators appears widespread, but such responses have not specifically been tested in sculpins (Kats and Dill, 1998). Ours is the first study to document that sculpins respond to chemical stimuli from predators. In our experiment, we observed that sculpins responded to brook trout regardless of the diet of the trout. Moreover, the intensity of response to the trout was similar regardless of the trout's recent diet. Some studies have documented that the diet of the predator influences the antipredator responses of prey animals. For example, Wilson and Lefcort (1993) showed that red-legged frog tadpoles respond more strongly to chemical stimuli from newts (*Taricha granulosa*) that have fed on tadpoles than newts that fed on invertebrates. Mathis and Smith (1993) showed that predator-naïve fathead minnows (*Pimephales promelas*) only respond to chemical stimuli from pike (*Esox lucius*) if the pike recently consumed minnows. In contrast, experienced minnows showed an antipredator response to chemicals from pike regardless of the pike's recent diet. The sculpins used in our study were wild-captured and hence experienced with brook trout. They responded to the trout regardless of its recent diet. This response makes good sense. Trout probably forage opportunistically on sculpins and hence are always a threat to them. A knowledge of the last meal eaten by the trout may provide little valuable information to the sculpin.

We found evidence that the presence of a damage-released alarm signal enhances the intensity of response to the predator. Scrimgeour et al. (1994) provided similar evidence for larval mayflies (*Baetis tricaudatus*). These results indicate that cues of the predator and alarm signals may compliment each other

to indicate a higher degree of threat than does either signal alone. The information received by the prey upon detecting predator chemicals is that there is a predator in the vicinity. When damage-released alarm cues are detected with predator chemicals, then the prey is aware of a predator that is actively hunting. The possibility exists that sculpins were not responding more intensely to the two cues because they compliment each other. Instead, the sculpins may have been responding to a quantitative difference in the amount of predation risk cue that they received. Additional experiments are needed to differentiate these possibilities.

Acknowledgments—We thank P. Altmann, J. Kobu, P. Lamothe, K. Mayer, and M. Puttlitz for their assistance. We also thank K. Edward and the Electron Microscopy Laboratory, University of Maine, for help and resources. Funding was provided by the University of Maine, the University of Saskatchewan, and NSERC of Canada. All experiments were completed in accordance with the University of Maine Institutional Animal Care and Use Committee Protocol #98-07-04.

REFERENCES

APPLETON, R. D., and PALMER, A. R. 1988. Water-borne stimuli released by predatory crabs and damaged prey induce more predator-resistant shells in a marine gastropod. *Proc. Natl. Acad. Sci. U.S.A.* 85:4387–4391.

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signaling in aquatic predator/prey interactions: a review and prospectus. *Ecoscience* 5:338–352.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, R. J. F. 1996. Damselfly larvae learn to recognize predators from chemical cues in the predator's diet. *Anim. Behav.* 52:315–320.

CHIVERS, D. P., PUTTLITZ, M. H., and BLAUSTEIN, A. R. 2000. Chemical alarm signaling by reticulate sculpins (*Cottus perplexus*). *Environ. Biol. Fish.* 57:347–352.

GIBERSON, R. T., and DEMAREE, R. S., JR. 1995. Microwave fixation: Understanding the variables to achieve rapid reproducible results. *Micros. Res. Techn.* 32:246–254.

HAZLETT, B. A. 1985. Disturbance pheromones in the crayfish *Orconectes virilis. J. Chem. Ecol.* 11:1695–1711.

HAZLETT, B. A. 1989. Additional sources of disturbance pheromone affecting the crayfish *Orconectes virilis. J. Chem. Ecol.* 15:381–385.

HAZLETT, B. A. 1990a. Disturbance pheromone in the hermit crab *Calcinus laevimanus* (Randolf, 1840). *Crustaceana* 58:314–316.

HAZLETT, B. A. 1990b. Source and nature of disturbance chemical system in crayfish. *J. Chem. Ecol.* 20:2263–2275.

HAZLETT, B. A. 1994. Alarm responses in the crayfish, *Orconectes virilis* and *Orconectes propinquus. J. Chem. Ecol.* 20:1525–1535.

HEWS, D. K. 1988. Alarm response in larval western toads, *Bufo boreas:* Release of larval chemicals by a natural predator and its effect on predator capture efficiency. *Anim. Behav.* 36:125–133.

HEWS, D. K., and BLAUSTEIN, A. R. 1985. An investigation of the alarm response in *Bufo boreas* and *Rana cascadae* tadpoles. *Behav. Neural Biol.* 43:47–57.

HOUTMAN, R., and DILL, L. M. 1994. The influence of substrate color on the alarm response of tidepool sculpins (*Oligocottus maculosus*; Pisces, Cottidae). *Ethology* 96:147–154.

HOWE, N. R., and SHEIKH, T. M. 1975. Anthopleurine: A sea anemone alarm pheromone. *Science* 189:386–388.

HUGIE, D. M., THURINGER, P. L., and SMITH, R. J. F. 1991. The response of the tidepool sculpin,

Oligocottus maculosus, to chemical stimuli from injured conspecifics, alarm signalling in the Cottidae (Pisces). *Ethology* 89:322–334.

HURYN, A. D., and CHIVERS, D. P. 1999. Contrasting behavioral responses by detritivorous and predatory mayflies to chemicals released by injured conspecifics and their predators. *J. Chem. Ecol.* 25:2729–2740.

JORDÃO, L. C., and VOLPATO, G. L. 2000. Chemical transfer of warning information in non-injured fish. *Behaviour.* 137:681–690.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Ecoscience* 5:361–394.

KEPPEL, G. 1982. Design & Analysis: A Researcher's Handbook. Prentice-Hall, Englewood Cliffs, New Jersey.

KIESECKER, J. M., CHIVERS, D. P., MARCO, A., QUILCHANO, C., ANDERSON, M. T., and BLAUSTEIN, A. R. 1999. Identification of a disturbance signal in larval red-legged frogs, *Rana aurora. Anim. Behav.* 57:1295–1300.

LAWRENCE, J. M. 1991. A chemical alarm response in *Pycnopodia helianthoides* (Echinodermata: Asteroidea). *Mar. Behav. Physiol.* 19:39–44.

LIMA, S. L., and DILL, L. M. 1990. Behavioral decisions made under the risk of predation: A review and prospectus. *Can. J. Zool.* 68:619–640.

MATHIS, A., and SMITH, R. J. F. 1993. Fathead minnows, *Pimephales promelas*, learn to recognize northern pike, *Esox lucius*, as predators on the basis of chemical stimuli from minnows in the pike's diet. *Anim. Behav.* 46:645–656.

PETRANKA, J. W., KATS, L. B., and SIH, A. 1987. Predator–prey interactions among fish and larval amphibians: Use of chemical cues to detect predatory fish. *Anim. Behav.* 35:420–425.

PFEIFFER, W. 1960. Über die Schreckreaktion bei Fischen und die Herkunft des Schreckstoffes. *Zeit. Vergl. Physiol.* 43:578–614.

SCHUTZ, F. 1956. Vergleichende Untersuchungen über die Schreckreaktion bei Fischen und deren Verbreitung. *Zeit. Vergl. Physiol.* 38:84–135.

SCRIMGEOUR, G. J., CULP, J. M., and CASH, K. J. 1994. Anti-predator responses of mayfly larvae to conspecific and predator stimuli. *J. North Am. Benth. Soc.* 13:299–309.

SIEGEL, S., and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral Sciences, 2nd ed. McGraw-Hill, New York, 399 pp.

SIH, A. 1987. Predator and prey lifestyles: An evolutionary and ecological overview, pp. 203–224, *in* W. C. Kerfoot and A. Sih (eds.). Predation: Direct and Indirect Impacts on Aquatic Communities. University Press of New England, Hanover, New Hampshire.

SMITH, R. J. F. 1976. Seasonal loss of alarm substance cells in North American cyprinoid fishes and its relation to abrasive spawning behaviour. *Can. J. Zool.* 54:1172–1182.

SMITH, R. J. F. 1979. Alarm reaction of Iowa and johnny darters (*Etheostoma*, Percidae, Pisces) to chemicals from injured conspecifics. *Can. J. Zool.* 57:1278–1282.

SMITH, R. J. F. 1982. Reaction of *Percina nigrofasciata. Ammocrypta beani* and *Etheostoma swaini* (Percidae, Pisces) to conspecific and intergeneric skin extracts. *Can. J. Zool.* 60:1067–1072.

SMITH, R. J. F. 1992. Alarm signals in fishes. *Rev. Fish Biol. Fish.* 2:33–63.

WILSON, D. J., and LEFORT, H. 1993. The effect of predator diet on the alarm response of red-legged frog, *Rana aurora*, tadpoles. *Anim. Behav.* 46:1017–1019.

WISENDEN, B. D., CHIVERS, D. P., and SMITH, R. J. F. 1995. Early warning of risk in the predation sequence: A disturbance pheromone in Iowa darters (*Etheostoma exile*). *J. Chem. Ecol.* 21:1469–1480.

WUDKEVICH, K., WISENDEN, B. D., CHIVERS, D. P., and SMITH, R. J. F. 1997. Reactions of *Gammarus lacustris* (Amphipoda) to chemical stimuli from natural predators and injured conspecifics. *J. Chem. Ecol.* 23:1164–1173.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

ONTOGENY CONSTRAINS SYSTEMIC PROTEASE INHIBITOR RESPONSE IN *Nicotiana attenuata*

NICOLE M. VAN DAM,[1,3] MARTIN HORN,[2] MICHAEL MAREŠ,[2] and IAN T. BALDWIN[1,*]

[1] *Max-Planck-Institute for Chemical Ecology*
Carl-Zeiss-Promenade 10
D-07745 Jena, Germany

[2] *Institute of Organic Chemistry and Biochemistry*
Academy of Sciences of the Czech Republic
Flemingovo nám. 2
16610 Prague-6, Czech Republic

(Received July 7, 2000; accepted October 29, 2000)

Abstract—Protease inhibitors (PIs) are plant compounds that can inhibit proteases of mammal, insect, or pathogen origin and are frequently induced by mechanical wounding, insect feeding, or pathogen infection. *Nicotiana attenuata* is a species that induces nicotine, volatiles, and phenolics in response to damage. Here we examine the distribution of PIs in *N. attenuata* to determine if they are part of the induced response in this species and if this response is ontogenetically constrained. We found that *N. attenuata* shoot extracts inhibited trypsin (Tryp) and chymotrypsin (Chym) activities, while root extracts inhibited Tryp, Chym, and the bacterial protease subtilisin (Sub). The highest TrypPI levels were found at midday in the source-sink transition leaf, while older or younger leaves contained lower TrypPI levels and did not show significant diurnal fluctuations. Rosette plants, bolting plants, and flowering plants all contained TrypPIs in leaves, stems, and flowers, while seed capsules, seeds, and young seedlings did not contain any PIs. PIs in *N. attenuata* rosette plants were induced by *Manduca sexta* larval feeding, methyl jasmonate (MeJA) treatment, wounding, and application of *M. sexta* oral secretion and regurgitant. The response to MeJA application was stronger and longer lasting than to mechanical wounding. The direction and magnitude of the systemic response to mechanical wounding or larval damage depended on the age of the leaf that was damaged and the frequency of wounding. The systemic signal for TrypPI induction appears to follow source-sink relations in the plant and to be regulated by the octadecanoid pathway. Interestingly, by

*To whom correspondence should be addressed. e-mail: baldwin@ice.mpg.de
[3] Current address: Netherlands Institute of Ecology, Centre for Terrestrial Ecology, Multitrophic Interactions Department, P.O. Box 40, 6666 ZG Heteren, The Netherlands.

0098-0331/00/0300-0547$19.50/0

the time plants reach the flowering stage, they had lost the ability to increase PI levels after MeJA treatment. We concluded that plant ontogeny constrains both constitutive and inducible PI production in *N. attenuata.*

Key Words—Induced responses, *Manduca sexta*, mechanical damage, methyl jasmonate, *Nicotiana attenuata*, octadecanoid pathway, ontogenetic constraints, protease inhibitors, source-sink relations.

INTRODUCTION

Protease inhibitors (PIs) are a class of compounds found in a wide range of plant families and are well studied for their activity as antiherbivore compounds (Ryan, 1990; Bode and Huber, 1992; Koiwa et al., 1997). Both in vitro experiments with insect gut extracts and *in planta* experiments with genetically transformed plants have shown that PIs can inhibit digestive enzymes and reduce growth of lepidopteran and coleopteran larvae (Johnson et al., 1989; McManus et al., 1994; Broadway, 1995; Heath et al., 1997). Additionally, some plants produce PIs that specifically inhibit proteases of pathogens (Geoffroy et al., 1990; Balandin et al., 1995). Many PIs have been found to be inducible after wounding, insect feeding, pathogen infection, or application of plant hormones, such as jasmonic acid (JA), abscisic acid (ABA), or ethylene (Peña-Cortes et al., 1988; Geoffroy et al., 1990; Farmer et al., 1992; Balandin et al., 1995; Koiwa et al., 1997).

Both constitutive and inducible PI levels in plants can be extremely variable, even within related plant species. Several types of PIs that differ in structure and activity are found within the genus *Nicotiana* alone. *N. tabacum*, for example, contains a PI that inhibits bacterial and fungal proteases and is a member of the potato PI-I family (Pot I) (Bode and Huber, 1992; Koiwa et al., 1997), and two PIs belonging to the potato PI-II family (Pot II) that inhibit trypsin (Tryp) and chymotrypsin (Chym) (Pearce et al., 1993; Balandin et al., 1995). *N. alata* and *N. plumbaginifolia* both produce PIs belonging to the Pot II family, which consist of a large precursor protein that is posttranslationally processed into smaller Tryp- or Chym-inhibiting subunits (Atkinson et al., 1993; Ausloos et al., 1995). Not all PIs are present constitutively (Fujita et al., 1993; Pearce et al., 1993; Balandin et al., 1995) and of those that are, not all are inducible (Hildmann et al., 1992; Botella et al., 1996).

The magnitude of PI induction may also vary depending on the amount of damage (Pearce et al., 1993; Alarcon and Malone, 1995), the type of herbivore that is feeding (Stout et al., 1998), or on the inducing agent that is applied (Botella et al., 1996). The induced PI response may be local and restricted to the treated leaves or organ (Jongsma et al., 1994; Balandin et al., 1995) or occur systemically throughout the plant (Pearce et al., 1993; Alarcon and Malone, 1995; Orians et al., 2000). Moreover, the ability of the plant to produce or induce PIs

may be age-dependent (Alarcon and Malone, 1995; Botella et al., 1996) or organ specific (Pearce et al., 1993; Ausloos et al., 1995), which suggests that in some plant species the expression of PIs is developmentally constrained. It is generally thought that this huge variation in types, distribution, and inducibility of PIs among plants is tightly linked with the ecological role of PIs in each species (Jongsma, 1995).

Nicotiana attenuata Torr. ex Watts (Solanaceae) is a native tobacco species that displays a remarkable array of inducible defenses in response to herbivores. Leaf damage and application of methyljasmonate (MeJA) significantly induce nicotine levels (Baldwin, 1999), volatile emissions (Halitschke et al., 2000; Kahl et al., 2000), and phenolic concentrations (M. Keinänen, personal communication) in this species. In addition to these defense compounds, it is likely that *N. attenuata* is also able to produce PIs. Closely related plant species, such as *N. tabacum* (Jongsma et al., 1994), *N. alata* (Heath et al., 1997), and *N. plumbaginafolia* (Ausloos et al., 1995), are known to produce several types of inducible PIs, all of which belong to the serine PIs (Koiwa et al., 1997). Hence, it may be expected that PIs are a significant part of the chemical changes causing increased resistance against herbivores after jasmonate treatment (Baldwin, 1998; van Dam et al., 2000).

Similar to other inducible defenses in native tobacco species, variations in induced PI patterns between and within *N. attenuata* plants may be developmentally constrained. For example, the systemic induction of both nicotine and volatiles in *Nicotiana* spp. depends on the developmental stage of the plant and the leaves. In *N. sylvestris*, a systemic increase in whole-plant nicotine is only elicited when old or mature leaves are wounded. This increase is due to increases in *de novo* nicotine synthesis. Once plants reach reproductive maturity at the elongating or flowering stage, they lose the ability to induce *de novo* nicotine production after damage (Ohnmeiss and Baldwin, 2000). The elicitation of systemic volatile emission in *N. attenuata* rosette plants is maximal when the second fully expanded leaf is damaged (Halitschke et al., 2000). Similar effects have been reported for systemic PI induction in poplar (Davis et al., 1991) and tomato (Orians et al., 2000), which suggests that plant ontogeny may account for some of the observed patterns in PI induction.

Here we show that *N. attenuata* contains different PIs in shoots and roots that can inhibit at least three different serine proteases. We found diurnal fluctuations in PI levels in the leaves of rosette plants, an increase in PI levels with development, and a decrease in PI inducibility in flowering plants. PIs in *N. attenuata* rosette plants can be induced by caterpillar feeding, MeJA application, wounding, and application of larval orval secretion and regurgitant, but the direction and magnitude of the systemic response depends on the age of the leaf that is damaged. We conclude that the systemic transport of the signal triggering PI induction follows source-sink relations in the plant and is at least partly regulated by the octadecanoid pathway.

METHODS AND MATERIALS

Plant Culture

Nicotiana attenuata Torr. ex Watts. (Solanaceae) seeds, from a bulk collection from several plants from a natural population in Utah (Baldwin, 1998), germinated and grew for two weeks in peat soil soaked in 50× diluted liquid smoke (House of Herbs, Passaic, New Jersey) to synchronize germination (Baldwin et al., 1994b). For hydroponic plants, seedlings were first transferred to communal hydroponic boxes filled with 0.292 g/liter. Peters Hydro Sol (Scotts Europe, Heerlen, The Netherlands), supplemented with 0.193 g/liter $Ca(NO_3)_2$, to synchronize growth and five days later to individual 1-liter containers filled with No-N solution, supplemented with 2 mmol/liter KNO_3 (Ohnmeiss and Baldwin, 1994). Unless otherwise indicated, hydroponic plants were used for experiments five to seven days after transfer to 1-liter containers. Soil-grown plants were transferred as seedlings to either 200 ml or 1-liter pots (depending on the duration of the experiment) filled with a peat-based substrate (TerraBril, Brill Substrate Co., Georgsdorf, Germany). Soil-grown plants were watered with demineralized water every other day. All plants were placed on tables in a growth room at 32°C/16-hr light, 27°C/8-hr dark, 65% relative humidity with 1000–1500 $\mu M/m^2/sec$ PAR at plant growing level.

Protease Inhibitor Extraction and Determination

Plant samples for PI analysis were weighed in an Eppendorf tube (~300 mg fresh wt), flash frozen in liquid N_2, ground with a pestle, and thawed on ice. If individual leaves were harvested, midribs were removed before weighing. After addition of 0.3 ml protein extraction buffer (Jongsma et al., 1994), the samples were vortexed and centifuged at 4°C for 20 min at 12,000*g*. The supernatant was transferred to a fresh Eppendorf tube and kept on ice until protein and PI analysis. Protein content of the samples was determined in triplicate using the method of Bradford (1976). PI activities were analyzed with the radial diffusion assay described by Jongsma et al. (1993, 1994), using either 42 nM bovine trypsin (Tryp; type III) (Fluka, Buchs, Switzerland), 42 nM bovine chymotrypsin (Chym; type II) (Sigma, St. Louis, Missouri) or 212 nM subtilisin Carlsbergh, (Sub; protease type VIII, bacteria) (Sigma) dissolved in agar. The active sites of all proteases were titrated to obtain the molar concentration of active enzyme before they were used for PI analysis (Jongsma et al., 1994). The detection limit of this method is ~0.2 μM PI (Jongsma et al., 1993). For TrypPI activities, a series of soybean Tryp inhibitor (STI) (Boehringer Mannheim, Mannheim, Germany) solutions were used to obtain a reference curve. To determine ChymPI and SubPI activities, we made reference extracts of *N. attenuata* plant material and determined their activity by titrating Chym or Sub with these extracts, using

benzoyl-tyrosine-*p*-nitroanilide (BTNA) (Bachem, Bubendorf, Switzerland) or *N*-acetyl-DL-phenylalanine-β naphthylester (APNE, Sigma) as substrate, respectively. Dilution series of these extracts with known ChymPI or SubPI activities were used to obtain a reference curve. PI activities are expressed as nanomoles per milligram of protein (Jongsma et al., 1994).

Types of PIs

In addition to the radial diffusion assay, we used spectrophotometric assays to test for the presence of inhibitors of five model target proteases belonging to the three different classes of proteases (serine, cysteine, and aspartic proteases) that have been described in the Solanaceae (Brzin et al., 1988; Mareš et al., 1989; Ryan, 1990). The inhibitory activity against Tryp, Chym, Sub (all serine proteases), papain (cysteine protease), and cathepsin D (aspartic protease) was measured in root, shoot, and seed extracts with assays that had an approximate detection limit of 0.02 μM. The TrypPI assay contained 4.6 pmol Tryp per well in 0.1 M Tris HC buffer with 10 mM $CaCl_2$, pH 7.6. After 15 min incubation at room temperature with inhibitor extract, 10 μl of a 50 mM benzoyl-argenine-*p*-nitroanilide (BANA) (Sigma) solution in dimethylsulfoxide (DMSO) was added to a final concentration of 2 mM BANA per well. After 30 min of incubation at 37°C the absorbance was measured at 405 nm. The ChymPI assay was performed similarly, but contained 11.8 pmol of Chym per well and was incubated for 1 hr at 37°C with 0.37 mM BTNA as substrate. SubPI activities were determined with 16.1 pmol Sub per well in 0.1 M Tris HCl containing 10 mM $CaCl_2$ and 0.05% Tween, APNE (0.48 mM) as substrate, and with an incubation time of 30 min at 37°C. Absorbance was measured at 520 nm immediately after adding 30 μl of a 1.6 mg/ml solution of Fast Blue (Fluka) in 0.1 M Tris HCl buffer, pH 7.6 Papain PI activity was measured similar to TrypPI activity, but in 0.1 M Na phosphate buffer, pH 6.0, containing 25 mM dithiothreitol, with papain (twice crystallized, Sigma) instead of trypsin and an incubation time of 1 hr at 37°C. PI assay with cathepsin D, isolated from bovine spleen (Fusek et al., 1992) was performed in Eppendorf tubes with 200 μl of preincubation mixture containing 25 pmol of cathepsin D and inhibitor extract. The pH of extract solutions was adjusted to 3.6 by adding 10 μl of 10% acetic acid per 100 μl extract solution. After 15 min of preincubation at room temperature, 1 ml of 2% hemoglobin in 0.1 M Na acetate buffer, pH 3.6, was added and incubation containued for 60 min at 37°C. The reaction was stopped by adding of 250 μl of 25% trichloracetic acid. The supernatant was collected by centrifugation (10 min at 10,000*g*) of the samples and its absorbance was measured at 280 nm.

Diurnal Fluctuations of PI Levels

Eighteen soil-grown rosette plants were selected two weeks after their transfer to 1-liter pots. On each plant, the leaf at node 0, defined as the leaf that is

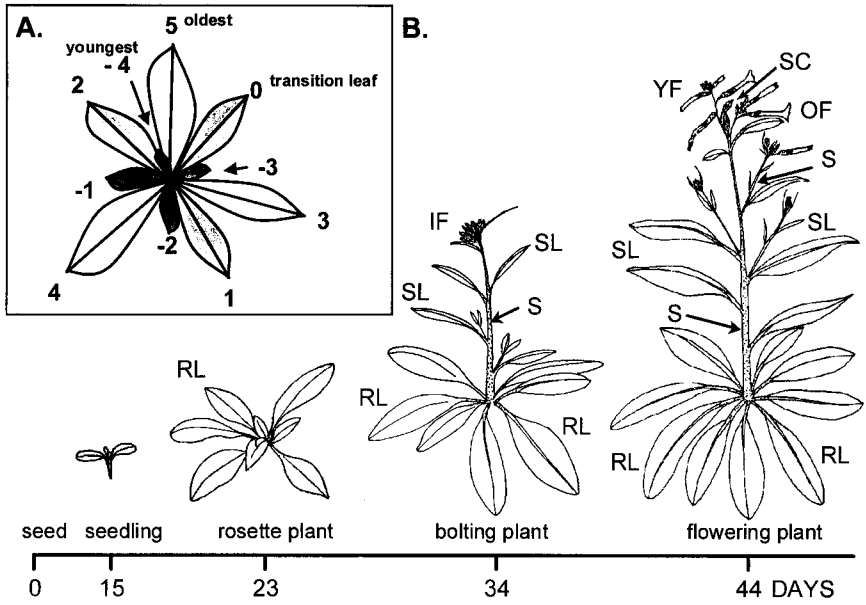


FIG. 1. (A) Numbering of the leaves at different phyllotaxic positions (nodes) on rosette plants of *N. attenuata* following Schittko et al. (2000). The leaf at node 0 has just completed the source-sink transition. (B) Schematic of the organs at different ontogenetic stages of *N. attenuata* plants. Time (in days) corresponds to the time line for the experiment measuring constitutive and induced PI activities in different developmental stages. RL = rosette leaves, SL = stem leaves, S = stems, IF = inflorescence, YF = young flower (unopened), OF = open flower, SC = seed capsules.

one phyllotaxic position younger than the first fully expanded leaf (Schittko et al., 2000) (Figure 1A) and the leaves at node 2 (older, fully expanded leaf) and node −2 (younger leaf) were labeled. The labeled leaves were harvested at 4-hr intervals (three plants per interval) during a 24-hr period to determine TrypPI activities.

Time Course of Induction

Plants were soil-grown as in the diurnal fluctuation experiment. On each plant, the leaf at node 0 was labeled and either damaged by removing a 2-mm strip perpendicular to the midrib with a scalpel each day (Jongsma et al., 1994), treated with 250 μg MeJA in lanolin (Baldwin, 1996), or left untreated. MeJA is deesterified to JA by plant esterases upon treatment and is a convenient way to introduce JA into plants. MeJA (Sigma) was close to its thermodynamic equilibrium (90.1% (1*R*,2*R*)-MeJA and 8.3% (1*R*,2*S*)-MeJA for the two naturally

occurring epimers. The labeled leaves were harvested at one, two, four, and eight days after the start of the experiment and extracted for TrypPI activities. Sampling took place at approx. 13:00 hr each harvest day (three plants per treatment per harvest). The plants whose labeled leaves were harvested were discarded. The TrypPI activities in the treated leaves were expressed relative to average activity of untreated leaves harvested at the same time.

Constitutive and Induced PI Activities in Different Developmental Stages

Minimal Seedling Size for PI Detection. Seeds were sown on day 0 and 14 days later, seedlings were transferred to 200-ml pots with soil. On days 18, 19, 20, and 21, a number of seedlings were excised with a scalpel at soil level to obtain 1–1.5 g fresh weight (FW) per sample (10, 6, 5, and 2 seedlings, respectively). The seedlings were extracted for TrypPI as above, with the addition of an extra concentration step in which proteins and PIs were precipitated with ice-cold, saturated $(NH_4)_2SO_4$ (Jongsma et al., 1994).

Developmental Changes. Seeds were sown as above (day 0). On day 15, seedlings were first transferred to communal hydroponic boxes to synchronize growth and five days later, to 2-liter pots filled with a high-N soil–Perlite mixture (van Dam and Baldwin, 1998). On day 23, three rosette plants (Figure 1B) were randomly selected to measure PI activities at the start of the experiment. Simultaneously, six plants were treated with 250 μg MeJA in 0.22 ml lanolin applied to two fully expanded leaves on each plant and three other plants were treated with 0.22 ml pure lanolin. On day 28, three MeJA-treated and three lanolin-treated rosettes were harvested by cutting of the shoot at the root–shoot interface. On day 35, three bolting plants (Figure 1B) were treated with MeJA, as above, and three other bolting plants were treated with pure lanolin. On day 39, we harvested the remaining three plants induced at the rosette stage on day 23, the three bolting plants induced on day 34, and the three bolting plants treated with lanolin on day 34. The entire shoot was cut off at the root–shoot interface and rosette leaves, stems, stem leaves, and inflorescences were separated (Figure 1B), weighed, and extracted for determination of TrypPI activity. On day 44, three flowering plants were treated with 250 μg MeJA on two rosette leaves and three other flowering plants were treated with pure lanolin. These plants were harvested on day 49 and separated into rosette leaves, stems, stem leaves, young unopened flowers, open flowers, and green seed capsules (Figure 1B). Additionally, we extracted three samples of seeds from the seed stock we used to grow the plants.

Local and Systemic Induction by MeJA Addition to Roots and Shoots

Nine hydroponically grown rosette plants were divided in groups of three and treated with either 250 μg MeJA in 0.02 ml lanolin paste on two fully expanded leaves (Baldwin, 1996), 250 μg MeJA as an aqueous solution to the

roots in the hydroponic solution (Lynds and Baldwin, 1998), or 0.02 ml pure lanolin paste to the shoot (control). Four days later, shoots and roots of the plants were harvested separately, flash frozen with liquid N_2, and ground with mortar and pestle. A 300-mg aliquot from each plant was extracted to determine TrypPI and ChymPI activities.

Local and Systemic Induction by Caterpillars

Six 2-day-old first instar *Manduca sexta* larvae (Carolina Scientific Supply Co, Burlington, North Carolina) were placed individually on the leaf at node 1 of six 2-week-old *N. attenuata* rosette plants. To prevent the larvae from moving to other leaves, leaves and larvae were enclosed in a 50-ml polystyrene container with a clear lid (Neupack Verpackungen, Hamburg, Germany). The petiole of the leaf was carefully slid through a slit in the side of the cup and the lids were perforated to allow for gas exchange and prevent condensation. Another six plants were fitted with similar cup over their node 1 leaf, but without a larva, to control for cup effects. Two days after the larvae had been placed on the plants, larvae and cups were removed from all plants and the damaged leaf at node 1, the leaf at node 3 (older than damaged leaf) and the leaf at node −1 (younger) of three control and three damaged plants were harvested. Before extraction, the leaves were photocopied on paper. The leaf area before feeding was estimated by cutting out the contour of the leaf as it was before damage and weighing the paper to the nearest 0.1 mg. Then the parts consumed by the larvae were cut out and the leaf copy was weighed again. The percentage leaf area consumed was calculated as [(mg before − mg after)/mg before] × 100%. The second group of three control and three damaged plants were harvested for TrypPI analysis four days after start of the experiment.

Local and Systemic Induction by Wounding at Different Leaf Positions

Twelve hydroponically grown plants in 1-liter pots were assigned randomly to one of four treatments (three plants each): (1) leaf at node 4 (older leaf) damaged, (2) leaf at node 0 (source-sink transition leaf) damaged, (3) leaf at node −4 damaged (younger leaf) damaged, or (4) no damage (control). The leaves were damaged daily by cutting off a 2-mm strip perpendicular to the midrib with a scalpel. Simultaneously with the first damage event, the plants were supplied with 1 mM KNO_3. Two days after the first damage event, the roots and the leaves at node 4, 0, and −4 were harvested on all plants for TrypPI and SubPI (roots only) analysis.

Local and Systemic Induction by Wounding or OS

Twenty hydroponically grown plants on 1-liter pots were assigned to the following treatments (four per treatment): (1) a single damage event and application of 15 μl water to the node 1 leaf; (2) as 1, but application of 15 μl undi-

luted, boiled, and filtered (0.45 μm filter pore size) *M. sexta* oral secretion and regurgitant (OS) (Schittko et al., 2000); (3) daily damage and daily application of 15 μl water; (4) as 3, but daily application of 15 μl *M. sexta* OS; and (5) control, leaf node 1 labeled at the start of the experiment but no damage treatment. Leaves were damaged by rolling a pattern wheel parallel to the midrib once on each side of the leaf, which created a standardized number of puncture wounds (Ohnmeiss and Baldwin, 1994). Water or OS was applied and rubbed in the leaf puncture wounds with a latex-gloved finger immediately after damage. Three days later, the damaged and treated leaves plus the leaves at node −1 were harvested to assess TrypPI activity.

Statistical Analysis

The data were analyzed using Statview (SAS Institute Inc., Cary, North Carolina) statistical software. Test values were calculated using type III SS and PI activity values were arcsine square root transformed before analysis. The PI levels of the time-course experiment and of the rosette plants in the different developmental stages experiment were analyzed by single ANOVA. Data sets with multiple dependent variables, such as PI levels in leaves and/or organs on the same plant, were analyzed by MANOVA. If the MANOVA produced a P value <0.05, it was followed by univariate ANOVAs for the individual effects and Fisher LSD post-hoc tests to detect significant differences between groups. Differences between treatment and organs in bolting and flowering plants in the developmental changes experiment were analyzed by separate ANOVAs with treatment and organ as main effects, because the data did not allow for MANOVA analysis due to lack of degrees of freedom. P values were corrected with the Bonferroni correction for multiple comparisons (Holm, 1979).

RESULTS

Types of PIs. All *N. attenuata* extracts exclusively contained serine PIs: we found TrypPI and ChymPI activity in the shoots and TrypPI, ChymPI, and SubPIs in the roots (Table 1). We detected neither PI activity directed against serine proteases in the seeds nor cysteine protease (papain) or aspartic protease (cathepsin D) inhibiting activity in any of the organs tested (Table 1). TrypPI activities in shoot extracts were consistently about four to five times higher than ChymPI activity in the same extract. Because TrypPI constituted the major part of the activity and was easier to detect, we used TrypPI as a proxy for total TrypPI and ChymPI activity in most of the following experiments.

Diurnal Fluctuations of PI Levels. The diurnal fluctuations in PI activity differed between leaves on different positions (MANOVA, $F_{15,28} = 2.05$, $P = 0.049$). TrypPI levels in the source-sink transition leaf (node 0) fluctuated sig-

TABLE 1. PRESENCE OF DIFFERENT TYPES OF CONSTITUTIVE PI ACTIVITIES IN ORGANS OF UNTREATED *N. attenuata* PLANTS MEASURED BY SPECTROPHOTOMETRIC METHODS[a]

Organ	TrypPI	ChymPI	SubPI	Papain PI	Cathepsin D PI
Roots	+	+	+	–	–
Shoots	+	+	–	–	–
Seeds	–	–	–	–	n.a.

[a] + = present, – = not detectable, n.a. = not analyzed.

nificantly during the day (ANOVA, $F_{5,12} = 3.767$, $P = 0.028$), being the highest in the middle of the light period and lowest at the end of the night (Figure 2A). Younger or older leaves had much lower PI levels during the daytime and showed no significant fluctuations (ANOVA, both $P > 0.1$). These data underscore the importance of controlling for diurnal and ontogenetic effects when quantifying PI dynamics.

Time Course of Induction. Both mechanical damage and application of 250 μg MeJA induced TrypPI levels locally in node 0 leaves above those measured in untreated controls (Figure 2B; ANOVA, treatment effect, $F_{1,15} = 57.113$, $P < 0.001$). TrypPI induction for both treatments was maximal after four days, but the effect of MeJA-application was signficantly stronger than that of damage (Figure 2B; treatment × harvest day effect, $F_{3,18} = 4.548$, $P = 0.0173$).

Constitutive and Induced PI Activities in Different Developmental Stages. Similar to seeds, very small seedlings do not contain detectable levels of TrypPI. We did not detect TrypPI activity until day 19 (six pooled seedlings, 1.0803 g FW). Seedlings harvested later, i.e., at day 20 and 21 also contained detectable TrypPI activities, and in subsequent experiments we only used plants that were 21 days old or older.

Rosette plants treated with MeJA had significantly higher TrypPI levels in their shoots than did untreated controls harvested at the same time or rosette plants harvested at the start of the experiment (Figure 3A; ANOVA, $F_{2,6} = 48.686$, $P < 0.001$). These MeJA-induced PI levls were lost in plants that were harvested 16 days later: bolting plants treated with MeJA as a rosette plant did not contain significantly higher PI levels than untreated bolting plants harvested at the same time (Figure 3B; Fisher LSD, $P = 0.41$). Bolting plants treated with MeJA five-days before harvest, however, contained significantly higher PI levels than did the other two groups (Figure 3B; ANOVA, treatment effect $F_{2,24} = 5.752$, $P = 0.009$, Fisher LSD comparisons with both other groups $P < 0.024$). Additionally, a significant difference in PI activity levels was found between organs on bolting plants (ANOVA, $F_{3,24} = 27.071$, $P < 0.001$), but the induced increase was similar in all organs (Figure 3B). Contrary to the patterns observed in younger plants, flowering plants that were treated with MeJA did not signifi-

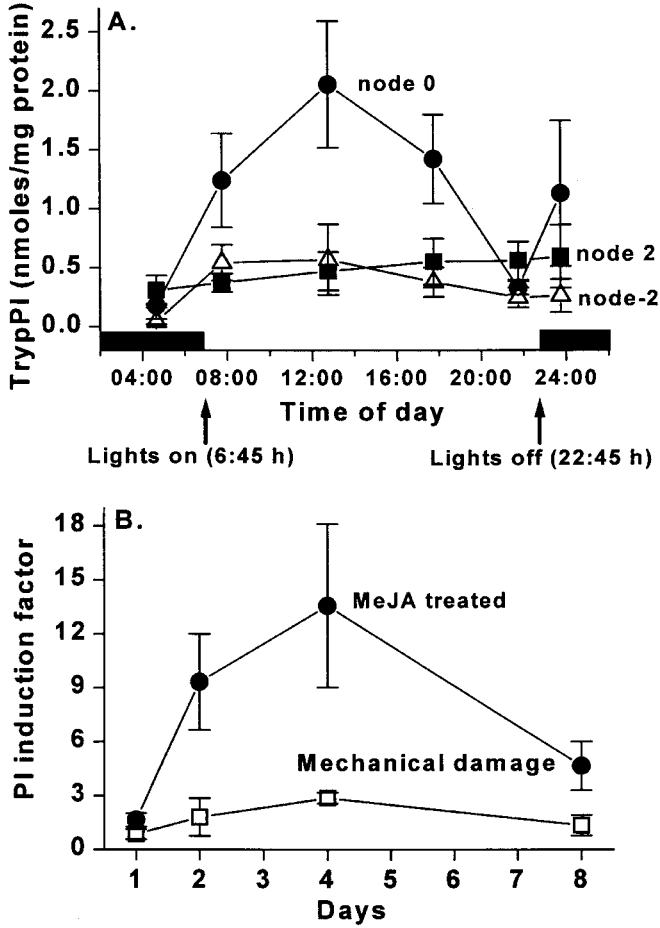


FIG. 2. (A) Diurnal fluctuations in TrypPI levels (mean ± SE, $N = 3$) in *N. attenuata* rosette plants in leaves at different phyllotaxic positions, harvested at 4-hr intervals. Circles: leaf at node 0 (source-sink transition leaf), triangles: leaves at node −2 (younger leaf), squares: leaf at node 2 (older leaf). (B) Relative increase of local TrypPI levels (means ± SE, $N = 3$) in time of the leaf at node 0 of *N. attenuata* rosette plants, after this leaf had been treated on day 0 with 250 μg MeJA or damaged daily by cutting with a scalpel. PI induction is expressed relative to the average PI level of node 0 leaves of untreated plants that were harvested at the same time intervals.

cantly increase TrypPI levels in any of their organs (Figure 3C; treatment effect $F_{1,24} = 0.741$, $P = 0.40$). Again, TrypPI levels differed significantly between organs ($F_{5,24} = 13.038$, $P < 0.001$). We did not detect any TrypPI activity in

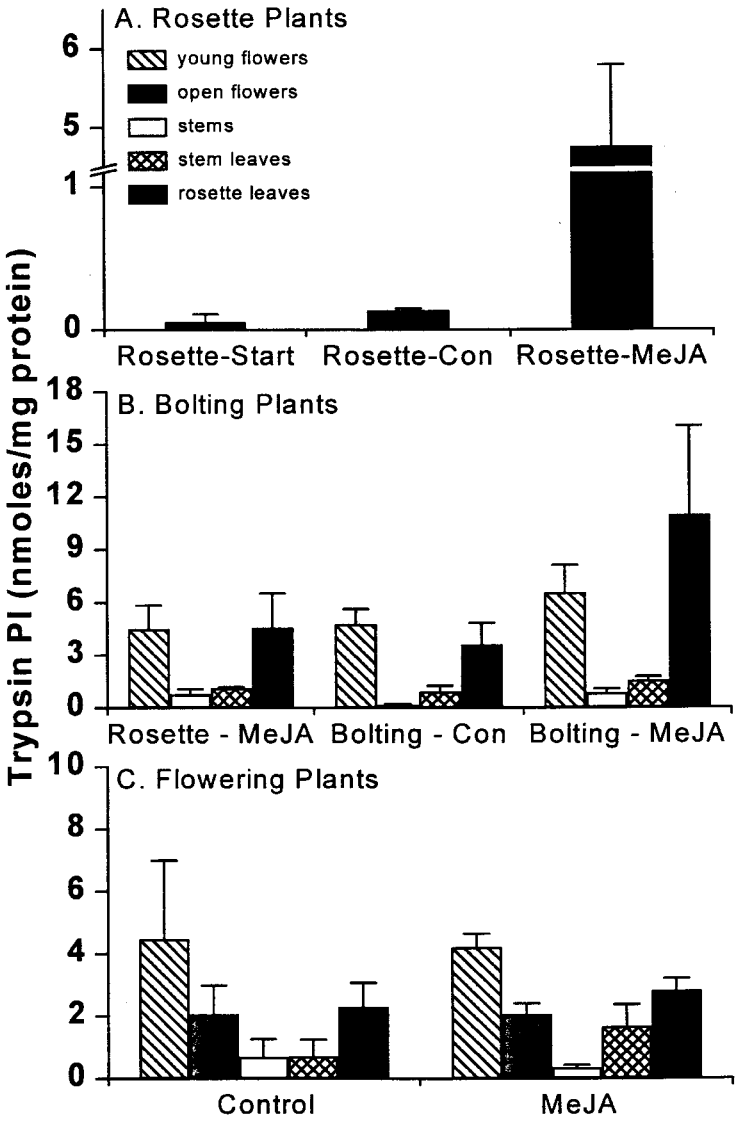


FIG. 3. TrypPI levels (mean ± SE, $N = 3$) in the different organs of control and MeJA-treated (250 μg/plant) *N. attenuata* plants in different developmental stages. (A) Rosette plants, harvested at day 23 (Start) or at day 28 (Con, MeJA). (B) Bolting plants, harvested on day 39, either treated with MeJA as rosette plants on day 23 (Rosette-MeJA), on day 34 when bolting (Bolting-MeJA) or treated with pure lanolin (Bolting-Con). (C) Flowering plants, treated with MeJA or pure lanolin (control) on day 44 and harvested on day 49.

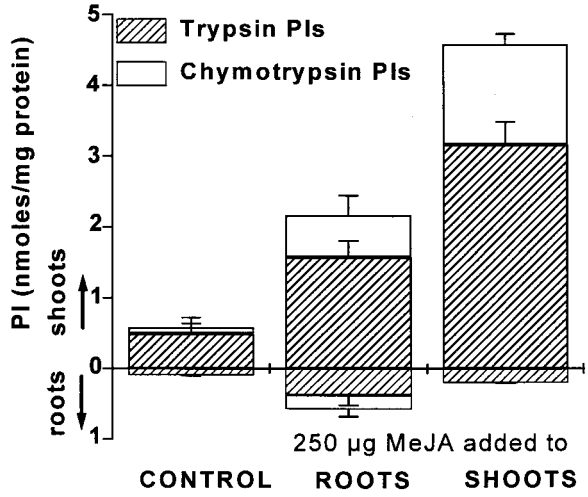


FIG. 4. Total PI activities, separated into TrypPI (hatched) and ChymPI (open) levels (mean ± SE, $N = 3$) in roots and shoots of hydroponically grown *N. attenuata* rosette plants two days after addition of MeJA to the roots or the shoots.

the seed capsules, which were still green at the time of harvest, nor in the seed samples (data not shown).

Local and Systemic Induction by MeJA Addition to Roots and Shoots. MeJA treatment significantly increased PI activities compared to those in control plants (Figure 4; MANOVA, treatment effect $F_{8,6} = 9.161$, $P = 0.0073$). Overall, leaf PI levels were significantly higher in the shoots than in the roots (Paired t test with Bonferroni correction for multiple comparisons, Tryp $t_8 = 5.121$, $P = 0.0018$, Chym, $t_8 = 3.482$, $P = 0.0083$). Although MeJA application also had a systemic effect, the strongest PI induction was in the treated organ (Figure 4). MeJA application to the roots induced total root PI activity levels to 6.0 times that of untreated roots, and leaf PI levels to 3.7 times that of control shoots (Figure 4, ROOT treatment). Leaf MeJA application, however, increased total leaf PI levels by a factor 8.2 and total root PI to only 2.1 times that of control plants (Figure 4, SHOOT treatment). Root ChymPI activity was only detectable in roots treated with MeJA and not in the roots of shoot-treated plants or control plants (Figure 4). SubPI levels in the roots did not significantly change in any of the MeJA treatments (data not shown).

Local and Systemic Induction by Caterpillars. On average, the *M. sexta* larvae consumed 13.2% (±3.7 SE) of the leaf they were confined to. This relatively small amount of damage significantly induced overall TrypPI activities in the plants they fed on (Figure 5, MANOVA treatment effect $F_{3,8} = 9.19$, P

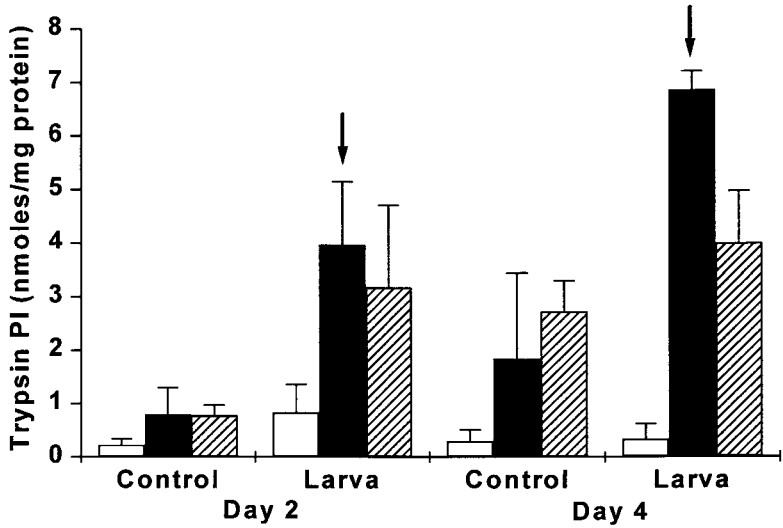


FIG. 5. TrypPI levels (mean ± SE, $N = 3$) after 2 and 4 days in leaves of *N. attenuata* rosette plants after 2 days of *M. sexta* larvae feeding on the leaf growing at node 1. Open bars: leaves at node 3 (older than damaged leaf), solid bars: leaves at node 1, hatched bars: leaves at node −1 (younger). Arrows identify the leaves that were damaged by the larvae.

= 0.012). The elevated PI levels were maintained for two days after the larvae had been removed (Figure 5, no significant treatment × day effect). Although the response was clearly systemic, the increase in PI activity was higher in damaged leaves and younger leaves than in older leaves (Figure 5). This indicates that the signal inducing PI activity levels is mainly transported acropetally within the shoot.

Local and Systemic Induction by Wounding at Different Leaf Positions. Mechanical damage significantly increased TrypPI levels in damaged shoots (Figure 6, MANOVA treatment effect $F_{9,15} = 2.845$, $P = 0.036$). There was both a local and a systemic effect, but within the shoot, systemic increased were detected only in leaves that were younger than the damaged leaf (Figure 6). For example, when node 0 leaves were damaged, TrypPI levels increased only in the damaged and younger leaves. When young leaves were damaged, none of the older leaves were significantly induced. This confirmed our previous observation that, within the shoots, the PI inducing signal is transported acropetally. Overall, the shoot response to damage on old leaves was the weakest (Figure 6). In the roots, however, both TrypPI and SubPI levels changed, although inversely, in response to damage (Figure 6, insert), but only TryPI levels did so significantly

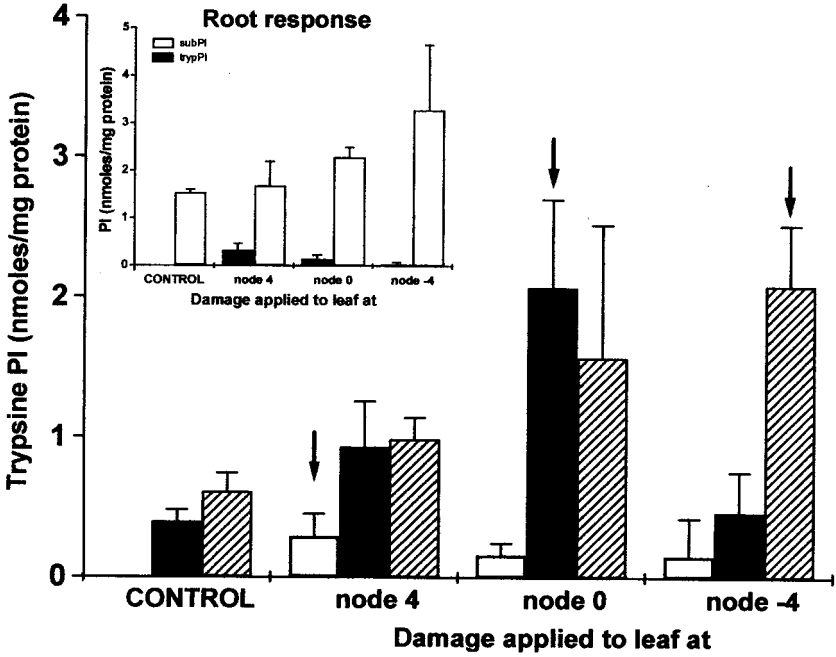


FIG. 6. TrypPI levels in shoots and TrypPI and SubPI levels (mean ± SE, $N = 3$) in roots (insert) of hydroponically grown *N. attenuata* rosette plants 2 days after the first damage. Different leaf positions were damaged daily with a scalpel. Open bars: leaf at node 4, solid bars: leaf at node 0, hatched bars: leaf at node −4. Arrows identify the leaf positions that were damaged in each group.

(ANOVA for TrypPI, treatment effect $P_{3,7} = 6.454$, $P = 0.02$). TrypPI levels were highest in roots of plants whose oldest leaves were damaged (Figure 6, insert). This indicates that in these plants some of the inducing signal was transported to the roots as well.

Local and Systemic Induction by Wounding and Regurgitant. Three days after the first treatment, TrypPI levels were significantly different between treatments (Figure 7, MANOVA $F_{8,28} = 3.111$, $P = 0.012$). The TrypPI levels in plants damaged once with or without application of OS were not significantly different from the control plants (Figure 7, Fisher LSD post-hoc test for both damaged and younger leaves, $P > 0.1$). Plants that were damaged repeatedly had significantly higher PI levels in the damaged leaves (Fisher LSD test on PI levels in damaged leaves, $P < 0.01$). Only when plants were repeatedly treated with OS were the PI levels in younger leaves higher than in those of the controls (Fisher LSD, $P = 0.0067$).

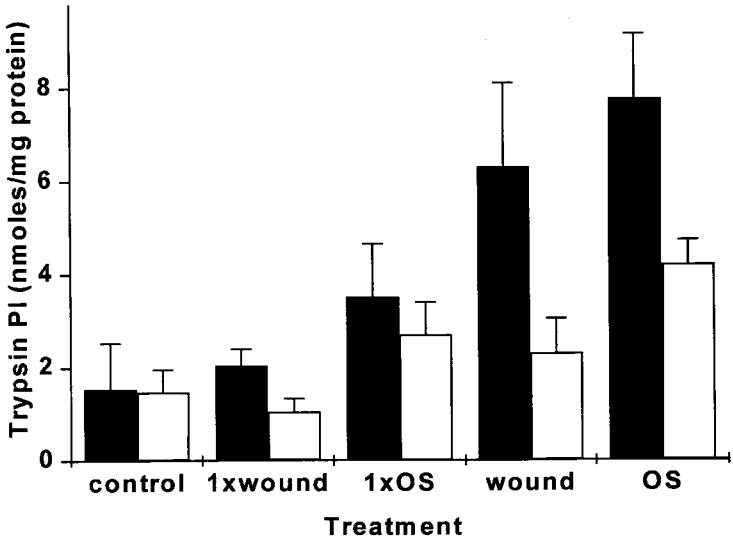


FIG. 7. TrypPI levels (mean ± SE, $N = 4$) of leaves on *N. attenuata* rosette plants 3 days after the first damage. 1 × wound = leaf at node 1 was damaged with a pattern wheel once on day 0 and 15 μl water was applied to the wound site, 1 × OS = same as 1 × wound plus 15 μl boiled and filtered *M. sexta* oral secretion and regurgitant (OS) was applied to the wound site, wound = leaf at node 1 was damaged and treated with 15 μl water daily for 3 days, OS = same as wound, but treated with 15 μl OS on the damaged site daily for 3 days. Solid bars: treated leaf (leaf at node 1), open bars: leaf at node −1 (younger).

DISCUSSION

Similar to what has been reported for other *Nicotiana* species, we found only inhibitors of serine proteases in *N. attenuata.* Preliminary results from affinity chromatography purification, followed by gel filtration and MALDITOF analysis indicate that the TrypPI/ChymPI activity from shoot extracts resides in a mixture of high and low MW proteins, the former weighing ~5.7 kDa (M. Horn and M. Mareš, unpublished results). This is similar to the structure of the Pot-II PI found in *N. alata.* This PI is synthesized as a 42-kDa precursor, which is proteolytically processed into five 6-kDa subunits, four of which inhibit Tryp and one Chym, and one, smaller, 5-kDa Chym-inhibiting unit (Atkinson et al., 1993; Heath et al., 1995; Lee et al., 1999). Our observation that the TrypPI activities were consistently 4–5 times higher than ChymPI activity in shoot extracts supports the proposition that the PIs in *N. attenuata* and *N. alata*, which belong to closely related sections within the genus *Nicotiana* (Goodspeed, 1954), share a

similar structure. The occurrence of similar types of PIs with different activities may provide broad-range plant resistance and possibly prevent herbivores from easily adapting their digestive enzymes and becoming resistant to plant defense (Broadway, 1995; Jongsma and Bolter, 1997).

Contrary to what has been found in *N. tabacum* (Geoffroy et al., 1990; Heitz et al., 1993), we did not find any Sub-inhibiting activity in shoots of *N. attenuata.* SubPI activity was found exclusively in the roots and was not significantly induced by either wounding or MeJA application. It is possible that SubPI activities increase specifically after pathogen infection (Geoffroy et al., 1990; Balandin et al., 1995) or elicitation by salicylic acid (SA) (Heitz et al., 1993). Affinity chromatography followed by gel filtration and SDS-PAGE revealed that the constitutive root SubPI consists of a 29-kDa protein that also inhibits Tryp (M. Horn and M. Mareš, unpublished results). It is generally thought that TrypPI/ChymPIs are targeted against insect or mammalian herbivores (Jongsma and Bolter, 1997), while SubPIs are considered defenses against pathogenic microorganisms (Geoffroy et al., 1990; Heitz et al., 1993). Given the abundance of microorganisms in the soil, the potential danger of attack by pathogens is much larger for roots than for shoots. This may explain our observation that *N. attenuata* contained relatively high constitutive SubPI in their roots.

We also found considerable variation in PI levels in time: within one photoperiod the PI level in source-sink transition leaves, growing at node 0, fluctuated dramatically. This variation suggests that PIs, in addition to being antiherbivore compounds, may be directly involved in the regulation of plant metabolism (Bode and Huber, 1992; Koiwa et al., 1997; Solomon et al., 1999).

On a larger time scale, PI levels in the plant changed during ontogeny. Seeds and very small seedlings did not contain any TrypPIs, while in rosette plants, PI levels increased with plant size. Within bolting and flowering plants, young, unopened flowers and rosette leaves had the highest PI levels. Apparently, these high PI levels in the flowers decrease after pollination, because open flowers contained lower PIs levels while seed capsules did not contain PIs at all. This is consistent with data on PI levels in reproductive organs of *N. plumbaginifolia* and *N. tabacum* (Pearce et al., 1993; Ausloos et al., 1995). In *N. plumbaginifolia*, PI levels were high in all floral parts, but dropped dramatically after pollination (Ausloos et al., 1995). No PI activity was found in the mature fruit or seeds of *N. tabacum* (Pearce et al., 1993; Ausloos et al., 1995). The high PI levels in the young flowers and flower parts may protect these valuable organs against herbivores and pathogens (Atkinson et al., 1993), while it was postulated that low PI level in mature fruits and seeds could facilitate seed dispersal by frugivores (Ausloos et al., 1995). In any case, the lack of PIs in the unripe fruits, seeds, and young seedlings of *N. attenuata* rules out a direct involvement in seed storage processes or germination (Koiwa et al., 1997).

TrypPI levels were locally and systemically induced by wounding, herbi-

vore feeding, and application of herbivore OS and MeJA, all of which are known to trigger the octadecanoid signaling pathway in plants (Koiwa et al., 1997). Alternatively, PI induction can also be enhanced (Fritig et al., 1998) or reduced (O'Donnell et al., 1996) by SA, which is involved in the systemic induction of pathogenesis-related proteins (Reymond and Farmer, 1998). Spray application of a 50 mM SA solution to shoots or of 1 mM SA to the roots, however, did not significantly change TrypPI levels in hydroponically grown *N. attenuata* plants (N. M. van Dam, unpublished results). These data indicate that for TrypPIs induction in *N. attenuata* JA may be the signal responsible for the systemic response: [^{14}C]JA tracer studies showed that JA applied to one fully expanded leaf is transported systemically throughout rosette plants (Zhang and Baldwin, 1997). Endogenous JA production increases with the number of wounds a leaf receives and results in proportional increases in whole-plant nicotine production (Baldwin et al., 1997). Similarly, we found that plants damaged daily with a pattern wheel had higher induced PI levels than plants subjected to a single damage event. The JA signaling pathway is also involved in systemic plant signaling after herbivore feeding. Application of *M. sexta* OS to *N. attenuata* leaves elicits both a localized JA burst (McCloud and Baldwin, 1997; Kahl et al., 2000) and a systemic signal that rapidly elicits JA amplification in leaf parts remote from the wound site (Schittko et al., 2000). In tomato plants JA is a specific systemic elicitor of both PI-I and PI-II mRNA transcription (Farmer et al., 1992). However, these results do not rule out that other signals, e.g., systemin-like molecules (Constabel et al., 1998), electrical signals (Wildon et al., 1992), ethylene (O'Donnell et al., 1996), or ABA (Peña-Cortes et al., 1988) may also be involved in systemic PI induction in *N. attenuata.*

Both local and systemic responses were influenced by induction treatment: TrypPI induction was stronger after MeJA treatment than after mechanical wounding. Replicated wounding, however, resulted in significantly higher local PI levels than a single wounding event, while additional application of *M. sexta* OS enhanced the systemic response. This indicates that the plant can discriminate between wounding and larval attack and possibly uses different signal pathways for local and systemic induction of PIs (Lightner et al., 1993; Koiwa et al., 1997; Reymond and Farmer, 1998). Application of *M. sexta* regurgitant also induces ethylene production in *N. attenuata* (Kahl et al., 2000), a plant hormone known to enhance the PI response to wounding (Heitz et al., 1993; O'Donnell et al., 1996) but to reduce the nicotine response (McCloud and Baldwin, 1997; Kahl et al., 2000). Such interactions between different signaling hormones may fine-tune the plant response towards specific attackers (Reymond and Farmer, 1998).

In contrast to induced increases in nicotine levels (Baldwin et al., 1994a), induction of TrypPI activity in *N. attenuata* is transient: 16 days after application of 250 μg MeJA, TrypPI levels were not different from those in untreated plants.

Possibly, PIs are metabolized to recycle valuable resources, e.g., N, that were used to construct them. This may be correlated with the resource costs of *de novo* production of PIs at induction, which may be even larger than the 6% of current N uptake invested in nicotine production (Baldwin et al., 1998). Similar to nicotine induction (Ohnmeiss and Baldwin, 2000), *de novo* PI induction is limited to the early stages of plant development: flowering plants treated with MeJA did not significantly increase local or systemic PI levels.

The within-plant pattern of systemic PI induction in *N. attenuata* rosette plants strongly suggests that the signal triggering remote PI induction follows source-sink relationships within the plant. When recently fully expanded, source leaves were damaged by herbivores or cutting, PI levels increased in all organs that receive photosynthetic products from these leaves, i.e., the younger leaves and, to a lesser extent, roots. If young sink leaves were damaged, PI levels increased only locally in this leaf and not in any other plant parts. Source-sink related induction patterns have been described in detail for PIs in poplar (Davis et al., 1991) and tomato (Orians et al., 2000). These results suggest that the systemic signal is transported in the phloem together with sucrose. Interestingly, damage to older leaves did not trigger such a strong systemic response in younger leaves as damage on younger source leaves, a pattern also reported from *N. tabacum* (Pearce et al., 1993). Possibly, these leaves export the majority of their photosynthetic products and systemic induction signals to the roots, which is consistent with the significantly larger TrypPI response in the roots when old leaves were damaged. Alternatively, older leaves may be less sensitive to leaf damage and produce less wound signal (Ohnmeiss and Baldwin, 2000).

PI induction is constrained by plant ontogeny, as has been described for the induction of nicotine (Ohnmeiss and Baldwin, 2000) and volatile emissions (Halitschke et al., 2000). *N. attenuata* is thus spatially and temporally limited in its ability to deploy certain defenses against herbivores. This ontogenetic constraint may be due to metabolic limitations: flowering plants preferentially allocate their resources to seed production and therefore may be physiologically prevented from allocating resources to functions that are not directly involved in fitness output, e.g., induced defense production. This may be an optimal strategy if leaf loss, e.g., to herbivores, in the flowering stage does not reduce lifetime seed output (Ohnmeiss and Baldwin, 2000). It remains a challenge to ecophysiologists to resolve the physiological and ecological significance of such ontogenetic constraints.

Acknowledgments—The authors thank M. Lim, G. Glawe, M. Ebbinghaus, and C. Müller for excellent technical assistance. N.M.D. thanks Dr. M. A. Jongsma and coworkers at CPRO-DLO in Wageningen, The Netherlands, for their hospitality and the private course in PI analysis. M.H. and M.M. were partially supported by grants GACR No. 522/00/1553 and GAAVCR No. IAA4055006. This research was supported by the Max Planck Society.

REFERENCES

ALARCON, J. J., and MALONE, M. 1995. The influence of plant age on wound induction of proteinase inhibitors in tomato. *Physiol. Plant.* 95:423–427.

ATKINSON, A. H., HEATH, R. L., SIMPSON, R. J., CLARKE, A. E., and ANDERSON, M. A. 1993. Proteinase inhibitors in *Nicotiana alata* stigmas are derived from a precursor protein which is processed into five homologous inhibitors. *Plant Cell* 5:203–213.

AUSLOOS, G. R. J., PROOST, P., VAN DAMME, J., VENDRIG, J. C. 1995. Proteinase inhibitor II is developmentally regulated in *Nicotiana* flowers. *Physiol. Plant.* 94:701–707.

BALANDIN, T., VANDERDOES, C., ALBERT, J. M. B., BOL, J. F., and LINTHORST, H. J. M. 1995. Structure and induction pattern of a novel proteinase inhibitor class II gene of toabacco. *Plant Mol. Biol.* 27:1197–1204.

BALDWIN, I. T. 1996. Methyl jasmonate-induced nicotine production in *Nicotiana attenuata:* Inducing defenses in the field without wounding. *Entomol. Exp. Appl.* 80:213–220.

BALDWIN, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U.S.A.* 95:8113–8118.

BALDWIN, I. T. 1999. Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *J. Chem. Ecol.* 25:3–30.

BALDWIN, I. T., KARB, M. J., and OHNMEISS, T. E. 1994a. Allocation of ^{15}N from nitrate to nicotine: Production and turnover of a damage-induced mobile defense. *Ecology* 75:1703–1713.

BALDWIN, I. T., STASZAK-KOZINSKI, L., and DAVIDSON, R. 1994b. Up in smoke: I. Smoke-derived germination cues for positive annual, *Nicotiana attenuata* Torr. Ex. Watson. *J. Chem. Ecol.* 20:2345–2371.

BALDWIN, I. T., ZHANG, Z. P., DIAB, N., OHNMEISS, T. E., MCCLOUD, E. S., LYNDS, G. Y., and SCHMELZ, E. A. 1997. Quantification, correlations and manipulation of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris. Planta* 201:397–404.

BALDWIN, I. T., GORHAM, D., SCHMELZ, E. A., LEWANDOWSKI, C. A., and LYNDS, G. Y. 1998. Allocation of nitrogen to an inducible defense and seed production in *Nicotiana attenuata. Oecologia* 115:541–552.

BODE, W., and HUBER, R. 1992. Natural protein proteinase inhibitors and their interaction with proteinase. *Eur. J. Biochem.* 204:433–451.

BOTELLA, M. A., XU, Y., PRABHA, T. N., ZHAO, Y., NARASIMHAN, M. L., WILSON, K. A., NIELSEN, S. S., BRESSAN, R. A., and HASEGAWA, P. M. 1996. Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. *Plant Physiol.* 112:1201–1210.

BRADFORD, M. N. 1976. A rapid and sensitive method for the quantization of microgram quantities of protein using the principle of dye binding. *Anal. Biochem.* 72:248–254.

BROADWAY, R. M. 1995. Are insects resistant to plant proteinase inhibitors? *J. Insects Physiol.* 41:107–116.

BRZIN, J., POPOVIC, T., DROBNIČ-KOŠOROK, M., KOTNIK, M., and TURK, V. 1988. Inhibitors of cysteine proteinases from potato. *Biol. Chem. Hoppe-Seyler* 363(Suppl.):233–238.

CONSTABEL, C. P., YIP, L., and RYAN, C. A. 1998. Prosystemin from potato, black nightshade, and bell pepper: Primary structure and biological activity of predicted system in polypeptides. *Plant Mol. Biol.* 36:55–62.

DAVIS, J. M., GORDON, M. P., and SMIT, B. A. 1991. Assimilate movement dictates remote sites of wound-induced gene expression in poplar leaves. *Proc. Natl. Acad. Sci. U.S.A.* 88:2393–2396.

FARMER, E. E., JOHNSON, R. R., and RYAN, C. A. 1992. Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol.* 98:995–1002.

FRITIG, B., HEITZ, T., and LEGRAND, M. 1998. Antimicrobial proteins in induced plant defense. *Curr. Opin. Immunol.* 10:16–22.

FUJITA, T., KOUCHI, H., ICHIKAWA, T., and SYONO, K. 1993. Isolation and characterization of a DNA that encodes a novel proteinase inhibitor I from a tobacco genetic tumor. *Plant Cell Physiol.* 34:137–142.

FUSEK, M., BAUDYŠ, M., and METCALF, P. 1992. Purification and crystallization of human cathepsin D. *J. Mol. Biol.* 226:555–557.

GEOFFROY, P., LEGRAND, M., and FRITIG, B. 1990. Isolation and characterization of a proteinaceous inhibitor of microbial proteinases induced during the hypersensitive reaction of tobacco to tobacco mosaic virus. *Mol. Plant Microbe Interact.* 3:327–333.

GOODSPEED, T. H. 1954. The genus *Nicotiana.* Orignis, relationships and evolution of its species in the light if their distribution, morphology and cytogenetics. Chronica Botanica Company, Walthan, Massachusetts.

HALITSCHKE, R., KEßLER, A., KAHL, J., LORENTZ, A., and BALDWIN, I. T. 2000. Eco-physiological comparison of direct and indirect defenses in *Nicotiana attenuata. Oecologia* 124:408–417.

HEATH, R. L., BARTON, P. A., SIMPSON, R. J., REID, G. E., LIM, G., and ANDERSON, M. A. 1995. Characterization of the protease processing sites in a multidomain proteinase inhibitor precursor from *Nicotiana alata. Eur. J. Biochem.* 230:250–257.

HEATH, R. L., MCDONALD, G., CHRISTELLER, J. T., LEE, M., BATEMAN, K., WEST, J., VANHEESWUCK, R., and ANDERSON, M. A. 1997. Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pests. *J. Insect Physiol.* 43:833–842.

HEITZ, T., GEOFFROY, P., STINTZI, A., FRITIG, B., and LEGRAND, M. 1993. cDNA cloning and gene expression analysis of the microbial proteinase inhibitor of tobacco. *J. Biol. Chem.* 268:16987–16992.

HILDMANN, T., EBNETH, M., PEÑA-CORTES, H., SÁNCHEZ-SERRANO, J. J., WILLMITZER, L., and PRAT, S. 1992. General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. *Plant Cell* 4:1157–1170.

HOLM, S. 1979. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 6:65–70.

JOHNSON, R., NARVAEZ, J., AN, G., and RYAN, C. 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. U.S.A.* 86:9871–9875.

JONGSMA, M. A. 1995. The resistance of insects to plant proteinase inhibitors. PhD thesis. Wageningen Agricultural University, Wageningen, The Netherlands.

JONGSMA, M. A., and BOLTER, C. 1997. The adaptation of insects to plant protease inhibitors. *J. Insect Physiol.* 43:885–895.

JONGSMA, M. A., BAKKER, P. L., and STIEKEMA, W. J. 1993. Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. *Anal. Biochem.* 212:79–84.

JONGSMA, M. A., BAKKER, P. L., VISSER, B., and STIEKEMA, W. J. 1994. Tyrpsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* 195:29–35.

KAHL, J., SIEMENS, D. H., AERTS, R. J., GÄBLER, R., KÜHNEMANN, F., PRESTON, C. A., and BALDWIN, I. T. 2000. Herbivore induced ethylene surpresses a direct defense but not a putative indirect defense against an adapted herbivore. *Planta* 210:336–342.

KOIWA, H., BRESSAN, R. A., and HASEGAWA, P. M. 1997. Regulation of protease inhibitors and plant defense. *TIPS* 2:379–384.

LEE, M. C. S., SCANLON, M. J., CRAIK, D. J., and ANDERSON, M. A. 1999. A novel two-chain proteinase inhibitor generated by circularization of a multidomain precursor protein. *Nat. Struct. Biol.* 6:526–530.

LIGHTNER, J., PEARCE, G., RYAN, C. A., and BROWSE, J. 1993. Isolation of signaling mutants of tomato (*Lycopersicon esculentum*). *Mol. Gen. Genet.* 241:595–601.

LYNDS, G. Y., and BALDWIN, I. T. 1998. Fire, nitrogen, and defensive plasticity in *Nicotiana attenuata. Oecologia* 115:531–540.

MAREŠ, M., MELOUN, B., PAVLÍK, M., KOSTKA, V., and BAUDYŠ, M. 1989. Primary structure of cathepsin D inhibitor from potatoes and its structure relationship to soybean trypsin inhibitor family. *FEBS* 251:94–98.

MCCLOUD, E. S., and BALDWIN, I. T. 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jamonic acid but not nicotine in *Nicotiana sylvestris. Planta* 203:430–435.

MCMANUS, M. T., WHITE, D. W. R., and MCGREGOR, P. G. 1994. Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. *Transgen. Res.* 3:50–58.

O'DONNELL, P. J., CALVERT, C., ATZORN, R., WASTERNACK, C., LEYSER, H. M. O., and BOWLES, D. J. 1996. Ehtylene as a signal mediating the wound response of tomato plants. *Science* 274:1914–1917.

OHNMEISS, T. E., and BALDWIN, I. T. 1994. The allometry of nitogren allocation to growth and an inducible defense under nitrogen-limited growth. *Ecology* 75:995–1002.

OHNMEISS, T. E., and BALDWIN, I. T. 2000. Optimal defense theory predicts the ontogeny of an induced nicotine defense. *Ecology* 81:1765–1783.

ORIANS, C. M., POMERLAU, J. J., and RICCO, R. 2000. Vascularachtecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. *J. Chem. Ecol.* 26:471–485.

PEARCE, G., JOHNSON, S., and RYAN, C. A. 1993. Purification and characterization from tobacco (*Nicotiana tabacum*) leaves of six small wound-inducible proteinase iso-inhibitors of the potato inhibitor II family. *Plant Physiol.* 102:639–644.

PEÑA-CORTES, H., SÁNCHEZ SERRANO, J., ROCHA SOSA, M., and WILLMITZER, L. 1988. Systemic induction of proteinase inhibitor-II gene expression in potato plants by wounding. *Planta* 174:84–89.

REYMOND, P., and FARMER, E. E. 1998. Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* 1:404–411.

RYAN, C. A. 1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.* 28:425–449.

SCHITTKO, U., PRESTON, C. A., and BALDWIN, I. T. 2000. Eating the evidence? *Manduca sexta* larvae can not disrupt specific jasmonate induction in *Nicotiana attenuata* by rapid communication. *Planta* 210:343–346.

SOLOMON, M., BELENGHI, B., DELLEDONNE, M., MENACHEM, E., and LEVINE, A. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell* 11:431–443.

STOUT, M. J., WORKMAN, K. V., BOSTOCK, R. M., and DUFFEY, S. S. 1998. Specificity of induced resistance in the tomato. *Lycopersicon esculentum. Oecologia* 113:74–81.

VAN DAM, N. M., and BALDWIN, I. T. 1998. Cost of jasmonate-induced responses in plants competing for limited resources. *Ecol. Lett.* 1:1–5.

VAN DAM, N. M., HADWICH, K., and BALDWIN, I. T. 2000. Induced responses in *Nicotiana attenuata* affect behavior and growth of the specialist herbivore *Manduca sexta. Oecologia* 122:371–379.

WILDON, D. C., THAIN, J. F., MINCHIN, P. E. H., GUBB, I., REILLY, A. J., SKIPPER, Y. D., DOHERTY, H. M., O'DONNELL, P. J., and BOWLES, D. J. 1992. Electrical signalling and systemic proteinase inhibitor induction in the wounded plant. *Nature* 360:62–65.

ZHANG, Z. P., and BALDWIN, I. T. 1997. Transport of [2-^{14}C]jasmonic acid leaves to roots mimics wound-induced changes in endogenous jasmonic acid pools in *Nicotiana sylvestris. Planta* 203:436–441.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

DEFENSIVE ROLE OF SECRETION OF ECTAL MANDIBULAR GLANDS OF THE WASP *Polistes dominulus*

ANGELO FORTUNATO,[1,4] ROLAND MAILE,[2] STEFANO TURILLAZZI,[1,*] E. DAVID MORGAN,[2] GLORIANO MONETI,[3] GRAEME R. JONES,[2] and GIUSEPPE PIERACCINI[3]

[1] *Dipartimento di Biologia Animale e Genetica*
Università di Firenze
via Romana 17, 50125 Firenze, Italy

[2] *Chemical Ecology Group, School of Chemistry and Physics*
Keele University
Staffordshire, ST5 5BG, UK

[3] *Centro Interdipartimentale di Spettrometria di Massa*
Università di Firenze
viale Pieraccini 6, 50100 Firenze, Italy

Received May 5, 2000; accepted November 15, 2000)

Abstract—Ectal mandibular glands of insects are exocrine glands localized in the anterior–lateral region of the head, close to the base of the mandibles. In social wasps, the gland is composed of secretory cells and a reservoir into which the secretion accumulates. At the time of emission the secretion flows onto a specialized cuticular area on the outside of the base of the mandibles. Secretion of *Polistes dominulus* is emitted only when wasps are greatly disturbed or in the presence of predators, and its function seems to be mainly defensive. Morphometric studies did not reveal any size differences between the glands of the queens and those of the workers. GC-MS analyses of the glands identified 32 compounds, mainly acids and aldehydes in the range C_2–C_{18}. The overall odor, caused by the mixture of aldehydes, is distinct. Workers do not respond strongly to the odor. The secretion probably serves as a warning signal to vertebrate predators.

Key Words—Ectal mandibular glands, colony defense, *Polistes dominulus*, social wasps, acids, aldehydes.

*To whom correspondence should be addressed. turillazzi@DBAG.UNIFI.IT
[4] Current address: Department of Ecology and Evolutionary Biology, 6100 Main Street, Houston, Texas 77005-1892, USA. E-mail: Fortang@ruf.rice.edu

0098-0331/00/0300-0569$19.50/0

INTRODUCTION

Exocrine glands are found widely among social insects where they have various functions. Twenty-one different glands have been described in social bees, 39 in ants, 11 in termites, and 23 in social wasps (Billen and Morgan, 1998; Downing, 1991; Fortunato et al., 1998, 2000a; Jeanne, 1996; Landolt and Akre, 1979; Nedel, 1960). Some of these glands are common to all social Hymenoptera, even though their functions may vary across species, castes, or ages. Exocrine glands can produce a great variety of organic compounds, from low-molecular-weight substances to proteins. These glandular substances can be utilized as nest materials, in immature brood rearing, in the defense against predators or microorganisms, and, quite frequently, in chemical communication (pheromones and allomones). Despite this wide range of products, exocrine glands belong to only three structural types, described by Noirot and Quennedey (1974). Secretory cells possess a great functional plasticity and can produce very different secretions with slight variations in their morphology and ultrastructural organization.

Ectal mandibular glands are cephalic glands located in the anterior-lateral region of the head and are the only head glands in wasps that possess a reservoir (Landolt and Akre, 1979; Downing, 1991). This reservoir constitutes the largest part of the gland and is surrounded (although not completely) by secretory cells of type 3. The secretion produced is stored in the reservoir, and a sclerotized glandular duct connects the reservoir with the outside, opening directly at the base of the internal mandibular surface (Fortunato et al., 2000b). The location of the exit hole is consistent with the function that the secretion has in most species of aculeate social Hymenoptera. In honeybees, these glands are particularly developed in queens, where they produce sexual pheromones during the nuptial flight and, later, secrete a complex mixture of compounds that has a function in colony control. In worker bees, these glands are less well developed and the function of their secretion varies with the age of the individual (Free, 1987). In males the glands are quite reduced (Lensky et al., 1985) and their function is unknown.

In stingless bees (Meliponini), mandibular glands produce trail-following pheromones (Lindauer and Kerr, 1960), and in *Trigona* (*Oxytrigona*) a caustic secretion is released by biting (Michener, 1974). In ants, the secretions of these glands are often used as alarm or defensive pheromones (Buschinger and Maschwitz, 1984). In bumble-bees, they are the source of the secretion that males lay on various substrata during patrolling flights and serve to attract females to mating sites (Bergman and Bergström, 1997; Roubik, 1989).

Little information exists on the function of these glands in social wasps (Landolt and Akre, 1979). Downing and Jeanne (1983) suggested that the function in *Polistes fuscatus* was related to dominance hierarchy. A role in reproductive behavior is hypothesized for males of *P. major* (Wenzel, 1987) and of

some Stenogastrinae (Fortunato, personal observations). *Polistes dominulus* is a species widely distributed in southern Europe, North Africa, the Middle East, and part of Asia. Since 1980 it has also begun to colonize the United States (Cervo et al., 2000). It is characterized by an annual colony cycle (Turillazzi, 1980) with nests founded by one or more mated females in early spring (Pardi, 1942). The first individuals to emerge from these new nests are workers; reproductives begin to appear in late July and August. We report here morphological measurements of the ectal mandibular glands and chemical analysis of the secretion of *Polistes dominulus*, together with behavioral data demonstrating the function of the glands.

METHODS AND MATERIALS

Morphometric and Histological Measurements. For this study we used individuals from laboratory and field colonies from 1995 to 1998. Morphological measurements were made on intact glands dissected from wasps collected in the field. Profiles of glands were drawn with a *camera lucida* under a stereoscopic light microscope at 50× magnification, and glandular surfaces were measured with a computer by using the Java software program. Measurements were normalized by dividing by the head surface of the same animal. Measurements were recorded in pixels, since the size relative to the head, not the absolute metric measure, was required. Cells were counted after fixing glands in Pampl's solution, rehydrating for 10 min, and coloring for 10 min in Emallume followed by a toning in fresh water for 15 min. This dye stains nuclei brown-red and cytoplasm pink. Finally, glands were dipped in Vaseline and cells were separated with micro-needles on a slide, covered with a cover glass, and counted under a light microscope at 400× magnification.

Chemical Analyses. Chemical analyses were made at the CISM laboratory of the University of Florence and at the Chemical Ecology Group at Keele. At Florence mandibular glands were dissected in distilled water and their reservoirs squeezed to expel their contents. The secretion was particularly evident as it floated on water and could be collected by means of a micro-syringe and then directly injected into the gas chromatograph. GC-MS analyses were performed on a Saturn 2000 instrument (Varian, Walnut Creek, California), equipped with a fused silica capillary column (30 m × 0.25 mm coated with a 0.25-μm-thick film of 5% phenyl–95% dimethylsiloxane), with helium carrier gas at constant head pressure (6.7 psi). The oven temperature was initially 40°C for 4 min, then increased to 100°C at a rate of 20°C/min, then at 5°C/min to 300°C and maintained there for 4 min. The injector and transfer line temperatures were 250°C and 280°C, respectively. Injections were in splitless mode, with the ion trap mass spectrometer in EI mode, acquiring the mass range from m/z 40 to m/z 400, or

in positive CI mode, using acetonitrile as the reactant gas, acquiring the mass range from m/z 70 to m/z 400. Compound identification was by comparison with standard mass spectral data bases, and molecular masses obtained from the CI spectra.

For the analyses at Keele, glands were dissected in Italy and directly inserted in glass capillaries that were then scaled in a flame according to the method of Morgan (1990) and sent to England by mail. At Keele samples were analyzed on a Hewlett-Packard 5890 gas chromatograph directly coupled to a 5970B quadrupole mass spectrometer by using 70 eV electron impact ionization, with control by a HP 5972/5971 Chemstation. Individual glands were chromatographed on a 15-m × 0.32-mm-ID column (bonded polyethylene glycol stationary phase, 0.25-μm thickness, Stabilwax, Restek) with quartz glass injection liners (produced in the lab at Keele) with a plug of Restek silanized glass wool. Samples were injected in splitless mode (injection temperature 250°C) by crushing the capillary tubes immediately inside the injector port after insertion, as described by Maile et al. (1998), with helium as the carrier gas at a flow of 1 ml/min, and an oven temperature programmed to increase from 40°C (3 min) at 11°C/min to a maximum of 200°C. The split valve was closed before crushing the sample and reopened 45 sec later. Mass spectra were scanned from m/z 35 to m/z 550 with a scanning time of about 2.4 sec.

Compounds were identified by comparing their mass spectra with standard mass spectral data bases. The identity of the simple acids was confirmed by coinjection of synthetic standards (Sigma-Aldrich Co. Ltd., Gillingham, UK). As the chromatograms were characterized by many coeluting compounds, single ion monitoring at m/z 60 was used to recognize all the acids. Because the detector response for this wide range of compounds varies greatly, for quantification, calibration curves of six different concentrations of solutions of all the even-numbered acids from acetic to stearic acid were constructed. These calibration curves were used for quantification for compounds of appropriate chain lengths by comparison of peak areas.

Behavioral Observations. Colonies in various stages of development were used for behavioral observation both in the lab and in the field. The characteristic aromatic odor of the ectal mandibular gland secretion is easily detectable, and this made it possible for us to know when it was emitted. We relied on this property to study the factors involved in its emission with the evident limitations of such a type of test. Field nests were studied at sites in the countryside around Florence. Laboratory colonies were reared in glass cages (15 × 15 × 15 cm) with a ceiling of glass and steel mesh and supplied with water and food ab libitum.

To evaluate the possible alarm function of the secretion, the glands were dissected from individuals belonging to the same or to alien colonies under a stereoscopic light microscope and were presented to the colonies by using forceps. In a second experiment, (one to four) dissected glands were crushed on

25-mm^2 pieces of blotting paper and brought close to the colonies. As a control, clean blotting paper squares (or with other crushed body glands) were presented before and after the experiment. Video recordings were made in daylight with a Sony Handicam 8 mm with a macro lens, while night video recording was possible with an infrared micro-video camera and a portable lamp with IR filter.

RESULTS

Morphometric Analysis. Mandibular gland size relative to body size as measured by head area is given in Table 1. There was no significant difference between foundresses and workers or between dominant and surbordinate foundresses taken from six different postemergence colonies. The size of glands dissected from preemergence foundresses, from workers collected in July–August, and from males collected on nests in September was statistically different only for workers versus males. This last difference could probably be due to the young age of the males. The number of type three secretory cells in foundresses was, on average (±SD) 98.38, ±11.187 (N = 21, range 80–121).

Chemical Analysis of Secretion. The secretion was complex and variable from one individual to another, but in each was dominated by a series of aliphatic acids from C_2 to C_{18} (Figure 1), with relatively more of the even-numbered carbon atoms (Table 2). None of the acids were identified as being responsible for the characteristic odor of the freshly discharged secretion. Except for acetic and butyric acids, they have little or no odor. Small amounts of C_7–C_{12} aldehydes and some alcohols related to these acids were also present. Where identifiable,

TABLE 1. GLAND SIZE, RELATIVE TO HEAD SIZE, MEASURED AS COMPUTER PIXELS, COLLECTED FOR GROUPS OF *Polistes dominulus*[a]

Group	N	Gland size (pixels ± SD)	Significance
Foundresses[*b]	12	121.381 ± 26.873	
Workers[*b]	12	116.425 ± 32.114	Student's t, NS
Dominant foundresses[*,b]	6	112.852 ± 19.003	
Subordinate foundresses[*,b]	6	129.910 ± 32.451	Student's t, NS
Pre-emergent foundresses[†]	14	1502 ± 91.726	Statistically different only for males vs. workers, ANOVA, F = 3.625, df = 2 and 50, P = 0.0339, Fisher's test, P = 0.0113
Workers collected July-August[†]	23	1544 ± 65.564	
Males on nests, September[†]	16	1476 ± 88.745	

[a] Data marked with [†] were measured under different computer conditions from those marked with [*]. Data collected by the two different methods cannot be compared.
[b] Wasps were taken from six different postemergent colonies.

TABLE 2. MAJOR CHEMICAL COMPOUNDS DETECTED IN MANDIBULAR GLAND SECRETION OF WORKERS AND FOUNDRESSES OF *Polistes dominulus* COLONIES AND THOSE DETECTED AS TRACES (t)[a]

Compound	Peak	Mean secretion [ng, (min–max)]	% of total
Acetic acid	1	11.1 (1.7–30.4)	5.1
Butanoic acid	2	4.8 (0–23.5)	1.4
Isovaleric acid	3	16.7 (0–69.9)	5.0
Hexanoic acid	4	15.2 (1.3–53.1)	2.5
Heptanal		t	
Heptanoic acid	5	18.4 (0.9–46.9)	3.4
Octanal		t	
Octanoic acid	6	64.3 (3.0–156.9)	12.7
Nonanal		t	
2-Nonenal		t	
4-Nonenal		t	
Nonanoic acid	7	46.8 (6.8–95.4)	9.6
2-Decen-1-ol		t	
Decanal		t	
2-Decenal		t	
4-Decenal		t	
Decanoic acid	8	24.4 (2.5–63.3)	3.9
(*x*)-Decenoic acid	9	5.7 (0–20.3)	0.9
(*y*)-Decenoic acid	10	14.2 (2.0–36.8)	3.1
Undecanal		t	
4-Undecenal		t	
Dodecanal		t	
(*x*)-Dodecenal		t	
Geranylacetone		t	
Geranylisopropanol		t	
Dodecanoic acid	11	26.6 (1.1–64.4)	4.3
Tetradecanoic acid	12	101.5 (1.8–311.3)	9.9
Pentadecanoic acid	13	61.9 (0–182.6)	5.3
Hexadecenal		t	
Hexadecanoic acid	14	335.6 (3.3–881.2)	25.9
Hexadecenoic acid	15	93.8 (0–296.2)	1.4
Heptadecanoic acid	16	12.4 (0–34.5)	0.8
Octadecanoic acid	17	329.2 (0–704.0)	3.4
Octadecenoic acid	18	416.3 (0–925.3)	1.4
Total		1599.3	100

[a] The amount of each compound detected in a whole gland (ng) and the relative proportion of each compound (%) are given. Peak numbers refer to Figure 1.

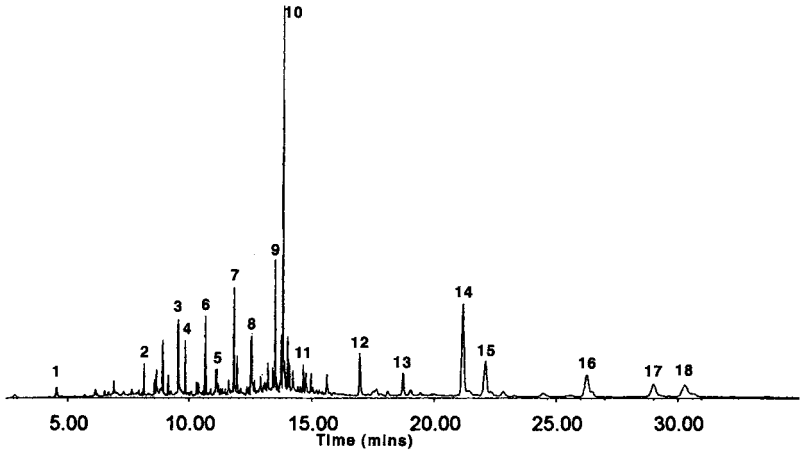


FIG. 1. Total ion chromatogram of the mandibular glands of a single worker of *Polistes dominulus*. The numbers correspond to those in Table 2.

these aldehydes are listed as traces in Table 1. The amounts of each component were in the low nanogram amounts, which made identification from the spectra difficult.

Behavior. Wasps release the secretion in a detectable quantity when isolated insects or colonies are greatly disturbed or attacked by a predator. Emission was perceived in 70 of 70 isolated females when they were trapped by their legs with forceps and in 50 of 50 colonies disturbed by the sudden approach of a hand or face to their cages. Individuals of any rank, caste, or sex can emit secretion at any moment of the colonial or life cycle (including hibernating females) both during the day or night. Emission occurs both from animals taken from nests or captured during foraging or mating flights.

Various factors can cause the emission of the secretion: capture, mechanical vibrations transmitted to the nest through the substrate, emission of CO_2 at the nest, presentation of possible predators to the colony (*Vespa crabro*), etc. Observations and video sequences showed that the approach of a predator to the nest first alerts the wasps, with raising of the wings and of the body. Rapid movements on the nests accompanied by mandible opening and wing beating follow, the latter producing an audible buzzing and circulation of air. The diffusion of the characteristic odor of the secretion, easily appreciated at this stage, is helped by the air disturbance by the wing beating. When the stimulus is particularly intense (this occurs especially after a flow of CO_2 over the nest) and at night, passage between all the various phases can be quite short. In the dark, wasps do not abandon the nest to attack, and this causes a cumulative higher release of secretion in the nest surroundings.

Wasps trapped by forceps and observed under a microscope are seen to open their mandibles to a maximum (more than 90°), uncover the basal internal surface (usually covered by the sides of the clypeus) and expose the specialized cuticular area with the glands opening (Fortunato et al., 2000b). In some cases, the emission of a little drop of secretion can be distinctly seen. Wasps can remain in this position for a few seconds to 1 min and can repeat the emission a number of times, even if the smell is less and less detectable.

After emission and when the colony is calm again, wasps begin intense and prolonged self-grooming.

Experiments on Possible Alarm Function of Secretion. In preliminary experiments, single mandibular glands were brought close to a colony on the tip of dissecting forceps. In no case was any particular alarm reaction observed from the wasps, and some of them only inspected and antennated the glands. In a second experiment, crushed glands on blotting paper were presented close to the colonies. The experiment was repeated on eight different colonies and in no case (0 of 8 trials) was any reaction observed different from the control. The same response occurred if mandibular glands were presented when crushed together with other glands (such as venom and salivary glands) (0 of 6 trials).

DISCUSSION

Morphometric analysis shows that there are no differences between dominant and subordinate females and between foundresses and workers of *P. dominulus* in the size of ectal mandibular glands. This is consistent with findings in *P. fuscatus* by Downing and Jeanne (1983), who measured the total size of the gland. However, this does not seem to be a good indicator of the actual functional activity of the glandular cells, as it is mainly determined by the size of the reservoir. On the other hand, it is known that glandular cells are particularly active in preemergence foundresses with respect to other phases of the seasonal cycle (Fortunato et al., 2000b). The preemergence phase is critical for *Polistes*, as nests are under heavy predation pressure (Yamane, 1996).

Chemical analysis of the secretion has identified a range of acids (Table 2), which are irritant and inclined to spread on insect cuticle. Many compounds that we identified have already been described as defensive secretions of other insects (Blum, 1981; Whitman et al., 1990). The characteristic odor of the fresh secretion is probably due to the mixture of aldehydes present but cannot be ascribed to any particular one. An interesting comparison can be made with the Nasonov secretion of *Apis mellifera*, which is produced in the glandular cells as a mixture of geraniol and nerol, which are oxidized enzymically to the corresponding aldehydes, and the aldehydes, on emission, are partly oxidized in the air to geranic and nerolic acids. The pheromone blend as detected is a mixture of alcohols, aldehydes, and acids (Pickett et al., 1981).

Wasps emit mandibular gland secretion only when a colony or a single animal is heavily disturbed; emission occurs at the end of a behavioral sequence that is typical of various social wasps (Starr, 1990). It can occur in any part of the day or of the colonial cycle. Rau reported a colony of *Polistes variatus* that, when disturbed, emitted a strong and sweetish odor easily detectable by the observer (Rau, 1939, page 37). There were no differences detectable between foundresses and workers in nest defense; while males seem to release the secretion only when captured. As our experiments indicate, the secretion does not possess an alarm function but, according to behavioral observations and to the characteristics of some of its compounds, it could be used as a defensive weapon. However, although this effect is still to be clarified, some hypotheses can be proposed. If the secretion is applied to an enemy body (other predatory insects) during a bite, it can act as an irritant; if it is vaporized in the air, it could act as an odorous warning mainly against vertebrate, macrosmatic, predators. The sting is efficient against predators only when it is used in contact with an aggressor. Wasps can prevent predators from approaching their colonies by attacking them in flight, but at night *Polistes* do not fly. A nocturnal predator such as a rat could get close enough to a nest to take advantage of the immature brood and to cause some damage to a nest before being repelled by the occupants. If stung by the wasps, the predators could connect the pain with the strong odor emitted by the insects during the defensive reaction and manage to avoid future possible unpleasant encounters. Thus, the odor (together with buzzing) would function in the dark the same way as the color and alarm movements act in the light. Experimental bioassays are necessary to confirm these hypotheses.

Acknowledgments—This work was supported by the European Commission through the Social Evolution network of the Universities of Aarhus, Firenze, Keele, Sheffield, Uppsala, Würzburg, and ETH Zürich, through the Training and Mobility of Researchers (TMR) program (contract ERBFMRXTC960072). Part of the research was also performed with funds from the Italian MURST (ex 60% and ex 40%). We thank Prof. Joan E. Strassmann and Dr. Rita Cervo who kindly revised an earlier version of this paper.

REFERENCES

BERGMAN, P., and BERGSTRÖM, G. 1997. Scent marking, scent origin, and species specificity in male premating behavior of two Scandinavian bumblebees. *J. Chem. Ecol.* 23:1235–1251.

BILLEN, J., and MORGAN, E. D. 1998. Pheromone communication in social insects: Sources and secretions, pp. 3–33, *in* R. K. Vander Meer, M. D. Breed, K. E. Espelie, and M. L. Winston (eds.). Pheromone Communication in Social Insects. Ants, Wasps, Bees, and Termites. Westview Press. Boulder, Colorado.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

BUSCHINGER, A., and MASCHWITZ, U. 1984. Defensive behaviour and defensive mechanisms in ants, pp. 95–150, *in* H. R. Hermann (ed.). Defensive Mechanisms in Social Insects. Praeger, New York.

CERVO, R., ZACCHI, F., and TURILLAZZI, S. 2000. *Polistes dominulus* (Hymenoptera, Vespidae) invading North America: Some hypotheses for its rapid spread. *Insectes Soc.* 47:In press.

DOWNING, H. A. 1991. The function and evolution of exocrine glands, pp. 540–569, *in* K. J. Ross and R. W. Matthews (eds.). The Social Biology of Wasps. Cornell University Press, Ithaca, New York.

DOWNING, H. A., and JEANNE, R. L. 1983. Correlation of season and dominance status with activity of exocrine glands in *Polistes fuscatus* (Hymenoptera: Vespidae). *J. Kans. Entomol. Soc.* 56:387–397.

FORTUNATO, A., ISIDORO, N., ROMANI, R., BIN, F., and TURILLAZZI, S. 1998. Ghiandole antennali in maschi di alcuni Vespidi e loro probabile ruolo nel riconoscimento tra i sessi. Atti XVIII Congresso Nazionale Italiano di Entomologia. Maratea, 208 pp.

FORTUNATO, A., DELFINO, G., and TURILLAZZI, S. 2000a. Exocrine glands in the parameres of male *Liostenogaster flavolineata* (Vespidae: Stenogastrinae). *Insect Social Life* In press.

FORTUNATO, A., TURILLAZZI, S., and DELFINO, G. 2000b. Ectal mandibular gland in *Polistes dominulus* (Christ) (Hymenoptera: Vespidae) ultrastructural modifications over the secretory cycle. *J. Morphol.* 224:45–55.

FREE, J. B. 1987. Pheromones of Social Bees. Chapman and Hall, London.

JEANNE, R. L. 1996. The evolution of exocrine gland function in wasps, pp. 144–160, *in* S. Turillazzi and M. J. West-Eberhard (eds.). Natural History and Evolution of Paper-Wasps. Oxford University Press, Oxford.

LANDOLT, P. J., and AKRE, R. D. 1979. Occurrence and location of exocrine glands in some social Vespidae. *Ann. Entomol. Soc. Am.* 72:141–148.

LENSKY, Y., CASSIER, P., DELORME-JOULIE, C., and LEVINSOHN, M. 1985. Pheromonal activity and fine structure of the mandibular glands of honeybee drones (*Apis mellifera* L.) (Insecta, Hymenoptera, Apidae). *J. Insect Physiol.* 31:265–276.

LINDAUER, M., and KERR, W. E. 1960. Communication between the workers of stingless bees. *Bee World* 41:29–41, 65–71.

MAILE, R., DANI, F. R., JONES, G. R., MORGAN, E. D., and ORTIUS, D. 1998. Sampling techniques for gas chromatographic-mass spectrometric analysis of long-chain free fatty acids from insect exocrine glands. *J. Chromatogr. A* 816:169–175.

MICHENER, C. D. 1974. The Social Behavior of the Bees. A Comparative Study. Harvard University Press, Cambridge, Massachusetts.

MORGAN, E. D. 1990. Preparation of small scale samples from insects for chromatography. *Anal. Chim. Acta* 236:227–235.

NEDEL, J. O. 1960. Morphologie und physiologie der mandibeldrüse eininger bienen-arten (Apidae). *Z. Morphol. Ökol. Tiere* 49:139–183.

NOIROT, C., and QUENNEDEY, A. 1974. Fine structure of insect epidermal glands. *Annu. Rev. Entomol.* 19:61–80.

PARDI, L. 1942. Ricerche sui Polistini. V. La poliginia iniziale in *Polistes gallicus* (L.). *Boll. Ist. Entomol. Univ. Bologna* 14:1–106.

PICKETT, J. A., WILLIAMS, I. H., SMITH, M. C., and MARTIN, A. D. 1981. Nasanov pheromone of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae). Part 3. Regulation of pheromone composition and production. *J. Chem. Ecol.* 7:543–554.

RAU, P. 1939. Studies in the ecology and behavior of *Polistes* wasps. *Bull. Brooklyn Entomol. Soc.* 34:36–44.

ROUBIK, D. W. 1989. Ecology and Natural History of Tropical Bees. Cambridge University Press, New York.

STARR, C. K. 1990. Holding the fort: Colony defence in some primitively social wasps. pp. 421–461 *in* D. L. Evans and J. O. Schmidt (eds.). Insect Defense: Adaptive Mechanisms and Strategies of Prey and Predators. State University of New York Press, Albany.

TURILLAZZI, S. 1980. Seasonal variation in size and anatomy of *Polistes gallicus* (L.) (Hymenoptera: Vespidae). *Monit. Zool. Ital.* 14:67–75.

WENZEL, J. W. 1987. Male reproductive behavior and mandibular glands in *Polistes major* (Hymenoptera: Vespidae). *Insectes Soc.* 34:44–57.

WHITMAN, D. W., BLUM, M. S., and ALSOP, D. W. 1990. Allomones: Chemicals for defence, pp. 280–351, *in* D. L. Evans and J. O. Schmidt, (eds.). Insect Defense: Adaptive Mechanisms and Strategies of Prey and Predators. State University of New York Press, Albany.

YAMANE, S. 1996. Ecological factors influencing the colony cycle of *Polistes* wasps. pp. 75–97, *in* S. Turillazzi and M. J. West-Eberhard (eds.). Natural History and Evolution of Paper-Wasps. Oxford University Press, Oxford.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

TRADE-OFFS IN ANTIHERBIVORE DEFENSES IN *Piper cenocladum:* ANT MUTUALISTS VERSUS PLANT SECONDARY METABOLITES

LEE A. DYER,[1,2,*] CRAIG D. DODSON,[2] JON BEIHOFFER,[3] and DEBORAH K. LETOURNEAU[2,4]

[1] *Department of Ecology and Evolutionary Biology*
Tulane University
New Orleans, Louisiana 70118

[2] *Western Colorado Center For Tropical Research*
Mesa State College
Grand Junction, Colorado 81501

[3] *US EPA, National Enforcement Investigation Center*
Bldg 53, Box 25227, DFC
Lakewood, Colorado 80225

[4] *Department of Environmental Studies*
University of California
Santa Cruz, California 95064

(Received November 16, 1999; accepted November 14, 2000)

Abstract—Ant–plant mutualisms may provide indirect evidence for costs of antiherbivore defenses when plants demonstrate trade-offs between allocating resources and energy into ant attractants versus chemical defenses. We tested the hypothesis that ecological trade-offs in defenses are present in *Piper cenocladum*. This plant possesses two distinct defenses: food bodies that attract predatory ants that destory herbivore eggs and amides that deter herbivores. Previous studies have demonstrated that the food bodies in *P. cenocladum* are an effective defense because the ants deter herbivory by specialist herbivores. Amides in other *Piper* species have been shown to have toxic qualities, but we tested the additional hypothesis that these amides have an actual defensive function in *P. cenocladum*. To test for ecological trade-offs between the two putative defenses, fragments of *P. cenocladum* were examined for the presence of amides both when the plant was producing food bodies and when it was not producing food bodies. Plants with active ant colonies had redundant defenses, producing food bodies and high levels of amides at the same time, but we detected a trade-off in that they had significantly lower levels of amides than did plants with no ants. To test for the defensive value

* To whom correspondence should be addressed.

0098-0331/00/0300-0581$19.50/0

of *P. cenocladum* amides, we used an ant bioassay and we examined herbivory results from previous experiments with plants that had variable levels of amides. These tests demonstrated that amides are deterrent to omnivorous ants, leaf cutting ants, and orthopterans. In contrast, the resident *Pheidole bicornis* ants are effective at deterring herbivory by specialist herbivores that oviposit eggs on the plant but not at deterring herbivory by nonresident omnivores. We concluded that although both amides and food body production appear to be costly, redundancy in defenses is necessary to avoid damage by a complex suit of herbivores.

Key Words—*Piper cenocladum*, ant plants, chemical defense, trade-offs, amides, herbivory, predation, Costa Rica, mutualism.

INTRODUCTION

Defenses against herbivores are assumed to be costly in terms of fitness-enhancing functions (Cates and Orians, 1975; Levin, 1976; Fox, 1981; Gould, 1983, 1988; Gershenzon, 1994; Sagers and Coley, 1995; Elle et al., 1999), but these costs are presumably outweighed by the benefits associated with lower levels of herbivory (Zangerl and Bazzaz, 1992; Simms, 1992). As a result of these costs, ecological and evolutionary defensive trade-offs are expected when resources are limited (Rehr et al., 1973; Simms, 1992). For example, a plant that invests heavily in trichomes as a defense should have low levels of chemical defenses compared to a similar species that has a low density of foliar trichomes or compared to an individual of the same species that has decreased its investment in trichome production. Such trade-offs can be difficult to detect (Simms, 1992), and some authors have even found that allocation of resources to multiple defenses is common in plants that need to defend against many different types of herbivores (e.g., Lindroth and Hwang, 1996).

While the presence of trade-offs is consistent with the hypothesis that defenses are costly, allocation of resources to multiple defenses, or redundancy in defenses (Romeo et al., 1996), does not imply absence of cost nor does it exclude the possibility of ecological trade-offs. Limited resources can still cause reduction of one redundant defense in response to an increase in another. Nevertheless, studies uncovering redundant defenses in ant–plant mutualisms have been used to argue that trade-offs between antiherbivore defenses do not occur and that the defenses are not costly because plants are able to allocate resources and energy into chemical defenses as well as ant attractants (biotic defenses). Steward and Keeler (1988) found that the mean number of different indole alkaloid compounds was not lower in species of *Ipomoea* that produce extrafloral nectaries compared to nonnectary plants; these results have been used as weak evidence that neither alkaloids nor nectaries are costly (Simms, 1992). Letourneau and Barbosa (1999) found that a mechanical defense (trichomes) in *Endospermum*

was induced both in the presence and absence of a biotic defense (ant defenders); this result implies that the mechanical defense is costly because it is induced (Baldwin, 1998), but that there is no trade-off between the mechanical and biotic defense. Janzen (1973) found that individuals of five *Cecropia* species found in areas without mutualistic *Azteca* ants did not produce mullerian bodies for these ants; he concluded that this biotic defense was metabolically expensive, but he did not examine the system for trade-offs with production of chemical defenses.

Only one study with ant–plant mutualisms has indicated that a trade-off may exist between biotic and chemical defenses, suggesting that both types of defenses are costly. Rehr et al. (1973) found that *Acacia* species without mutualistic *Pseudomyrmex* ants had leaves with cyanogenic glycosides, whereas a species with resident ants did not have cyanogenic glycosides. Furthermore, diets made from the nonant species adversely affected the feeding efficiency of *Prodenia eridania* (Noctuidae) larvae. These results have been used as evidence to support the hypothesis that there are costs associated with biotic defenses as well as chemical defenses (Simms, 1992). However, HCN did not affect the larvae in this study (Rehr et al., 1973), thus their conclusions rely on the assumption that the nonant *Acacias* contained an unidentified defensive compound that affects herbivores; it is equally plausible that nutritional factors other than plant defenses affected larval feeding efficiency.

We tested the general hypothesis of trade-offs in energy and resource allocation to competing defenses using an ant plant that facultatively produces food bodies in the presence of a specific ant species and that varies in levels of defensive secondary compounds. *Piper cenocladum* (Piperaceae) is an understory shrub that produces lipid- and amino acid-rich food bodies for a resident ant, *Pheidole bicornis* (Formicidae: Myrmicinae), which protects the plants from specialist herbivores (Letourneau, 1983). Plants in the Piperaceae commonly have high levels of secondary compounds that have antifeedant activities (reviewed by Parmar et al., 1997), and *P. cenocladum* has high levels of three amides that may be deterrent to herbivores (Dodson et al., 2000; Dyer and Letourneau, 1999a). We conducted a study to test the hypothesis that production of food bodies and secondary compounds is metabolically expensive so that high investment in one defense will lead to absence or lower levels of the other defense. Regardless of the presence of trade-offs, the fact remains that this plant species has redundancy in its antiherbivore defenses; thus we also tested the hypothesis that the multiple defenses are necessary because amides in *P. cenocladum* deter those consumers that are not deterred by ants: generalist herbivores and omnivores.

METHODS AND MATERIALS

Study System. Piper cenocladum is an understory shrub (usually less than 4 m tall) common in lowland wet forests in Costa Rica (Burger, 1971). The species

reproduces both vegetatively through layering (fallen shrubs root adventitiously) or fragmentation (petioles and twigs break off and root) and through seed (Gartner, 1989; Greig, 1993). The plant is commonly found as a shrub as well as a fragment (Gartner, 1989), and the biology of the two architectures can be very different (Dyer and Letourneau, 1999b). The leaves are large (most leaves fall within the range of 86–430 cm^2 when fully expanded) and long-lived (approximately 2 years), and opalescent food bodies are produced on the adaxial surface of sheathing leaf bases (petiolar cavities) when occupied by *Pheidole bicornis* ants.

The herbivores most commonly found feeding on *Piper* ant plants at our study site are specialist lepidopterans and coleopterans. Feeding damage from generalist herbivores, including leafcutter ants (Hymenoptera: Formicidae: *Atta cephalotes*) and orthopterans (Orthoptera: Tetigoniidae, Acrididae: *Microtylopteryx hebardi*, and Eumasticidae: *Homeomastax robertsi*) is occasionally found. The main herbivores that eat leaf tissue are geometrid moth larvae (Lepidoptera: Geometridae: *Cambogia* sp. and *Eois* sp.), skippers (Lepidoptera: Hesperiidae: *Quadrus cerealis*), saddleback caterpillars (Lepidoptera: Limacodidae), weevils (Coleoptera: Curculionidae: *Ambates* spp.), and at least 10 species of flea beetles (Coleoptera: Chrysomelidae: *Physimera* spp.) (Marquis, 1991; Dyer, Letourneau, and G. Gentry unpublished data).

Pheidole bicornis is a small, dimorphic species that occupies *Piper cenocladum*, harvests the food bodies produced by the plant, and removes insect eggs, some vines, and small phylloplane particles from the leaves (Risch et al., 1977; Letourneau, 1983, 1998). Production of food bodies by the plant is induced by an unknown compound produced by the ants (Dodson et al., unpublished data), and the food bodies are produced continually as a source of nutrition for the ants, in contrast to the occasional herbivores ants collect from the plant surface (Letourneau, unpublished data). Other species of *Pheidole* and other ants are occasionally found in the petiolar cavities of *P. cenocladum*, but they do not induce food body production by the plant.

The phytochemistry of the family Piperaceae and the genus *Piper* is well documented. Members of the genus *Piper* are known to produce alkaloids, aromatic hydrocarbons, oxygenated cyclohexanes, terpenes, chalcones, flavones, phenyl propenes, lignans, neolignans, and amides of a characteristic type sometimes referred to as *Piper* amides (reviewed by Parmar et al., 1997). These amides contain a phenyl moiety with a variable length carbon side chain (typically with at least one unsaturation) ending in a carbonyl carbon. The nitrogen-containing portion of the amide is derived from piperidine, pyrrole, or an isobutyl group and may contain an unsaturation and/or a carbonyl group. *Piper cenocladum* contains several amides at high concentrations (up to 0.58% dry weight): piplartine, 4′-desmethyl piplartine, and cenocladamide (Dodson et al., 2000). To our knowledge, none of the other *Piper* ant plants (*P. sagittifolium* C.DC., *P.*

obliquum Ruiz & Pavon, and *P. fimbriulatum* C.DC.) have been investigated for secondary compounds. A closely related species without ant mutualists, *Piper imperiale*, also contains high levels of amides (C. Dodson and J. Searcy, unpublished data). Many *Piper* amides that have been investigated for biological activity have been demonstrated to be insecticidal (Gbewonyo et al., 1993; Su and Horvat, 1981; Miyakado et al., 1989) or deterrent to leaf-cutting ants (Capron and Wiemer, 1996). One of the amides in *P. cenocladum*, piplartine, is known to be cytotoxic in vitro (Duh et al., 1990).

Study Site. Plant and insect collection areas were located at the La Selva Biological Station, Heredia Province, Costa Rica, located at 10°25′N, 84°05′W at ca. 100 m elevation on the Caribbean slope (Hartshorn and Hammel, 1994). The natural history of this lowland wet forest is described in great detail in McDade et al. (1994). We used plant fragments from previous experiments that had been established on a hectare of primary forest at about 1400 m on the Sendero Jaguar trail at La Selva. The soil at this site is the poorest of soil types where *P. cenocladum* grows at La Selva (Sollins et al., 1994), and the understory vegetation is similar to other primary and secondary forest areas where shrubs and fragments are found.

Quantification of Amides. We examined amides in experimental fragments. Plant samples were prepared as described in Dodson et al. (2000) and included 1-g replicate samples from individual plant fragments from La Selva. We harvested leaves from a two-year experimental study where fragments had been established in the forest (see Dyer and Letourneau, 1999a, for complete methods). The leaves were harvested from five samples of *P. cenocladum* fragments with ants (each sample included a homogenate of plants, with a mean of 4.4 plants per sample) and five samples of *P. cenocladum* fragments from that ants had been excluded (a mean of 3.8 plants per sample). We chose most recently expanded leaves that were similar in size and that had low levels of herbivory (<10%) and low epiphyll loads. Ant exclusion was accomplished by applying to the petioles one to two drops of dilute Diazinon insecticide (0.85 mg wettable powder per liter of distilled water) every two to three months for 1.8 years (see Dyer and Letourneau, 1999a for methods). Experiments examining the effects of these dilute insecticide applications indicated no measurable effects of the insecticides on plant biomass, herbivory, epiphyll cover, and other variables (Letourneau and Dyer, 1998; Letourneau, Dyer, and G. Vega, unpublished observations). When the plant fragments were harvested, mean number of ants per plant per sample was 4 ± 3 SE for the ant exclusion plants and 129 ± 56 SE for the unmanipulated ant plants. None of these 10 samples were manipulated in any other way (i.e., no fertilizer was added to the soil, light was not manipulated).

Leaves were dried at room temperature, ground, and extracted with 95% ethanol; the amides being isolated, as well as most putative *Piper* plant defenses, are stable at room temperature (Dodson et al., 2000; Parmar et al., 1997). The

crude extract residue was resuspended in 3 : 1 water–ethanol, which was then exhaustively extracted with chloroform and quantitatively analyzed by GC-MS using commercially available piperine as an internal standard at the 80 μg/ml level. Five point calibrations (50, 100, 200, 300, and 500 μg/ml) were prepared using synthetic piplartine (r^2 = 1.000) and synthetic 4′-desmethylpiplartine (r^2 = 0.999). Cenocladamide is unstable in solution, so its structural isomer, 4′-desmethylpiplartine was used as a standard, thus all concentrations we report for cenocladamide are estimates (Dodson et al., 2000).

We used multivariate analysis of variance (MANOVA) to examine the effects of ant exclusion on the concentrations of the three amides. We used profile analysis (*sensu* Tabachnick and Fidell, 1996) to detect any differences in responses of the three amides to the ant exclusion. The Wilks lambda statistic was used for all hypothesis tests.

Assays for Other Compounds. To examine the possibility of trade-offs between ant defense and other chemical defenses, we assayed for other common defensive compounds. TLC and GC-MS of all crude residues and ^{1}H NMR of the other fractions obtained in previously described chromatography (Dodson et al., 2000) were conducted to detect phenyl propanoids, aromatic hydrocarbons, oxygenated cyclohexanes, lignans, and neolignans in bulk samples of naturally occurring shrubs with and without ants. A separate sample of crude extract was screened for alkaloids (differential pH partitioning and TLC with iodoplatinic acid visualization).

Deterrence of Amides to Omnivores and Generalist Herbivores. Leaves from bulk shrub samples with high (1.36% dry weight) versus low (0.41% dry weight) levels of total amides were tested for palatability to insects using a bioassay with the giant tropical ant, *Paraponera clavata* (Formicidae: Ponerinae). The two distinct levels of amides were obtained by using naturally occurring shrubs without ants (high amides) and shrubs with ants (low amides). The *Paraponera* bioassay has successfully identified deterrent compounds in caterpillars and herbivore toxins in plants such as nicotine and solapalmatine in normal plant concentrations (Dyer, 1995, 1997; Dodson, Dyer, and G. Gentry, *unpublished data*). The bioassay only uncovers very deterrent compounds because the ants are less selective than other arthropods with respect to diet (Dyer, 1997), and extracts that deterred the ants were even more deterrent to wasps and bugs (Dyer, 1997). Complete methods for the bioassay are in Dyer (1995). Briefly, 15 g dry weight of each plant treatment (ants versus no ants) was extracted in 250 ml methanol. A 6.7% solution of this extract in 20% (by weight) sugar water and a control (6.7% methanol in 20% sugar water) were offered simultaneously to ants in 2.4-ml microcentrifuge tubes. Tubes were weighed before and after being exposed to foraging ants for 1 hr. A total of 150 extracts (each offered with a control tube) per plant treatment were offered to 15 colonies found between 2000 and 3200 m along the Sendero Tres Rios trail at La Selva. Adjusted consump-

tion differences (*sensu* Dyer, 1995) were calculated for each offering. This value compares consumption of extract versus control vials, adjusting for total consumption; for unpalatable extracts, a higher positive adjusted consumption difference indicates greater unpalatability. Mean adjusted consumption differences for the two plant treatments (high versus low amides) were compared using a t test ($N = 15$ colonies).

We conducted new analyses on folivory data from concurrent experiments (Dyer and Letourneau, 1999a,b) to test the hypothesis that *P. cenocladum* amides are deterrent to generalist herbivores. In one experiment (Dyer and Letourneau, 1999a,b) all leaves from 47 experimental plant fragments with ants and all leaves from 44 fragments from which ants had been removed were examined for damage from various orthopterans. This is the same experiment from which leaves were harvested from fragments for the chemical analysis. In a second experiment (Dyer and Letourneau, 1999b), all leaves from 40 shrubs with ants and all leaves from 37 shrubs from which ants had been excluded were examined for damage from *Atta cephalotes* and various orthopterans. We used chi-square statistics to test hypotheses of association between presence of ants and presence of herbivore damage.

RESULTS

Assays with the shrub samples for potential defenses other than amides were negative. TLC and GC-MS of all crude residues and ^{1}H NMR of the other fractions obtained in the previously described chromatography (Dodson et al.) indicates the absence of phenyl propanoids, aromatic hydrocarbons, oxygenated cyclohexanes, lignans, and neolignans. The screen for alkaloids was also negative.

Plant fragments from which ants were excluded had significantly higher concentrations of total amides (Table 1, $F_{1,8} = 8.3$, $P = 0.02$), and the profile analysis revealed that the magnitude of this increase did not differ between the three amides ($P > 0.05$ for all comparisons). For plant fragments maintained with insecticide applications to remove ant colonies, overall amide concentrations were 0.2 times greater than in plants with ants, which is a smaller difference than that reported for naturally occurring shrubs (3.3 times greater levels of amides in shrubs without ants) (Dodson et al., 2000). The overall levels of amides in fragments appear to be higher than those reported for naturally occurring shrubs (Dodson et al., 2000), but other differences between the fragments and shrubs were not controlled (i.e., many of the shrubs came from a different site).

The bioassays demonstrated that leaves from shrubs without ants were significantly more unpalatable than leaves from shrubs with ants. The mean adjusted

TABLE 1. CONCENTRATIONS OF *Piper* AMIDES IN *P. cenocladum* EXPERIMENTAL FRAGMENTS WITH AND WITHOUT *Pheidole bicornis* ANTS[a]

Compound	Concentration (% dry mass)	
	Ants present	Ants excluded
Piplartine	0.37	0.38
4′-desmethylpiplartine	0.39	0.43
Cenocladamide	0.64	0.93
Total amides	1.40	1.74

[a] Exclusion of ants was accomplished by applying dilute insecticide (Dyer and Letourneau, 1999a).

consumption difference was 0.055 for ant leaves and 0.266 for nonant leaves ($t_{28} = 2.8$, $P = 0.008$). Both of these values are relatively high, and the value for nonant leaves is not significantly different from plants with high levels of toxic compounds such as nicotine from cigarette tobacco (Dodson, Dyer, and G. Gentry, unpublished data).

Orthopteran damage was found on 18 of 91 *P. cenocladum* plants in the fragments experiment, and 78% (14) of these were plants with ants ($\chi^2_1 = 6.1$, $P = 0.01$). In the shrub experiment, orthopteran damage was found on 43 of 77 plants, and 27 (63%) of these were plants with ants ($\chi^2_1 = 4.6$, $P = 0.03$). Damage from the leaf cutting ant, *A. cephalotes*, is generally rare on *P. cenocladum*, but for the shrub experiment, 5 of 77 plants had ant damage, and all of these were plants with ants ($\chi^2_1 = 4.9$, $P = 0.03$).

DISCUSSION

A trade-off was evident between biotic and chemical defenses in experimental fragments of *P. cenocladum*. Overall levels of amides were higher when ants were either absent or at very low numbers in *P. cenocladum*. These experimental data corroborate correlational data that also demonstrated a defensive trade-off: naturally occurring shrubs without ants had 3.3 times higher concentrations of total amides than shrubs with ants (Dodson et al., 2000). Such a trade-off suggests that both biotic and chemical defenses are costly. If both defenses were low in material and energy costs, the plant would experience minimum tissue loss from herbivores by having both the ants and high levels of the amides, since it is likely that together they protect the plant against a broad range of different herbivores. Letourneau (1983; also see Letourneau and Dyer, 1998) has demonstrated that *Pheidole bicornis* ants cause lower lepidopteran folivory by removing eggs from the surface of the leaf. However, there is no evidence that these ants are able to decrease levels of herbivory by orthopterans and *Atta cephalotes*

(leaf cutting ants), since they are too small to deter such large herbivores. *Piper* amides could deter orthopteran herbivory by decreasing their feeding efficiency and deter *A. cephalotes* herbivory (Capron and Weimer, 1996) through some other mechanism such as antifungal properties.

Examination of leaves from previous experiments (Dyer and Letourneau, 1999a,b) corroborated the hypothesis that *P. cenocladum* amides are deterrent to generalist herbivores. Damage from leaf cutting ants and orthopterans was more common on plants with ants, and we have demonstrated that *P. cenocladum* shrubs and fragments with ants have lower levels of amides than those without ants. Furthermore, the bioassays demonstrate that plants without ants are more unpalatable than plants with ants. For the orthopteran data and the bioassay data, the plants were the same plants that we analyzed for amides, with ant-exclusion plants having higher levels of amides; thus, higher amide concentrations are present in plants that are avoided more by orthopterans and ants. However, orthopteran damage was still low when ants were present, and the levels of amides in ant-containing plants were still high enough to deter ants in the bioassay, so it is likely that plants are protected from generalists by amides whether or not they have ants—the protection is simply enhanced when the levels of amides increase. These results support recent hypotheses that stress that importance of redundancy in defenses when faced with a diverse herbivore assemblage (Romeo et al., 1996).

Since each mole of each amide contains a mole of nitrogen, the concentrations found in both our manipulated plants and the naturally occurring shrubs represent a considerable investment (Gershenzon, 1994) by these plants, which are nutrient limited (Dyer and Letourneau, 1999a). Results from other studies with *P. cenocladum* suggest that not only are amides costly, but they are more expensive to synthesize than food bodies. Plants grown in an experiment where all herbivores were excluded had a greater total biomass when ants were present and the plants were presumably producing lower levels of amides (Dyer and Letourneau, 1999a). *Piper* amides (Dodson et al., 2000) and food bodies (Risch and Rickson, 1981) both represent considerable investments of nitrogen and carbon, but it is possible that the costs of biosynthetic machinery, storage structures and enzymes, and overall investment of raw materials (reviewed by Gershenzon, 1994) for the amides is higher per plant than for the food bodies. One alternative mechanism for this result, that the ants provide nutrients to the plants in the form of nitrogenous wastes, has not been supported by experiments and field observations (Letourneau, 1983). It has not escaped our notice that other secondary metabolites may also increase when ants are excluded. We have eliminated this possibility for all probable candidates except the terpenes and flavanoids.

We cannot completely rule out the possibility that greater levels of herbivory on the ant-excluded plants induced increased levels of amides in those plants. However, the correlational results for shrubs (Dodson et al., 2000) only

included plants with low levels of herbivory (less than 10%) that had the same leaf size, levels of damage, and epiphyll load. For the fragments, which had lower levels of herbivory for ant versus nonant plants (Dyer and Letourneau, 1999a), the differences in amide concentrations were not as great as they were in the shrubs, which suggests that induction was not an important mechanism in our results. It is also possible that stressing the plants by adding insecticide to the petioles induced an increase in defensive compounds, but we have evidence that Diazinon has minimal physiological effects on *P. cenocladum* (Letourneau and Dyer, 1998; Dyer and Letourneau, 1999b), and we did not add insecticides to any of the shrub petioles.

Thus, in this ant–plant system, there appear to be trade-offs between investment in chemical defenses and ant attractants. Both shrubs and fragments utilize the two defenses and both exhibit the trade-off. Redundancy in defenses is also important to deter a diverse suite of herbivores, and the plant will invest in both defenses if possible.

Acknowledgments—This study was funded by NSF grant DEB-9318543 to D.K.L., faculty research grants from the UCSC Academic Senate and Social Sciences Division to D.K.L., and an NSF REU grant to D.K.L., L.A.D., and C.D.D. A portion of the study was funded by an Earthwatch grant and a National Geographic grant to L.A.D. We thank the Organization for Tropical Studies for use of their facilities and for valuable logistical help. Excellent technical assistance was provided by G. Vega, R. Krach, J. Sorensen, G. Gentry, J. Searcy, J. Jay, Z. Wright, and 12 Earthwatch volunteers. We are grateful to G. Gentry for comments that improved previous drafts of the manuscript.

REFERENCES

BALDWIN, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U.S.A.* 95:8113–8118.

BURGER, W. 1971. Flora Costaricensis. *Field. Bot.* 35:1–227.

CAPRON, M. A., and WIEMER, D. F. 1996. Piplaroxide an ant-repellent piperidine epoxide from *Piper tuburculatum. J. Nat. Prod.* 59:794–795.

CATES, R. G., and ORIANS, G. H. 1975. Successional status and the palatability of plants to generalized herbivores. *Ecology* 56:410–418.

DODSON, C. D., DYER, L. A., SEARCY, J., WRIGHT, Z., and LETOURNEAU, D. K. 2000. Cenocladamide, a dihydropyridone alkaloid from *Piper cenocladum. Phytochemistry* 53:51–54.

DUH, C. Y., WU, Y. C., and WANG, S. K. 1990. Cytotoxic pyridine alkaloids from *Piper aborescens. Phytochemistry* 29:2689–2691.

DYER, L. A. 1995. Tasty generalists and nasty specialists? A comparative study of antipredator mechanisms in tropical lepidopteran larvae. *Ecology* 76:1483–1496.

DYER, L. A. 1997. Effectiveness of caterpillar defenses against three species of invertebrate predators. *J. Res. Lepid.* 35:1–6.

DYER, L. A., and LETOURNEAU, D. K. 1999a. Relative strengths of top-down and bottom-up forces in a tropical forest community. *Oecologia* 119:265–274.

DYER, L. A., and LETOURNEAU, D. K. 1999b. Trophic cascades in a complex, terrestrial community. *Proc. Natl. Acad. Sci. U.S.A.* 96:5072–5076.

ELLE, E., VAN DAM, N. M., and HARE, J. D. 1999. Cost of glandular trichomes, a "resistance" character in *Datura wrightii* Regel (Solanaceae). *Evolution* 53:22–35.

FOX, L. R. 1981. Defense and dynamics in plant-herbivore systems. *Am. Zool.* 21:853–864.

GARTNER, B. L., 1989. Breakage and regrowth of *Piper* species in rain forest understory. *Biotropica* 21:303–307.

GBEWONYO, W. S. K., CANDY, D. J., and ANDERSON, M. 1993. Structure–activity relationships of insecticidal amides from *Piper quieneese. Root Pestic. Sci.* 37:57–66.

GERSHENZON, J. 1994. The cost of plant chemical defense against herbivory: A biochemical perspective, pp. 105–173, *in* E. Bernays (ed.). Insect–Plant Interactions, Vol. V. CRC Press, Boca Raton, Flordia.

GOULD, F. 1983. Genetics of plant–herbivore systems: Interactions between applied and basic study, pp. 599-653, *in* R. Denno, and B. McClure (eds.). Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York.

GOULD, F. 1988. Genetics of pairwise and multispecies plant-herbivore coevolution, pp. 13–55, *in* K. C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, San Diego, California.

GREIG, N. 1993. Predispersal seed predation on five *Piper* species in a tropical rainforest. *Oecologia* 93:412–420.

HARTSHORN, G. S., and HAMMEL, B. E. 1994. Vegetation types and floristic patterns, pp. 73–89, *in* L. A. McDade, K. S. Bawa, H. A. Hespenheide, and G. S. Hartshorn (eds.). La Selva. Ecology and Natural History of a Neotropical Rainforest. University of Chicago Press, Chicago.

JANZEN, D. H. 1973. Comments on host-specificity of tropical herbivores and its relevance to species richness, pp. 201–211, *in* V. H. Heywood (ed.). Taxonomy and Ecology. Academic Press, London.

LEVIN, D. A. 1976. The chemical defenses of plants to pathogens and herbivores. *Annu. Rev. Ecol. Syst.* 7:121–159.

LETOURNEAU, D. K. 1983. Passive aggression: An alternative hypothesis for the *Piper–Pheidole* association. *Oecologia* 60:122–126.

LETOURNEAU, D. K. 1998. Ants, stem-borers, and fungal pathogens: Experimental tests of a fitness advantage in *Piper* ant-plants. *Ecology* 79:593–603.

LETOURNEAU, D. K., and BARBOSA, P. 1999. Ants, stem borers, and pubescence in *Endospermum* in Papua New Guinea. *Biotropica* 31:295–302.

LETOURNEAU, D. K., and DYER, L. A. 1998. Experimental test in lowland tropical forest shows top-down effects through four trophic levels. *Ecology* 79:1678–1687.

LINDROTH, R. L., and HWANG, S. Y. 1996. Diversity, redundancy, and multiplicity in chemical defense systems of aspen, pp. 25–56, *in* J. T. Romeo, J. A. Saunders, and P. Barbosa (eds.). Phytochemical Diversity and Redundancy in Ecological Interactions. Plenum Press, New York.

MARQUIS, R. J. 1991. Herbivore fauna of *Piper* (Piperaceae) in a Costa Rican wet forest: Diversity, specificity, and impact, pp. 179–208, *in* P. W. Price, T. M. Lewinsohn, G. W. Fernandes, and W. W. Benson (eds.). Plant–Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. John Wiley & Sons, New York.

MCDADE, L. A., BAWA, K. S., HESPENHEIDE, H. A., and HARTSHORN, G. S. 1994. La Selva. Ecology and Natural History of a Neotropical Rainforest. University of Chicago Press, Chicago.

MIYAKADO, M., NAKAYAMA, I., and OHNO, N. 1989. Insecticidal unsaturated isobutylamides, pp. 173–187, *in* J. T. Arnason, B. J. R. Philogene, and P. Morand (eds.). Insecticides of Plant Origin. American Chemical Society, Washington, D.C.

PARMAR, V. S., JAIN, S. C., BISHT, K. S., JAIN, R., TANEJA, P., JHA, A., TYAGI, O. D., PRASAD, A. K., WENGEL, J., OLSEN, C. E., and BOLL, P. M. 1997. Phytochemistry of the genus *Piper. Phytochemistry* 46:597–673.

REHR, S. S., FEENY, P. P., and JANZEN, D. H. 1973. Chemical defence in Central American non-ant-acacias. *J. Anim. Ecol.* 42:405–416.

RISCH, S. J., and RICKSON, F. R. 1981. Mutualism in which ants must be present before plants produce food bodies. *Nature* 291:149–150.

RISCH, S., MCCLURE, M., VANDERMEER, J., WALTZ, S. 1977. Mutualism between three species of tropical *Piper* (Piperaceae) and their ant inhabitants. *Am. Midl. Nat.* 98:433–444.

ROMEO, J. T., SAUNDERS, J. A., and BARBOSA, P. (eds.). 1996. Phytochemical Diversity and Redundancy in Ecological Interactions. Plenum Press, New York.

SAGERS, C. L., and COLEY, P. D. 1995. Benefits and costs of defense in neotropical shrub. *Ecology* 76:1835–1843.

SIMMS, E. L. 1992. Costs of plant resistance to herbivory, pp. 392–495, *in* R. S. Fritz, and E. L. Simms (eds.). Ecology and Evolution of Plant Resistance. University of Chicago Press, Chicago.

SOLLINS, P., SANCHO, M. F., MATA Ch.R., SANFORD, R. 1994. Soils and soil process research, pp. 34–53, *in* L. A. McDade, K. S. Bawa, H. A. Hespenheide, and G. S. Hartshorn (eds.). La Selva. Ecology and Natural History of a Neotropical Rainforest. University of Chicago Press, Chicago.

STEWARD, J. L., and KEELER, K. H. 1988. Are there trade-offs among antiherbivore defenses in *Ipomoea* (Convolvulaceae)? *Oikos* 53:79–86.

SU, H. C. F., and HORVAT, R. 1981. Isolation, identification, and insecticidal properties of *Piper nigrum* amides. *J. Agric. Food Chem.* 29:115–118.

TABACHNICK, B. G., and FIDELL, L. S. 1996. Using Multivariate Statistics, 3rd ed. Harper Collins, New York.

ZANGERL, A., and BAZZAZZ, F. A. 1992. Theory and pattern in plant defense allocation, pp. 363–391, *in* R. S. Fritz and E. L. Simms (eds.). Ecology and Evolution of Plant Resistance. University of Chicago Press, Chicago.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

PLANT DENSITY AND NUTRIENT AVAILABILITY CONSTRAIN CONSTITUTIVE AND WOUND-INDUCED EXPRESSION OF TRYPSIN INHIBITORS IN *Brassica napus*

DONALD F. CIPOLLINI[1,*] and JOY BERGELSON[2]

[1] *Department of Biological Sciences*
Wright State University
3640 Colonel Glenn Highway
Dayton, Ohio 45435-0001

[2] *Department of Ecology and Evolution*
University of Chicago
1101 East 57th Street
Chicago, Illinois 60637

(Received July 7, 2000; accepted November 14, 2000)

Abstract—We investigated the effects of plant density on plant size, leaf total soluble protein content, and constitutive and wound-induced levels of proteinaceous trypsin inhibitors in pot-grown *Brassica napus* seedlings in two greenhouse studies. We manipulated plant density by varying the number of intraspecific neighbors surrounding a target plant in the center of each pot. In general, constitutive and induced levels of trypsin inhibitors were significantly reduced by competition in a density-dependent manner, to the extent that induction was greatly reduced or abolished in target plants surrounded by six neighbors. To investigate whether the effects of plant density on inhibitor production were mediated by nutrient availability, we manipulated the concentration of a complete fertilizer applied to target plants surrounded by six neighbors in two greenhouse studies. In general, constitutive and wound-induced levels of inhibitors in plants surrounded by six neighbors were increased by nutrient addition in a dose-dependent manner, such that wound-induction was completely restored in competing plants under conditions of high nutrient availability. Leaf total soluble protein content, measured only in the second trial of each experiment, was not affected by any of the treatments. The effects of plant density, nutrient addition, and wounding on inhibitor levels in all experiments were independent of their effecs on above-ground plant size at the time of wounding. Overall, our results suggest that decreasing nutrient availability mediates the density-dependent reductions in inhibitor levels in *B. napus* seedings.

* To whom correspondence should be addressed. e-mail: don.cipollini@wright.edu

0098-0331/00/0300-0593$19.50/0

Key Words:—Adaptive phenotypic plasticity; *Brassica napus*; competition; constraints; costs of denfense; induced defense; insect herbivory; proteinase inhibitors; trypsin inhibitors; wounding.

INTRODUCTION

Induced defenses to enemy attack have been demonstrated in over 100 plant–herbivore and plant–pathogen systems and appear to be a form of adaptive phenotypic plasticity (Karban and Baldwin, 1997; Cipollini, 1998; Baldwin, 1999; Agrawal 1999). If defense production is costly to fitness in plants, phenotypic plasticity in defense production may have evolved as a cost-saving strategy that enables close phenotype–environment matching and maximizes plant fitness. However, many factors can constrain the induction of defense, potentially placing limits on the benefits of inducibility and preventing perfect phenotype-environment matching (DeWitt et al., 1998). For example, the degree of the induction of defense traits and/or biological resistance in plants can vary with such factors as plant size and age, type and timing of damage, and environmental conditions (Broadway and Missurelli, 1990; Karban; 1993; Baldwin and Schmelz, 1994; Jongsma et al., 1994; Alarcon and Malone, 1995; Gianoli and Niemeyer, 1996; Baldwin, 1999; Cipollini and Redman, 1999; Thaler et al., 1999).

Competition for limited resources is an important environmental factor known to influence many plant processes from the individual to the population and community level (Grace and Tilman, 1990). In terms of insect and disease resistance, some plant populations have been found to be more susceptible to insect attack or disease outbreaks when growing at high density (Colhoun, 1973; Burdon, 1987; Reader, 1992; but see Karban, 1993; Morrison, 1996). For example, we have found that interspecific competition with weeds increases herbivore damage and aphid numbers on *Brassica napus* plants in the field (D. Cipollini and J. Bergelson, unpublished data). This general phenomenon is thought to be partly explained by the presence of favorable microenvironmental conditions (e.g., high humidity, shelter from predators) and plant spatial distributions that facilitate the rapid build-up and spread of insects or disease inoculum among plants at high density (Colhoun, 1973; Reader, 1992). While these effects are undoubtedly important, plants may be unable to fully express constitutive and wound-induced defenses and/or biological resistance under resource-limiting conditions (like high density), thus contributing to their increased susceptibility to natural enemy attack (Clay, 1990). For example, Karban et al. (1989) showed that both reduced soil volume and increased plant density increasingly reduced the induction of biological resistance to mites and *Verticillium* fungi in pot-grown cotton. However, no measures of physiological or morphological defense traits were made in this study to address possible mechanisms.

Serine proteinase inhibitors are one class of constitutive and inducible defense found widely throughout the plant kingdom. Serine proteinase inhibitors (which are proteins) function by binding specifically with the protein binding site of proteinases (e.g., trypsin and chymotrypsin of insects and mammals), thereby competitively inhibiting their ability to bind and cleave proteins (see Koiwa et al., 1997, for review). Although their effectiveness as a defense against herbivores can vary by plant and herbivore species (Bolter and Jongsma, 1995; Broadway, 1995), proteinase inhibitors have been shown to reduce the growth and survival of many insects when present in artificial diets (e.g., Broadway and Duffey, 1986) and to reduce both insect feeding rate and performance when expressed in transgenic plants (e.g., Johnson et al., 1990; McManus et al., 1994). Moreover, the induction of serine proteinase inhibitors by wounding or insect attack, along with the induction of other defense proteins and compounds, is commonly associated with induced resistance to insects (Broadway et al., 1986; Thaler et al., 1996; Stout et al., 1998b).

We investigated some of the potential ecological constraints on the expression of a chemical defense by examining the effect of increasing plant density on constitutive and wound-induced levels of trypsin inhibitors in *Brassica napus* L. To investigate whether effects of plant density were mediated by nutrient availability, we examined the effects of increasing nutrient availability on constitutive and wound-induced levels of trypsin inhibitors in plants grown at high density. We also examined the extent to which variation in above-ground growth or total leaf soluble protein content explained the effects of plant density and nutrient availability on inhibitor production. We found that constitutive and local wound-induced levels of inhibitors were reduced by the presence of competitors in a density-dependent fashion and that nutrient addition could increasingly restore constitutive levels and wound-inducibility in plants grown at high density. Effects of plant density and nutrient availability on inhibitor production were independent of their effects on above-ground growth and were not associated with total leaf soluble protein content.

METHODS AND MATERIALS

Plant Growth. *Brassica napus* L. (Brassicacae) is a self-compatible annual plant that is grown commercially as an oilseed and forage crop that can occasionally escape cultivation and become naturalized in some environments (Duke, 1983; D. Cipollini, personal observation). We used an agronomic cultivar of *B. napus* (cv. Westar), commonly used in agroecological and physiological studies (e.g., Bodnaryk, 1992; Cipollini and Bergelson 2000), in our experiments. Plants were grown in ProMix BX potting soil from seed in 300 ml square pots (7 × 7 cm) in a greenhouse. To reduce genetic variation among replicate plants, we used

seeds gathered from one self-pollinated maternal plant that had been grown under greenhouse conditions very similar to those used in this experiment. Greenhouse photoperiod during experiments was controlled at 16L : 8D. Natural daylengths were extended as necessary with sodium vapor lamps, which were programmed to turn on whenever natural light levels dipped below 400 μmol photons/m^2/sec [photosynthetically active radiation (PAR)] during the 16-hr light period. Mean daytime irradiance during these experiments was ~1000 μmol photons/m^2/sec PAR. Temperatures averaged 26 ± 3°C during the light period and 21.5 ± 2°C during the dark period. Plants were watered daily with tap water and fertilized as indicated in each experiment. The first trial of each of our experiments was performed in the greenhouse at the University of Chicago (UC), while the second trial of each of our experiments was performed in the greenhouse at Wright State University (WSU). The major difference in conditions between the two trials was that daylengths were extended with cool-white fluroescent lighting at WSU, but other unknown differences certainly existed. Because of these differences, each trial was statistically analyzed separately to determine whether the pattern of responses to the treatments in each experiment would be similar under similar (but not identical) growing conditions.

Experiment 1: Effects of Plant Density on Growth and Trypsin Inhibitor Production. In this experiment, we manipulated plant density within pots around a target plant. This approach, termed the "target-neighbor" approach, has been commonly used to examine the competitive strength of individual plants under intra- or interspecific competition (Gibson et al., 1999). We grew target plants from seed in the center of each pot at four densities: (1) alone, (2) surrounded by two neighbors, (3) surrounded by four neighbors, or (4) surrounded by six neighbors. These pot densities correspond to field densities of ~15, 46, 75, and 105 plants/m^2, which are realistic for wild *Brassica* populations in the field (D. Cipollini, personal observation). Two to three seeds were sown at each position in each pot, and plants were selected for uniformity in size and thinned to the proper density and spacing three days after germination. Neighbors in each density treatment were spaced equidistant from the target plant and from each other. All plants in each pot were of the same age. Plants were fertilized once on day 7 following thinning with 50 ml of 200 ppm 15 : 16 : 17 N-P-K (plus micronutrients) soluble fertilizer (Peter's Peat-Lite Special).

Ten days after thinning, we randomly assigned half the target plants in each density treatment to be wounded. We wounded plants by crushing the distal one tenth of the first true leaf with sterilized needle-nosed pliers. Leaf crushing has been widely used in studies on the regulation of proteinase inhibitors in plants (e.g., Jongsma et al., 1994) and enables careful control of the amount and timing of damage applied to each leaf. We determined previously that this minimal amount of damage could effectively induce trypsin inhibitors in this cultivar of *B. napus* (Cipollini and Bergelson, 2000). The induction of trypsin inhibitor

activity in *Brassica oleraceae* (Broadway and Missurelli, 1990), and *Brassica rapa* (D. Cipollini, J. Busch, K. Stowe, E. Simms, and J. Bergelson, unpublished data) by this type of mechanical wounding closely simulates that produced in response to feeding by larval *Trichoplusia ni*. We expect that trypsin inhibitor induction by mechanical wounding simulates induction by natural herbivory in *B. napus* to a large degree, but the absence of herbivore saliva in mechanical wounds and the lack of spatially and temporally variable feeding patterns typical of herbivores certainly influences the degree to which this is true. Prior to wounding on day 10, we measured plant height and length of the first true leaf on all damaged and undamaged target plants. Three days following wounding, the first true leaves from wounded and unwounded target plants in each density treatment were harvested, placed individually in 1.7 ml microfuge tubes, and frozen immediately in liquid nitrogen. Thus, this study focused on locally induced, rather than systemically induced defenses. Leaf samples were stored at −20°C until analysis for trypsin inhibitor activity and soluble protein content as described below. Plants were grown in a completely randomized design on the greenhouse benches and randomly moved twice before wounding and once following wounding. Treatments were replicated 6–10 times.

We analyzed height and leaf length data taken on day 10 with a one-way ANOVA with density as the main effect. We analyzed trypsin inhibitor data with a two-way ANOVA with density (four levels), wounding (+ or −), and their interaction as effects in the model, with and without leaf length and plant height on day 10 as covariates. Because of unequal cell sample sizes, tests of significance were based on type III sums of squares. Data in all experiments were analyzed with SAS using the General Linear Models procedure (Release 6.12, SAS Institute Inc., Cary, North Carolina).

This entire experiment was repeated in a second trial, using five replicate plants per treatment combination. In addition to all of the measures above, total soluble protein content of the first true leaves (which required leaf weighing) was measured for each target seedling in the second trial, as described below. This was done to determine the relationship between trypsin inhibitor content (a soluble protein) and total soluble protein content in first true leaves. Growth and inhibitor data were analyzed separately for each trial. Total leaf protein data generated in the second trial were statistically analyzed in the same way as trypsin inhibitor data.

Experiment 2: Effects of Nutrient Availability on Growth and Trypsin Inhibitor Production. In this experiment, we manipulated nutrient availability to plants through additions of 15 : 16 : 17 N-P-K (plus micronutrients) soluble fertilizer to examine whether nutrient addition could ameliorate the effects of high plant density on inhibitor production. Target plants in each pot were grown surrounded by six neighbors, as described above. The soil used in this study contains a small amount of nutrients that can be depleted by plants in the absence of

additional fertilization, as evidenced by eventual growth limitation (D. Cipollini, personal observation). We fertilized plants 7 and 10 days after thinning with a 100-ml solution of fertilizer at one of the following four concentrations: 0, 50, 100, or 200 ppm. Ten days after thinning, we measured all target plants and wounded half of them in each nutrient treatment as described above. First true leaves were harvested from all target plants three days following wounding and stored until analysis of trypsin inhibitor activity and soluble protein content. Treatments were replicated 8–10 times.

We analyzed height and leaf length data taken on day 10 with a one-way ANOVA with nutrient availability (four levels) as the main effect. We analyzed trypsin inhibitor data with a two-way ANOVA with nutrient availability (four levels), wounding (+ or −), and their interaction as effects in the model, with and without leaf length and plant height on day 10 as covariates. Again, because of unequal cell sample sizes, tests of significance were based on type III sums of squares.

This experiment was also repeated a second time with five replicate plants per treatment combination. Total soluble protein contents of first true leaves were measured in addition to all of the other measures, and total leaf protein data were analyzed in the same way as inhibitor data. Data were analyzed separately for each trial.

Protein Extraction and Analysis of Trypsin Inhibitor Activity and Total Protein Content. To extract soluble proteins, we first ground individual leaf samples directly in the microfuge tubes without buffer, using a Teflon minipestle. A 0.150-ml aliquot of ice-cold 1 mM HCl (Broadway and Missurelli, 1990) was then placed in each tube, and the tubes were vortexed for 10 sec. Samples were then centrifuged as 12,000 *g* for 12 min in a microfuge cooled to 4°C. After centrifugation, the cleared supernatants were transferred into new tubes and used as the soluble protein extracts for both the trypsin inhibitor and total soluble protein content measurements. Extracts were kept on ice throughout the entire procedure and used immediately in chemical analyses.

We analyzed the trypsin inhibitor content of individual protein extracts using a radial diffusion assay through a trypsin-containing agar (Jongsma et al., 1993, 1994), as modified for *B. napus* in Cipollini and Bergelson (2000). In both trials, soluble protein contents of each extract were quantified in all experiments by the method of Bradford (1976), using the Bio-Rad protein dye reagent and bovine serum albumin as the standard. We expressed trypsin inhibitor content of each extract as micrograms of trypsin inhibitor/mg extract protein. This measure should be interpreted as the amount of trypsin inhibitor present per unit extractable soluble protein in a leaf. In the second trial of each density and nutrient experiment, we measured the fresh weight of leaf samples prior to protein extraction in order to determine total soluble protein content of leaf samples, which we expressed as mg total protein/g leaf fresh weight.

RESULTS

Effects of Density on Growth, Inhibitor Production, and Protein Content. In the first trial of this experiment (UC), we found that plant density affected both height ($F_{3,59} = 19.97$, $P < 0.001$), and length of the first true leaf ($F_{3,59} = 6.90$, $P < 0.001$) of 10-day-old *B. napus* seedlings. Height of plants in this experiment increased with increasing density, while leaf length decreased with increasing density (Figure 1A). In the second trial (WSU), plant density affected height ($F_{3,36} = 3.29$, $P = 0.0316$), and length of the first true leaf ($F_{3,36} = 4.68$, $P = 0.007$), in a similar pattern as in the first trial, although plants were slightly smaller overall (Figure 1B).

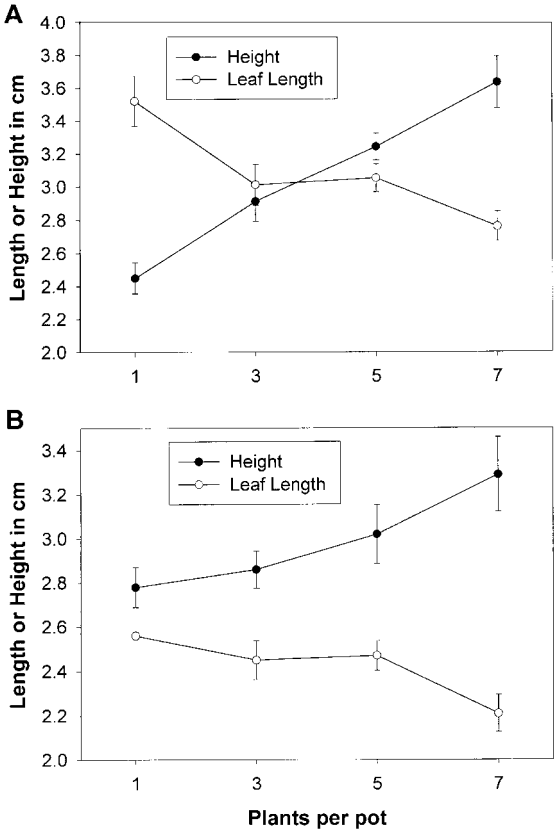


FIG. 1. Effects of plant density on plant height and length of the first true leaf in *B. napus* seedlings. In trial 1 (A), each point is the mean (±SE) of 6–10 plants; in trial 2 (B), each point is the mean (±SE) of 5 plants.

TABLE 1. UNIVARIATE ANOVA OF EFFECTS OF DENSITY, WOUNDING, AND THEIR INTERACTION ON TRYPSIN INHIBITOR LEVELS IN *B. napus* SEEDLINGS (TRIAL 1)

Source	*df*	MS	*F*	*P*
Density	3	508.86	56.9	<0.001
Wounding	1	223.48	25.0	<0.001
Density × wounding	3	41.342	4.62	0.006
Error	55	491.66		

In turn, we found that plant density, wounding, and their interaction all affected trypsin inhibitor levels in *B. napus* seedlings in either one or both trials (Tables 1 and 2). In unwounded plants, there was a density-dependent decline in constitutive trypsin inhibitor levels in the first trial (Figure 2A), that was not apparent in the second trial (Figure 2B). Wounding increased trypsin inhibitor levels in *B. napus* seedlings in both trials, as expected, but the degree of induction was dependent on plant density. In the first trial, inhibitor levels were induced by ~25% by wounding in plants grown alone, but the degree of induction declined with density to the extent that induction was completely abolished in plants surrounded by six neighbors (Figure 2A). Although (perhaps because) absolute levels of inhibitors were lower overall in the second trial, inhibitor levels were induced by ~170% by wounding in plants grown alone, but declined in a density-dependent fashion (i.e., significant wounding × density interaction), to the extent that inhibitor levels were induced by only ~80% in plants surrounded by six neighbors (Figure 2B).

In the second trial of this experiment, total soluble protein content of first true leaves was not affected by density ($F_{3,32} = 1.48$, $P = 0.239$), wounding ($F_{1,32} = 1.38$, $P = 0.249$), or their interaction ($F_{3,32} = 0.30$, $P = 0.823$) (data not shown).

We also found that effects of density and wounding on inhibitor production were largely independent of the effects of these factors on aboveground plant

TABLE 2. UNIVARIATE ANOVA OF EFFECTS OF DENSITY, WOUNDING, AND THEIR INTERACTION ON TRYPSIN INHIBITOR LEVELS IN *B. napus* SEEDLINGS (TRIAL 2)

Source	*df*	MS	*F*	*P*
Density	3	9.5875	2.56	0.072
Wounding	1	217.01	57.9	<0.001
Density × wounding	3	13.625	3.64	0.023
Error	32	3.7428		

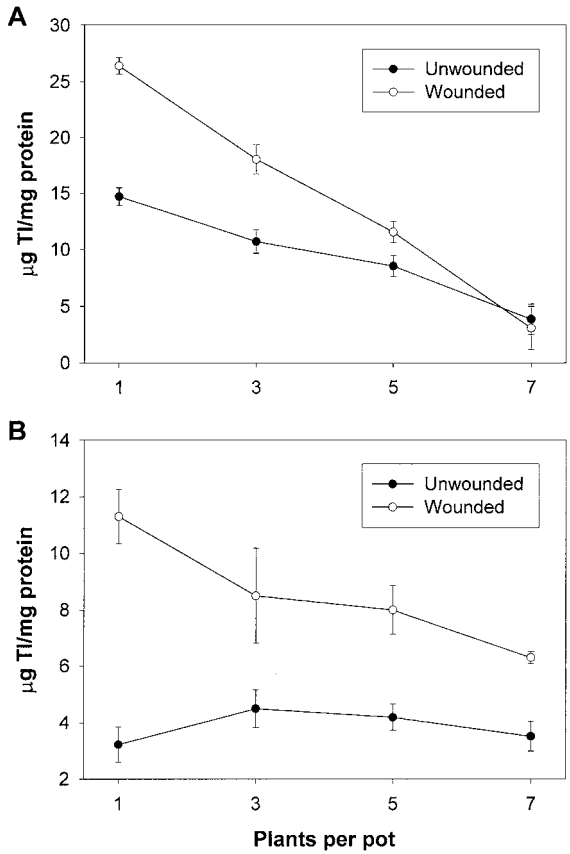


FIG. 2. Effects of plant density on constitutive and wound-induced TI activity in *B. napus* seedlings. In trial 1 (A) each point is the mean (±SE) of 6–10 plants; in trial 2 (B), each point is the mean (±SE) of 5 plants.

size in either trial. When either height or leaf length (on the day plants were wounded) were used as covariates in the ANOVA, significant effects of plant density, wounding, and their interaction on inhibitor levels were still apparent (data not shown).

Effects of Nutrient Availability on Growth and Inhibitor Production. In the first trial of this experiment, we found that nutrient availability had small but significant effects on height ($F_{3,68} = 2.83$, $P = 0.045$) and leaf length ($F_{3,68} = 16.51$, $P < 0.001$) of plants surrounded by six neighbors. Plants were shorter under no or low fertilization than at the highest fertilization level (Figure 3A). Leaves were slightly longer at the highest nutrient level than in all of the other

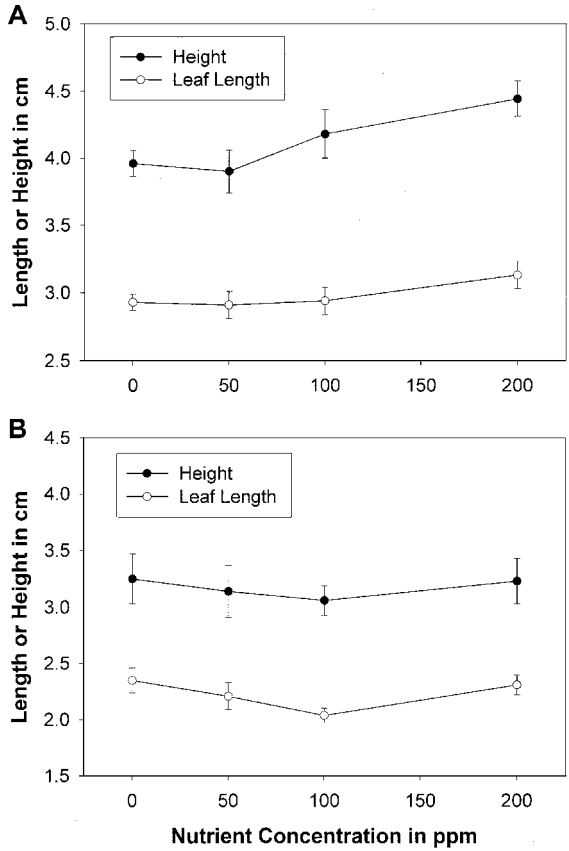


FIG. 3. Effects of nutrient availability on plant height and length of the first true leaf in *B. napus* seedlings grown at high density. In trial 1 (A), each point is the mean (±SE) of 8–10 plants; in trial (B), each point is the mean (±SE) of 5 plants.

treatments. In the second trial, nutrient availability had no effect on height ($F_{3,36} = 0.82$, $P = 0.492$) or leaf length ($F_{3,36} = 1.54$, $P = 0.224$) (Figure 3B), and plants were smaller than in trial 1 (Figure 3b).

In turn, we found that nutrient availability and wounding affected trypsin inhibitor levels in plants surrounded by six neighbors. In the first trial (Table 3), inhibitor levels increased with nutrient availability in a dose-dependent fashion and were higher in wounded plants (Figure 4A). In addition, the degree of induction of inhibitors by wounding was increased by nutrient availability in an additive fashion. While there was little or no induction at low nutrient availability, induction was completely restored by high nutrient availability to

TABLE 3. UNIVARIATE ANOVA OF EFFECTS OF NUTRIENT AVAILABILITY (NUTRIENT), WOUNDING, AND THEIR INTERACTION ON TRYPSIN INHIBITOR LEVELS IN HIGH-DENSITY GROWN *B. napus* SEEDLINGS (TRIAL 1)

Source	*df*	MS	*F*	*P*
Nutrient	3	124.44	10.4	<0.001
Wounding	1	157.65	13.2	<0.001
Nutrient × wounding	3	24.143	2.02	0.122
Error	55	657.14		

levels found in plants grown alone (Figure 4A). In the second trial (Table 4), inhibitor levels increased with nutrient availability in a dose-dependent fashion and were higher in wounded plants (Figure 4B). However, the degree of induction of inhibitors by wounding was increased interactively by nutrient availability. While inhibitors were induced by ~30% in unfertilized seedlings, inhibitors were induced by ~167% in fully-fertilized seedlings, a degree of induction nearly identical to that found in plants grown alone (Figure 4B).

In the second trial of this experiment, total soluble protein content of first true leaves was not affected by nutrient ($F_{3,32} = 0.34$, $P = 0.798$), wounding ($F_{1,32} = 0.001$, $P = 0.962$), or their interaction ($F_{3,32} = 1.17$, $P = 0.335$) (data not shown).

As above, we found that effects of nutrient availability on inhibitor production in this experiment were independent of the effects of these factors on aboveground plant size in either trial. When either height or leaf length on the day plants were wounded was used as the covariate in the ANOVA, significant effects of nutrient availability and wounding on inhibitor levels were still apparent (data not shown).

DISCUSSION

In this study, increased plant density and reduced nutrient availability were found to be important constraints on the constitutive and wound-induced expression of trypsin inhibitors in pot-grown *B. napus* seedlings. While wounding substantially induced inhibitors in plants either grown alone or at high density with ample fertilization, induction was either completely abolished or greatly reduced in plants grown at high density and low levels of fertilization. Constitutive levels of inhibitors were also low in competing plants without ample fertilization. Although plants were slightly smaller and levels of inhibitors were lower overall in the second trial of each of our experiments, the pattern of effects was largely the same as in the first trial. Our findings suggest that high density and/or reduced nutrient availability may be important ecological constraints on the abil-

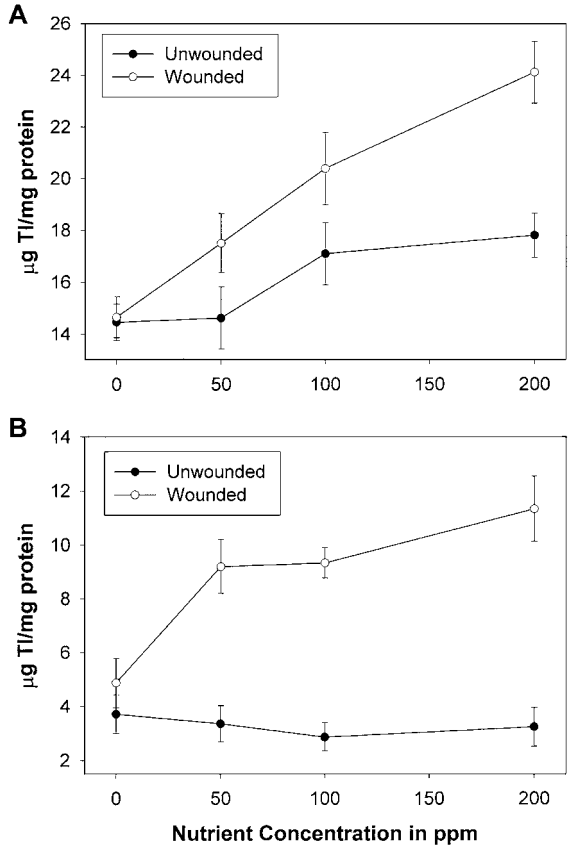


FIG. 4. Effects of nutrient availability on constitutive and wound-induced TI activity in first true leaves of *B. napus* seedlings grown at high density. In trial 1 (A), each point is the mean (±SE) of 8–10 plants; in trial 2 (B), each point is the mean (±SE) of 5 plants.

TABLE 4. UNIVARIATE ANOVA OF EFFECTS OF NUTRIENT AVAILABILITY (NUTRIENT), WOUNDING, AND THEIR INTERACTION ON TRYPSIN INHIBITOR LEVELS IN HIGH-DENSITY GROWN *B. napus* SEEDLINGS (TRIAL 2)

Source	*df*	MS	*F*	*P*
Nutrient	3	15.360	4.39	0.011
Wounding	1	294.84	84.3	<0.001
Nutrient × wounding	3	22.564	6.45	0.002
Error	32	3.4993		

ity of a plant to actively express chemical defenses to natural enemies. If this finding is also true in the field, it may lead to increased herbivore damage under such conditions. Furthermore, if the expression of chemical defenses is sensitive to environmental conditions, then not only the benefits, but also the costs of induced resistance in plants will likely vary across a range of environmental variation in the field (Baldwin, 1998; Baldwin et al., 1998). For example, Bergelson has shown that fitness costs of the constitutive expression of resistance in both wild lettuce (Bergelson, 1994b) and *Arabidopsis thaliana* (Bergelson 1994a) can vary with plant density and/or nutrient availability. Likewise, van Dam and Baldwin (1998) have shown that fitness costs of jasmonate-induced defense in tobacco are more apparent when induced plants compete with an uninduced neighbor. Baldwin and Hamilton (2000) have shown that such costs are associated with a reduced ability of jasmonate-induced plants to compete for nitrogen with uninduced neighbors. To more fully understand the range of costs and benefits of plant resistance, relevant environmental variables, such as competition, should be incorporated more often into studies on the regulation and ecological role of induced defenses.

In studies of the effects of environmental factors on the induction of defense, it is important to distinguish whether the environmental factors directly affect induction (e.g., through a resource-based constraint) or indirectly affect induction through modulation of plant size or developmental stage. This is particularly important when induction is developmentally regulated, as is true for the induction of trypsin inhibitors in *Brassica oleracea* (Broadway and Missurelli, 1990) and the induction of oxidative enzymes in tomato (Cipollini and Redman, 1999). Gianoli and Niemeyer (1996) have shown that increased temperature reduced the induction of defensive hydroxamic acids by aphid feeding in wheat. However, the effect of temperature on induction disappeared when its effect on plant growth rate was statistically controlled. In our experiment, significant effects of nutrient availability and plant density on inhibitor levels were independent of the effect of these factors on above-ground plant size at the time of wounding. When compared at a common size, increased plant density and reduced nutrient availability still constrained constitutive and induced inhibitor production. The relationship between inhibitor production and below ground growth is unknown, however. In turn, the effects of plant density, nutrient availability, and wounding on inhibitor levels were not associated with effects of these factors on leaf total soluble protein content, which were not significant. This finding is important in order to demonstrate that the environmental control of the constitutive or wound-induced production of inhibitors (which are soluble proteins) is not simply passively tied to total soluble protein production and that differences in inhibitor production among treatments are not an artifact of the manner in which we expressed inhibitor content (micrograms of trypsin inhibitors per milligram of extract protein).

The finding that plant density and nutrient availability could regulate inhibitor production independently from their effects on plant growth or total protein production suggests that inhibitor production is tuned to internal resource levels. Inhibitor induction in plants at high density and low nutrients levels may have been passively limited by resource availability. If this were the case, however, we would expect that total leaf soluble protein contents also would have been affected by high density or low nutrient levels, but they were not. On the other hand, if inhibitor production is costly to fitness, plants may have prioritized resources to important functions other than inhibitor production under conditions of resource stress (perhaps toward the synthesis of proteins that improve nutrient uptake) that may serve in some way to maximize growth or competitive ability. Indeed, inhibitor production was much more greatly affected by density or nutrient availability in our study than was above-ground growth. We do not know if reduced inhibitor production and declines in inducibility are the direct result of active suppression of inhibitor synthesis, the indirect result of resource limitation caused by allocation of resources to other functions, or the result of some other mechanism. If induced defense in plants is a form of adaptive phenotypic plasticity and is produced optimally, as has been suggested (Cipollini, 1998; Agrawal 1999; Baldwin, 1999), the suppression of allocation to induced defenses in certain environments to the benefit of growth could be an ecological cost-saving strategy with adaptive value. This possibility has rarely been considered, in part because the degree of wound induction of some defense compounds, such as furanocoumarins in parsnip (Zangerl and Berenbaum, 1994/95) and nicotine in *Nicotiana attenuata* (Lynds and Baldwin, 1998), can show little sensitivity to nutrient and/or light availability. In the only study we are aware of that investigated this possibility, Baldwin and Hamilton (2000) demonstrated that induction of nicotine synthesis in competing *Nicotiana sylvestris* plants with methyl jasmonate led to reduced fitness of plants grown with uninduced neighbors. To apparently minimize such costs, however, competing jasmonate-induced plants utilized a greater proportion of their acquired nitrogen in growth and reproduction than in nicotine synthesis relative to uninduced neighbors, which is consistent with the mechanism that we suggest is driving our findings. If defense cost savings under resource stress is the adaptive mechanism driving such results, it will be interesting and important to identify situations under which such cost savings ultimately translate into fitness benefits in the field.

While it is not known which nutrient may have been most limiting to inhibitor production in our experiments, proteinase inhibitors are nitrogen-rich proteins. Bolter et al. (1998) have shown that nitrogen deprivation severely reduced the wound induction of cysteine proteinase inhibitors in potato and tomato plants. In contrast, Stout et al. (1998a) have shown that nitrogen availability had little effect on the induction of chymotrypsin inhibitor activity in tomato, although inhibitor activity was not adjusted for extract protein con-

tent in their study. Unfortunately, the relationship between the effects of nitrogen availability on induced inhibitor levels and plant growth in either of these studies is not known. In our high-density treatments, plants exhibited a morphological response indicative of the shade avoidance syndrome (e.g., increased stem elongation at the expense of leaf area production) (Dudley and Schmitt, 1996; Cipollini and Schultz, 1999). While this suggests that light limitation may have partially mediated the results in our density experiments, it has been shown that plants can induce the shade avoidance response using light cues reflected from neighbors before significant light limitation occurs (Ballaré et al., 1990). Based on the relative lack of mutual shading among plants grown at high density in our study, we do not think that light was the primary limiting factor on inhibitor production in our experiment. Instead, because nutrient addition could increasingly restore constitutive and wound-induced levels of inhibitors in high density plants to levels found in plants grown alone, we believe that the effects of density on inhibitor production in our study were largely mediated by nutrient availability (and possibly nitrogen availability in particular). Nonetheless, increased light levels have been shown to increase serine proteinase inhibitor production in wounded tomato plants (Gustafson and Ryan, 1976), and light limitation would likely become more important as plants grow larger and the degree of mutual shading increases.

Regardless of the underlying physiological mechanisms, our results suggest that the ability of a brassicaceous plant to defend itself from natural enemy attack by using proteinase inhibitors (or other chemical defenses) may be severely constrained when plants are growing at high density and/or under conditions of low nutrients in the field, which could lead to increased herbivore damage. We recently found that weed competition can increase herbivore damage and aphid number on this cultivar of *B. napus* in the field (D. Cipollini and J. Bergelson, unpublished data), which may be related to dimished chemical defense production at high density. The ecological consequences of such findings may be compounded by the fact that plants are often less tolerant of leaf area removal when growing at high density or at low resource levels (Lee and Bazzaz, 1980; Lentz and Cipollini, 1998) and the fact that increased density can favor rapid herbivore and pathogen population growth through mechanisms unrelated to plant tissue quality (Reader, 1992). Moreover, our finding that plant density can profoundly influence the expression of a chemical defense is ecologically relevant to brassicaceaous plants in the field, which exhibit only short-distance seed dispersal, and thus often germinate and grow surrounded by a dense population of intraspecific neighbors (D. Cipollini, personal observation).

Because the effect of plant density on plant defensive chemistry has rarely been measured, it is not known the extent to which other classes of compounds, either in brassicaceous plants or in other plants, show the same lability as trypsin inhibitors in our study. For example, brassicaceous plants also rely to some

degree on a variety of glucosinolates for defense against natural enemies (Bodnaryk, 1992), but the effect of plant density on either constitutive or wound-induced glucosinolate levels has never been studied to our knowledge. Hjalten et al. (1994) have demonstrated that total phenolic concentration of birch seedlings declines with increasing plant density, but inducibility of phenolics was not examined in their study. In turn, although we can speculate on the ecological consequences of our findings in the greenhouse, the effect of plant density on induced biological resistance in the field has rarely been studied. In one study in which it has, however, induced resistance to leaf miners in wild cotton by early-season caterpillar damage tended to be more effective in plants grown at a reduced density relative to those grown at an endemic density (Karban, 1993). The ecological consequences of our findings at the biochemical level on the resistance of brassicaceous plants in the field are currently being investigated.

Acknowledgments—We thank Jennifer Olecziewski, Sylvia Woods, Jeremiah Busch, Sandra Suwanski, John Zdenek, and Ken Yliniemi for technical assistance. Suggestions by Tom Juenger, Kendra Cipollini, Jim Coleman, Colin Orians, and one anonymous reviewer substantially improved this manuscript. We acknowledge financial support from Wright State University (to D.F.C.) and the Packard Foundation (to J.B.)

REFERENCES

AGRAWAL, A. A. 1999. Induced plant defense: Evolution of induction and adaptive phenotypic plasticity, pp. 251–268, *in* A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defense Against Pathogens and Herbivores: Biochemistry, Ecology, and Agriculture. APS Press, St. Paul, Minnisota.

ALARCON, J.-J., and MALONE, M. 1995. The influence of plant age on wound induction of proteinase inhibitors in tomato. *Physiol. Plant.* 95:423–427.

BALDWIN, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. U.S.A.* 95:8113–8118.

BALDWIN, I. T. 1999. Inducible nicotine production in native *Nicotiana* as an example of adaptive phenotypic plasticity. *J. Chem. Ecol.* 25:3–30.

BALDWIN, I. T., and HAMILTON III, W. 2000. Jasmonate-induced responses of *Nicotiana sylvestris* result in fitness costs due to impaired competitive ability for nitrogen. *J. Chem. Ecol.* 26:916–952.

BALDWIN, I. T., and SCHMELZ, E. A., 1994. Constraints on an induced defense: The role of leaf area. *Oecologia* 97:424–430.

BALDWIN, I. T., GORHAM, D., SCHMELZ, E. A., LEWANDOWSKI, C. A., and LYNDS, G. Y. 1998. Allocation of nitrogen to an inducible defense and seed production in *Nicotiana attenuatea. Oecologia* 115:541–552.

BALLARÉ, C. L., SCOPEL, A. L., and SANCHEZ, R. A. 1990. Far-red radiation reflected from adjacent leaves: an early signal of competition in plant canopies. *Science* 247:329–332.

BERGELSON, J. M. 1994a. Changes in fecundity do not predict invasiveness: a model study of transgenic plants. *Ecolog* 75:249–252.

BERGELSON, J. M. 1994b. The effects of genotype and the environment on costs of resistance in lettuce. *Am. Nat.* 143:349–359.

BODNARYK, R. P. 1992. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31:2671–2677.

BOLTER, C. J., and JONGSMA, M. A. 1995. Colorado potato beetles (*Leptinotarsa decenlinpata*) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate, *J. Insect Physiol.* 41:1071–1078.

BOLTER, C. J., LATOSZEK-GREEN, M., and TENUTA, M. 1998. Dependence of methyl jasmonate- and wound-induced cysteine proteinase inhibitor activity on nitrogen concentration. *J. Plant Physiol.* 152:427–432.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

BROADWAY, R. M. 1995. Are insects resistant to plant proteinase inhibitors? *J. Insect Physiol.* 41:107–116.

BROADWAY, R. M., and DUFFEY, S. S. 1986. Plant proteinase inhibitors: Mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua. J. Insect Physiol.* 32:827–833.

BROADWAY, R. M., and MISSURELLI, E. L. 1990. Regulatory mechanisms of tryptic inhibitory activity in cabbage plants. *Phytochemistry* 29:3721–3725.

BROADWAY, R. M., DUFFEY, S. S., PEARCE, G., and RYAN, C. A. 1986. Plant proteinase inhibitors: A defense against insects? *Entomol. Exp. Appl.* 41:33–38.

BURDON, J. J. 1987. Diseases and Plant Population Biology. Cambridge University Press, New York.

CIPOLLINI, D. F. 1998. Induced defenses and phenotypic plasticity. *Trends Ecol. Evol.* 13:200.

CIPOLLINI, D. F., and BERGELSON, J. 2000. Environmental and developmental regulation of trypsin inhibitor activity in *Brassica napus J. Chem. Ecol.* 26:1411–1422.

CIPOLLINI, D. F., and REDMAN, A. M. 1999. Age-dependent effects of jasmonic acid treatment and wind exposure on foliar oxidase activity and insect resistance in tomato. *J. Chem. Ecol.* 25:271–281.

CIPOLLINI, D. F., and SCHULTZ, J. C. 1999. Exploring cost constraints on stem elongation in plants using phenotypic manipulation. *Am. Nat.* 153:236–242.

CLAY, K. 1990. The impact of parasitic and mutualistic fungi on competitive interactions among plants, pp. 391–413, *in* J. Grace, and D. Tilman (eds.). Perspectives on Plant Competition. Academic Press, New York.

COLHOUN, J. 1973. Effects of environmental factors on plant disease. *Annu. Rev. Phytopathol.* 11:343–364.

DEWITT, T. J., SIH, A., and WILSON, D. S. 1998. Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* 13:77–81.

DUDLEY, S. A., and SCHMITT, J. 1996. Testing the adaptive plasticity hypothesis: density-dependent selection on manipulated stem length in *Impatiens capensis. Am. Nat.* 147:445–465.

DUKE, J. 1983. Handbook of Energy Crops. Unpublished, Purdue University Library.

GIANOLI, E., and NIEMEYER, H. M. 1996. Environmental effects on the induction of wheat chemical defenses by aphid infestation. *Oecologia* 107:549–552.

GIBSON, D. J., CONNOLLY, J., HARTNETT, D. C., and WEIDENHAMER, J. D. 1999. Designs for greenhouse studies of interactions between plants. *J. Ecol.* 87:1–16.

GRACE, J., and TILMAN, D. 1990. Perspectives on Plant Competition. Academic Press, New York.

GUSTAFSON, G., and RYAN, C. A. 1976. The specificity of protein turnover in tomato leaves: The accumulation of proteinase inhibitors, induced with the wound hormone PIIF. *J. Biol. Chem.* 251:7004–7010.

HJALTEN, J., DANELL, K., and ERICSON, L. 1994. The impact of herbivory and competition on the phenolic concentration and palatability of juvenile birches. *Oikos* 71:416–422.

JOHNSON, R., NARVAEZ, J., AN, G., and RYAN, C. A. 1990. Expression of proteinase inhibitors I and

II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae. *Proc. Natl. Acad. Sci. U.S.A.* 86:9871–9875.

JONGSMA, M. A., BAKER, P. L., and STIEKEMA, W. J. 1993. Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. *Anal. Biochem.* 212:79–84.

JONGSMA, M. A., BAKKER, P. L., VISSER, B., and STIEKEMA, W. J. 1994. Trypsin inhibitor activity in mature tomato and tobacco plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* 195:29–35.

KARBAN, R. 1993. Induced resistance and plant density of a native shrub, *Gossypium thurberi*, affect its herbivores. *Ecology* 74:1–8.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, Illinois.

KARBAN, R., BRODY, A. K., and SCHNATHORST, W. C. 1989. Crowding and a plant's ability to defend itself against herbivores and disease. *Am. Nat.* 134:749–760.

KOIWA, H., BRESSAN, R. A., and HASEGAWA, P. M. 1997. Regulation of proteinase inhibitors and plant defense. *Trends Plant Sci.* 2:379–384.

LEE, T. D., and BAZZAZ, F. A. 1980. Effects of defoliation and competition on growth and reproduction in the annual plant *Abutilon theophrasti. J. Ecol.* 68:813–821.

LENTZ, K., and CIPOLLINI, D. F. 1998. Effect of light and simulated herbivory on growth of the endangered northeastern bulrush, *Scirpus ancistrochateus. Plant Ecol.* 139:125–131.

LYNDS, G. Y., and BALDWIN, I. T. 1998. Fire, nitrogen, and defensive plasticity in *Nicotiana attenuata. Oecologia* 115:531–540.

MCMANUS, M. T., WHITE, D. W. R., and MCGREGOR, P. G. 1994. Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. *Trans. Res.* 3:50–58.

MORRISON, J. A. 1996. Infection of *Juncus dichotomus* by the smut fungus *Cintractia junci:* An experimental field test of the effects of neighboring plants, environment, and host plant genotype. *J. Ecol.* 84:691–702.

READER, R. J. 1992. Herbivory as a confounding factor in an experiment measuring competition among plants. *Ecology* 73:373–376.

STOUT, M. J., BROVONT, R. A., and DUFFEY, S. S. 1998a. Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, *Lycopersicon esculentum. J. Chem. Ecol.* 24:945–963.

STOUT, M. J., WORKMAN, K. V., BOSTOCK, R. M., and DUFFEY, S. S. 1998b. Stimulation and attenuation of induced resistance by elicitors and inhibitors of chemical induction in tomato (*Lycoperiscon esculentum*) foliage. *Entomol. Exp. Appl.* 86:267–279.

THALER, J. S., STOUT, M. J., KARBAN, R., and DUFFEY, S. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* 22:1767–1781.

THALER, J. S., FIDANTSEF, A. L., DUFFEY, S. S., and BOSTOCK, R. M. 1999. Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *J. Chem. Ecol.* 25:1597–1609.

VAN DAM, N. M., and BALDWIN, I. T. 1998. Costs of jasmonate-induced responses in plants competing for limited resources. *Ecol. Lett.* 1:30–33.

ZANGERL, A. R., and BERENBAUM, M. R. 1994/95. Spatial, temporal, and environmental limits on xanthotoxin induction in wild parsnip foliage. *Chemoecology* 5/6:37–42.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

IDENTIFICATION AND EFFECTS OF INTERACTION PHYTOTOXIC COMPOUNDS FROM EXUDATE OF *Cistus ladanifer* LEAVES

N. CHAVES,* T. SOSA, J. C. ALÍAS, and J. C. ESCUDERO

Department of Ecology, Faculty of Science
University of Extremadura
06080 Badajoz, Spain

(Received May 31, 2000; accepted November 11, 2000)

Abstract—Eleven allelochemicals (ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, hydroxycinnamic acid, methyl propionate, oxalic acid, methylmalonic acid, *p*-anisic acid, butyric acid, 3-hydroxybutyric acid, and azulene) were identified in the exudate of *Cistus ladanifer* L. We studied the effect of each on germination, cotyledon emergence, root length, and cotyledon length of *Rumex crispus*. Three groups were distinguished with respect to phytotoxic activity: compounds with low activity (ferulic acid, 4-hydroxybenzoic acid, oxalic acid, methylmalonic acid, *p*-anisic acid, hydroxybutyric acid, and azulene), with intermediate activity (cinnamic acid and hydroxycinnamic acid), and with high activity (methyl propionate and butyric acid). The effect of the interaction of the compounds was studied. When acting conjointly, all combinations tested produced a more negative effect on both germination and seedling growth than when acting alone. The interaction affected cotyledon emergence and root length more negatively than germination and cotyledon length. When hydroxycinnamic acid and cinnamic acid were added to these mixtures there was an enhancement in the phytotoxic activity, accentuating the effect of the other allelochemicals.

Key Words: Allelopathy, allelochemicals, phenolic compounds, *Cistus ladanifer*, *Rumex crispus*.

INTRODUCTION

Understanding the development, evolution, and structure of communities is a major objective of ecology. For plants, the questions are little understood.

*To whom correspondence should be addressed.

0098-0331/00/0300-0611$19.50/0

Among the interactions, it has been suggested that allelopathy, defined as chemical plant–plant interaction whether positive or negative (Rice, 1984), is a major factor in regulating plant community structure (Smith and Martin, 1994). It could be regarded as a competitive phenomenon, and in habitats with limiting environmental conditions, there may be an enhancement of competition in general.

Cistus ladanifer is involved in the reduction of the richness and diversity of herbs in Mediterranean communities where it is present (Malato-Belíz et al., 1992). The cause may be responses to competition for nutrients, chemical interference, litterfall build-up, and penetration of light (Kuuluvaine, 1994). As we have shown in earlier work (Chaves and Escudero, 1997), the exudate of *Cistus ladanifer* leaves has phytotoxic activity that may be a cause of the reduction of herbs growing near this species.

Different types of secondary metabolites act as allelochemicals, among them phenols, terpenes, alkaloids, polyacetylenes, fatty acids, and steroids (Carballeira, 1980; Ballester et al., 1982; Kil and Yim, 1983; 1992; Wacker et al., 1990; Odén et al., 1992; Macías et al., 1993; Li et al., 1993; Gallardo et al., 1998). In many cases, compounds are found together in the growth medium, and their effect on plant development may be the sum of their interactions. Although there is little information on the interaction of phenols or other compounds as to how they affect germination and seedling growth, there is evidence that a mixture of phenols enhances their inhibitory action (Li et al., 1993).

The goal of the present work was to contribute to understanding the role of *Cistus ladanifer* in determining the structure of the communities it dominates. To this end, as we had shown previously that there are compounds in its exudate that act negatively on the germination and subsequent development of herbs (Chaves and Escudero, 1997), the next step was to determine the possible compounds that are involved in this process. In particular, we set out to demonstrate the potential of the identified compounds, determine the concentrations at which they are active, and understand the possible effects deriving from the interactions of the allelochemicals identified in the *Cistus ladanifer* exudate.

METHODS AND MATERIALS

Extraction of Exudate and Identification of Compounds

The exudate was extracted with chloroform. After evaporation, it was redissolved in hot methanol (10 ml/mg extract) (Vogt and Gülz, 1991), and the solution was stored at −20°C for 12 hr, thereby provoking the precipitation of methanol-insoluble compounds, mainly hydrocarbons and waxes. The soluble material was directly chromatographed on Sephadex LH-20 (12.5 g Sephadex LH-20 in a 25-cm-long × 1.5-cm-diam. column), with methanol as eluent. This procedure separates the flavonoids (Chaves et al., 1998) from the rest of the

compounds that are eluted first. It is this fraction that inhibits the germination of the herb species tested (Chaves and Escudero, 1997). On analysis by GC-MS, we identified the following compounds: ferulic acid (**1**), cinnamic acid (**2**), 4-hydroxybenzoic acid (**3**), hydroxycinnamic acid (**4**), methyl propionate (**5**), oxalic acid (**6**), methylmalonic acid (**7**), *p*-anisic acid (**8**), butyric acid (**9**), 3-hydroxybutyric acid (**10**), and azulene (**11**).

Germination Bioassay

Chemically pure reagents were obtained from Sigma Chemical Co. The species used to measure the effect of the compounds was *Rumex crispus* and was chosen because its seed germination and seedling growth are affected negatively when it is watered with the fraction of the exudate from which the compounds have been identified (Chaves and Escudero, 1997). *Rumex crispus* is found in the habitat of *Cistus ladanifer*. The seeds used for the bioassay were gathered in their natural habitat at the end of summer.

We placed 50 seeds (four replicates for a total of 200 seeds) in 9-cm-diam Petri dishes on Whatman No. 118 filter paper. The trial was initiated by adding 5 ml of the test solution or control (Milli Q water). The dishes were placed in a culture chamber with controlled temperature and lighting: 25°C constant temperature, and a 16L : 8D photoperiod. Every two days, 1 ml of the corresponding solutions (test or Milli Q water for the controls) was added to the dishes. Germinated seeds were counted daily (germination was when the seed coat had ruptured and the radicle had emerged). The experiment was continued for 10 days. We also counted cotyledon emergence, and at the end of the experiment measured the cotyledon length and root length of 10 seedlings from each dish (Jaderlund et al., 1996).

Several concentrations were tested for each compound, from the highest concentration of 1 mM down to that where we had observed no effect on any of the parameters. This lowest concentration depended on the compound: it was 0.5 mM for compounds **1**, **6**, **7**, **8**, **10**, and **11**; 0.1 mM for compounds **3** and **4**; 0.05 mM for **2**; and 0.001 mM for **5** and **9**.

The effect of the interaction of the compounds was studied by considering the action that each exerted separately, mixing those that acted negatively on germination, cotyledon emergence, root length, and cotyledon length, as well as a mixture of all compounds together. The seeds were watered with equimolar concentrations of 1 and 0.5 mM of the different mixtures. The following mixtures were tested:

Mixture 1: Compounds that inhibit root length when tested alone (**3**, **8**, **11**). Two solutions were prepared with equimolar concentrations of each of the compounds: 1 mM + 1 mM + 1 mM and 0.5 mM + 0.5 mM + 0.5 mM.

Mixture 2: Compounds of mixture 1 plus hydroxycinnamic acid. Two solu-

tions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 3: Compounds of mixture 1 plus cinnamic acid. Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 4: Compounds of mixture 1 plus hydroxycinnamic and cinnamic acids. Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 5: Compounds that inhibit cotyledon length when tested alone (**1**, **3**, **6**, **7**, **8**, **10**). Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and at 0.5 mM.

Mixture 6: Compounds of mixture 5 plus hydroxycinnamic acid. Two solutions were preared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 7: Compounds of mixture 5 plus cinnamic acid. Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 8: Compounds of mixture 5 plus hydroxycinnamic and cinnamic acids. Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 9: Mixture of hydroxycinnamic and cinnamic acids. Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

Mixture 10: Mixture of all the compounds. Two solutions were prepared with equimolar concentrations of each of the compounds at 1 mM and 0.5 mM.

To eliminate effects of pH and osmotic potential, we measured these parameters for each solution. Osmotic pressure was measured with an HR-33T dewpoint microvoltmeter. The pH varied between 4.7 and 5.2 from one solution to another, and the osmotic potential was low in all solutions, varying between 0.2 and 0.5 bars. There were no significant differences in pH or osmotic pressure among solutions.

The results for germination, cotyledon emergence, inhibition of root length, and inhibition of cotyledon length are expressed as percentages of the control values. The significance level of comparisons among treatments was estimated by using the Mann-Whitney U test. Differences were taken as significant with $P < 0.05$.

RESULTS

Effect of Individual Allelochemicals on Germination, Cotyledon Emergence, and Seedling Growth. For each compound, the lowest concentration tested was

that at which there was no significant ($P > 0.05$) effect on the parameters being studied. Three groups were distinguished with respect to phytotoxic activity: compounds with low activity (**1**, **3**, **6**, **7**, **8**, **10**, and **11**); with intermediate activity (**2** and **4**); and with strong activity (**5** and **9**) (data not shown).

With respect to germination and cotyledon emergence, compounds **1**, **3**, **6**, **7**, **8**, **10**, and **11** showed no significant effect ($P > 0.05$). They affected seedling growth negatively, since they inhibited root length and cotyledon length ($P < 0.05$). Compounds **3**, **8**, and **11** inhibited root length at 1 mM concentration, and compound **11** at 0.5 mM. All compounds, with the exception of **11**, had a negative action on cotyledon length. The most effective were **1** and **6**, which had an inhibitory effect from 0.5 mM. (data not shown).

Compounds **2** and **4** were more active than the previous group. Compound **2** inhibited germination at 1 mM and cotyledon emergence was negatively affected by both: from 0.5 mM in the case of **2**, and only at 1 mM for **4**. For both allelochemicals, we observed an increase in the percentage inhibition of cotyledon emergence with concentration, but only **2** showed a significant positive correlation with concentration ($r^2 = 0.99$). (data not shown).

With respect to root length, compounds **4** and **2** were strong inhibitors at 1 mM, but the effect decreased at lower concentrations. For both compounds, the inhibition of root length was concentration-dependent ($r^2 = 0.98$ and $r^2 = 0.9$ for **4** and **2**, respectively). The cotyledon length was inhibited by **4** at only the highest concentration and down to 0.5 mM for **2**. There was also a concentration-dependent relationship in this case (data not shown).

Both of the most active compounds, **5** and **9**, completely inhibited germination down to 0.05 mM and had a strong effect at 0.01 mM. They were even more effective in inhibiting cotyledon emergence, being active down to 0.001 mM for **5** and 0.01 mM for **9** (data not shown).

The root length was inhibited by **5** at concentrations above 0.01 mM, and above 0.0001 mM for **9**. Likewise, cotyledon length was affected at above 0.001 mM for **9**, and 0.05 mM for **5** (data not shown).

Effect of Interactions of Allelochemicals on Rumex crispus *Germination and Seedling Growth.* The results given in Tables 1–4 (below) show the effect on germination of the conjoint action of the allelochemicals identified. All combinations tested produced a greater inhibition of seedling growth than when acting alone.

Mixture 1, made up of **3**, **8**, **11**, showed a negative effect (at 1 mM) on germination (Table 1), reducing germination by practically 60%. This effect disappeared at 0.5 mM. When hydroxycinnamic acid was added to this mixture, there was an enhancement of the inhibition, and this was enhanced further with the addition of cinnamic acid where the inhibitory action of the other compounds was augmented even at 0.5 mM.

Mixture 2, made up of compounds **1**, **3**, **6**, **7**, **8**, and **10**, non of which inhib-

TABLE 1. EFFECT OF TWO CONCENTRATIONS (1 mM AND 0.5 mM) OF MIX OF ALLELOCHEMICALS IDENTIFIED FROM *C. ladanifer* EXUDATE ON *R. crispus* GERMINATION, EXPRESSED AS PERCENTAGE RELATIVE TO CONTROL

Treatment[a]	Germination (%)	
	1 mM	0.5 mM
1: 4-Hydroxybenzoic acid, P-anisic acid and azulene	40.3	97.3[b]
+ Hydroxycinnamic acid	20.8	74.3
+ Cinnamic acid	3.8	39.4
+ Cinnamic acid + hydroxycinnamic acid	4.5	34.4
2: Ferulic acid, 4-hydroxybenzoic acid, oxalic acid, methylmalonic acid, p-anisic acid and 3-hydroxybutyric acid	10.0	49.7
+ Hydroxycinnamic acid	7.9	38.3
+ Cinnamic acid	0.6	20.5
+ Cinnamic acid + hydroxycinnamic acid	0.0	18.5
3: Cinnamic acid and hydroxycinnamic acid	15.4	72.2
4: Ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, hydroxycinnamic acid, oxalic acid, methylmalonic acid, *p*-anisic acid, 3-hydroxybutyric acid and azulene.	0.6	7.4

[a] 1: Mix of compounds that inhibit root length when tested alone; 2: mix of compounds that inhibit cotyledon length; 3: mix of compounds that inhibit cotyledon birth and (weakly) germination; 4: mix of all compounds.
[b] Not significantly different ($P > 0.05$) from control (Mann-Whitney U test).

ited germination individually, afected germination negatively when they acted jointly (Table 1). As in the previous case, the addition of hydroxycinnamic acid (**4**), enhanced the inhibitory action, reducing germination practically to zero.

Cotyledon emergence (Table 2) was strongly affected by the compounds acting conjointly. The interaction affected cotyledon emergence more negatively than germination. As with germination, the addition of cinnamic acid enhanced the effect, reducing cotyledon emergence practically to zero. At a lower concentration (0.5 mM), the effect was attenuated, except for the mixture of all the allelochemicals.

Root length and cotyledon length were affected differently. Root length was negatively affected by all mixtures (Table 3). At the 1 mM concentration, the reduction was so great that the seedlings were practically nonviable. The cotyledon length, however, as can be seen from Table 4, in those treatments where there was cotyledon emergence, was less negatively affected; mixture 1 (compounds **3**, **8**, and **11**, at 1 mM did not significantly affect this parameter. Hydroxycinnamic acid, when added to mixture 1, greatly enhanced the inhibitory activity (at 1 mM).

TABLE 2. EFFECT OF TWO CONCENTRATIONS (1 mM AND 0.5 mM) OF MIX OF ALLELOCHEMICALS IDENTIFIED FROM *C. ladanifer* EXUDATE ON EMERGENCE OF COTYLEDONS OF *R. crispus*, EXPRESSED AS PERCENTAGE RELATIVE TO CONTROL

Treatment[a]	Cotyledons emergence (%)	
	1 mM	0.5 mM
1: 4-Hydroxybenzoic acid, P-anisic acid and azulene	36.7	76.0
+ Hydroxycinnamic acid	2.1	53.3
+ Cinnamic acid	1.4	24.4
+ Cinnamic acid + hydroxycinnamic acid	0.0	20.5
2: Ferulic acid, 4-hydroxybenzoic acid, oxalic acid, methylmalonic acid, p-anisic acid and 3-hydroxybutyric acid	5.2	39.8
+ Hydroxycinnamic acid	0.0	19.0
+ Cinnamic acid	0.0	6.0
+ Cinnamic acid + hydroxycinnamic acid	0.0	4.9
3: Cinnamic acid and hydroxycinnamic acid	5.3	49.0
4: Ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, hydroxycinnamic acid, oxalic acid, methylmalonic acid, *p*-anisic acid, 3-hydroxybutyric acid and azulene.	0.0	0.0

[a] 1: Mix of compounds that inhibit root length when tested alone; 2: mix of compounds that inhibit cotyledon length; 3: mix of compounds that inhibit cotyledon birth and (weakly) germination; 4: mix of all compounds.

DISCUSSION

In earlier studies (Chaves and Escudero, 1997) we saw the allelopathic potential of the exudate secreted by *cistus ladanifer* leaves. In this work, we tried to understand this species' activity by determining the chemical nature of the compounds and by quantifying their phytotoxic activity. At least 11 compounds were found to possess activity, although at different levels of effectiveness. Two of them, methyl propionate and butyric acid, inhibited germination even at low concentrations. These compounds also are present in the exudate at low concentrations (Chaves et al., unpublished). The remaining compounds mostly affect seedling growth, inhibiting root length and cotyledon length. This effect may have equally negative consequences, since root growth is important for any species to become established under natural conditions.

The concentrations at which these compounds are effective are consistent with the findings of other workers (Kuiters, 1989; Li et al., 1993), who reported that some phenolic acids, such as cinnamic acid, have the same effect and activity on germination and seedling growth. The effective concentration is around 0.1–1 mM in inhibiting seedling growth or germination. Those authors found that

TABLE 3. EFFECT OF TWO CONCENTRATIONS (1 mM AND 0.5 mM) OF MIX OF ALLELOCHEMICALS IDENTIFIED FROM *C. ladanifer* EXUDATE ON *R. crispus* ROOT LENGTH, EXPRESSED AS PERCENTAGE RELATIVE TO CONTROL

Treatment[a]	Root length (%)	
	1 mM	0.5 mM
1: 4-Hydroxybenzoic acid, P-anisic acid and azulene	8.7	79.0
+ Hydroxycinnamic acid	3.9	18.0
+ Cinnamic acid	3.9	12.0
+ Cinnamic acid + hydroxycinnamic acid	3.9	3.9
2: Ferulic acid, 4-hydroxybenzoic acid, oxalic acid, methylmalonic acid, p-anisic acid and 3-hydroxybutyric acid	3.9	10.8
+ Hydroxycinnamic acid	6.8	6.5
+ Cinnamic acid	0.9	3.9
+ Cinnamic acid + hydroxycinnamic acid	0.0	4.9
3: Cinnamic acid and hydroxycinnamic acid	4.6	37.5
4: Ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, hydroxycinnamic acid, oxalic acid, methylmalonic acid, *p*-anisic acid, 3-hydroxybutyric acid and azulene.	1.2	7.5

[a] 1: Mix of compounds that inhibit root length when tested alone; 2: mix of compounds that inhibit cotyledon length; 3: mix of compounds that inhibit cotyledon birth and (weakly) germination; 4: mix of all compounds.

ferulic acid, however, inhibits germination at 1 mM, whereas in our experiment it inhibited seedling growth only, not germination. Blum and Rebbeck (1989) report that while ferulic acid inhibits root length, the roots are thicker, and they suggest that this compound may stimulate the initiation of secondary roots. We did not observe this phenomenon in our trials, nor any stimulatory effect on germination at low concentrations (Milborrow, 1984). The effect of these compounds has been shown to be species-dependent (Haugland and Brandsaeter, 1996), and this effect may be due to the difference in seed size, permeability, differential uptake, and metabolism.

Phenolic acids have been shown to inhibit the length of roots more than shoots (Li et al., 1993). We observed this phenomenon for cinnamic and hydroxycinnamic acids, with there also being a concentration-dependent relationship ($r^2 = 0.98$ and 0.92, respectively). This was not found to be the case, however, with ferulic and 4-hydroxybenzoic acids. Ferulic acid did not inhibit this parameter but did inhibit cotyledon length, and 4-hydroxybenzoic acid, while having an inhibitory effect, had more influence on cotyledon length. In contrast, azulene, which is not a phenolic, had a strong effect on root length but none on cotyledon length.

Compounds that have phytotoxic activity usually derive from various

TABLE 4. EFFECT OF TWO CONCENTRATIONS (1 mM AND 0.5 mM) OF MIX OF ALLELOCHEMICALS IDENTIFIED FROM *C. ladanifer* EXUDATE ON LENGTH OF *R. crispus* COTYLEDONS, EXPRESSED AS PERCENTAGE RELATIVE TO CONTROL

Treatment[a]	Length of cotyledon (%)	
	1 mM	0.5 mM
1: 4-Hydroxybenzoic acid, P-anisic acid and azulene	90.0[b]	92.0[b]
+ Hydroxycinnamic acid	19.0	82.0
+ Cinnamic acid	40.0	61.0
+ Cinnamic acid + hydroxycinnamic acid	0.0	82.0
2: Ferulic acid, 4-hydroxybenzoic acid, oxalic acid, methylmalonic acid, p-anisic acid and 3-hydroxybutyric acid	34.0	102.0[b]
+ Hydroxycinnamic acid	0.0	59.0
+ Cinnamic acid	0.0	52.0
+ Cinnamic acid + hydroxycinnamic acid	0.0	22.0
3: Cinnamic acid and hydroxycinnamic acid	52.8	86.1[b]
4: Ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, hydroxycinnamic acid, oxalic acid, methylmalonic acid, *p*-anisic acid, 3-hydroxybutyric acid and azulene.	0.0	0.0

[a] 1: Mix of compounds that inhibit root length when tested alone; 2: mix of compounds that inhibit cotyledon length; 3: mix of compounds that inhibit cotyledon birth and (weakly) germination; 4: mix of all compounds.
[b] Not significantly different ($P > 0.05$) from control (Mann-Whitney U test).

sources, and it is a complicated task to determine their origin. Their effect, however, is determined by their conjoint action, and, as we found here, this action is more effective than when they act alone. Most previous reports of the interaction among phenols have not been subjected to rigorous examination. The present work has shown that a mixture of compounds, even of those with a weak negative effect, phytotoxic accentuates effects. This effect is enhanced when cinnamic and hydroxycinnamic acids are present. Li et al. (1993) have demonstrated synergy in the action of such phenolic acids as caffeic, ferulic, and cinnamic acids. This synergy implies that a high concentration is probably unnecessary for them to be active. Many of them have been identified in soils at low concentrations (Blum et al., 1989; Lyu et al., 1990; Lehman et al., 1994; Inderjit and Dakshini, 1991, 1992), and synergy may compensate for the low concentrations.

There is a growing understanding of the function and mode of action of these compounds. Neither pH nor osmotic pressure seem to be responsible for the observed negative effects (Rikala and Jozefek, 1990; Nilsson, 1992). Other theories are defended by Pandey (1994), Jaderlund et al. (1996), and Gallardo et al. (1998). For example, plants consume less water in the presence of such compounds, leading to root dysfunction, and the action of phenolic compounds

might be involved in lowering membrane integrity and disrupting enzymatic pathways.

Acknowledgments—The skilled assistance of Dr. J. Luis Rios (Instituto de la Grasa, CSIC, Sevilla, Spain) with the gas-mass spectroscopy measurements is gratefully acknowledged. We are grateful to the anonymous reviewer for contributing to the improvement of our manuscript.

This research was supported by the Ministerio de Educación y Cultura, CICYT project number: PB97-0372.

REFERENCES

BALLESTER, A., VIEITEZ, A. M., and VIEITEZ, E. 1982. Allelopathic potential of *Erica vagans*, *Calluna vulgaris*, and *daboecia cantabrica. J. Chem. Ecol.* 8:851–857.

BLUM, U., and REBBECK, J. 1989. Inhibition and recovery of cucumber roots given multiple treatments of ferulic acid in nutrient culture. *J. Chem. Ecol.* 15:917–928.

BLUM, U., GERIC, T. M., and WEED, S. B. 1989. Effects of mixture of phenolic acids on leaf area expansion of cucumber seedling grown in different pH Portsmouth A1 soil materials. *J. Chem. Ecol.* 15(10):2413–2423.

CARBALLEIRA, A. 1980. Phenolic inhibitors in *Erica australis* L. and in associated soil. *J. Chem. Ecol.* 6(3):593–596.

CHAVES, N., and ESCUDERO, J. C. 1997. Allelopathic effect of *Cistus ladanifer* on seed germination. *Funct. Ecol.* 11:432–440.

CHAVES, N., RÍOS, J. J., GUTIÉRREZ, C., ESCUDERO, J. C., and OLÍAS, J. M. 1998. Analysis of secreted flavonoids of *Cistus ladanifer* L. By high-performance liquid chromatography-particle beam mass spectrometry. *J. Chromatogr. A.* 799:111–115.

GALLARDO, T. M., MARTÍN, B. B., and MARTÍN, D. F. 1998. Inhibition of water fern *Salvinia minima* by cattail (*Typha domingensis*) extracts and by 2-chlorophenol and salicylaldehyde. *J. Chem. Ecol.* 24(9):1483–1490.

HAUGLAND, E., and BRANDSAETER, L. O. 1996. Experiments on bioassay sensitivity in the study of allelopathy. *J. Chem. Ecol.* 22(10):1845–1859.

INDERJIT, and DAKSHINI, K. M. M. 1991. Hesperetin 7-rutinoside (hesperidin) and taxifolin 3-arabinoside as germination and growth inhibitors in the soils associated with the weed, *Pluchea lanceolata* (DC) C. B. Clarke (Asteraceae). *J. Chem. Ecol.* 17:1585–1591.

INDERJIT, and DAKSHINI, K. M. M. 1992. Formononetin 7-*O*-glucoside, an additional inhibitor from the soil associated with the weed, *Pluchea lanceolata* (DC) C.B. Clarke (Asteraceae). *J. Chem. Ecol.* 18:713–718.

JADERLUND, A., ZACKRISSON, O., and NILLSSON, M. C. 1996. Effects of bilberry (*Vaccinium myrtillus* L.) litter on seed germination and early seedling growth of four boreal tree species. *J. Chem. Ecol.* 22(5):973–986.

KIL, B. S., and YIM, Y. J. 1983. Allelopathic effects of *Pinus densiflora* on undergrowth of red pin forest. *J. Chem. Ecol.* 9(8):1135–1151.

KIL, B. S., and YUN, K. W. 1992. Allelopathic effects of water extracts of *Artemisia princeps* var. *orientalis* on selected plant species. *J. Chem. Ecol.* 18(1):39–51.

KUITERS, A. T. 1989. Effects of phenolic acids on germination and early growth of herbaceous woodland plants. *J. Chem. Ecol.* 15:467–479.

KUULUVAINE, T. 1994. Gap disturbance, ground microtopography, and the regeneration dynamics of boreal coniferous forest in Finland: A review. *Ann. Zool. Fenn.* 31:35–51.

LEHMAN, M. E., BLUM, U., and GERIG, T. M. 1994. Simultaneous effects of ferulic and *p*-coumaric acids on cucumber leaf expansion in split-root experiments. *J. Chem. Ecol.* 20:1773–1782.

LI, J., INOUE, M., NISHIMURA, H., MIZUTANI, J., and TSUZUKI, E. 1993. Interactions of *trans*-cinnamic acid, its related phenolic allelochemicals, and abscisic acid in seedling growth and seed germination of lettuce. *J. Chem. Ecol.* 19(8):1775–1787.

LYU, S. W., BLUM, U., GERIC, T. U., and O'BRIEN, T. E. 1990. Effects of mixtures of phenolic acid on phosphorus uptake by cucumber seedlings. *J. Chem. Ecol.* 16(8):2559–2567.

MACÍAS, F. A., GALINDO, J. C. G., MASSANET, J. M., RODRIGUEZ-LUIS, F., and ZUBIA, E. 1993. Allelochemicals form *Pilocarpus goudotianus* leaves. *J. Chem. Ecol.* 19(7):1371–1379.

MALATO-BELÍZ, J. ESCUDERO, J. C., and BUYOLO, T. 1992. Application of traditional indices and of diversity to an ecotonal area of different biocoenes. The State of the Art in Vegetatio Science. International Association for Vegetation Science (IAVS), Toledo, Spain. Abstracts, 75 pp.

MILBORROW, B. V. 1984. Inhibitors, pp. 77–110, *in* M. B. Wilkins (ed.). Advanced Plant Physiology. Pitman Publishing, London.

NILSSON, M.-C. 1992. The mechanisms of biologicla interference by *Empetrum hermaphroditum* on tree seedling establishment in boreal forest ecosystem. PhD thesis. Swedish University of Agricultural Sciences, Umeå, Sweden. ISBN 91-576-4640-6.

ODÉN, P. C., BRANDTBERG, P. O., ANDERSON, R. GREG, R., ZACKRISSON, O., and NILSSON, M. C. 1992. Isolation and characterization of a germination inhibition from leaves of *empetrum hermaphroditum* Hagerup. *Scand. J. For. Res.* 7:497–502.

PANDEY, D. K. 1994. Inhibition of salvinia (*Salvinia molesta* Mitchell) by parthenium (*Parthenium hysterophorus* L.). I. Effect of leaf residue and allelochemicals. *J. Chem. Ecol.* 20:3111–3122.

RICE, E. L. 1984. Allelopathy. Academic Press, Orlando, Florida.

RIKALA, R., and JOZEFEK, H. J. 1990. Effects of dolomite lime and wood ash on peat substrate and development of tree seedlings. *Silvia Fenn.* 24(4):323–334.

SMITH, A. E., and MARTIN, L. D. 1994. Allelopathic characteristics of 3 cool-season grass species in the forage ecosystem. *Agron. J.* 86:243–246.

VOGT, T., and GÜLZ, P. G. 1991. Isocratic column liquid chromatographic separation of a complex mixture of epicuticular flavonoid aglycones and intracellelar flavonol glycosides from *Cistus laurifolius* L. *J. Chromatogr.* 537:453–459.

WACKER, T. L., SAFIR, G. R., and STEPHENS, C. T. 1990. Effects of ferulic acid on *Glomus fasciculatum* and associated effects on phosphorus uptake an growth of asparagus (*Asparagus officinalis* L.). *J. Chem. Ecol.* 16:901–909.

Journal of Chemical Ecology, Vol. 27, No. 3, 2001

PLANT GROWTH INHIBITING FLAVONOIDS IN EXUDATE OF *Cistus ladanifer* AND IN ASSOCIATED SOILS

N. CHAVES,* T. SOSA, and J. C. ESCUDERO

Department of Ecology, Faculty of Science
University of Extremadura
06080 Badajoz, Spain

(Received April 28, 2000; accepted November 5, 2000)

Abstract—Of the aglycone flavonoids identified in the exudate of *cistus ladanifer*, two, the flavone apigenin-4′-(*O*)-methyl and the flavonol kaempferol-3,7-di(*O*)-methyl inhibit development of the seedlings of *Rumex crispus* at 0.5 and 1 mM. Additive effects were observed between the major flavonols of the exudate kaempferol-3-(*O*)-methyl and kaempferol-3,7-di-(*O*)-methyl in inhibiting the size of the cotyledons and delaying the germination and cotyledon emergence. The presence of apigenin-4′-(*O*)-methyl, kaempferol-3,7-di-(*O*)-methyl, and kaempferol-3-(*O*)-methyl was detected in the soils associated with *C. ladanifer* during the summer and autumn months. That these compounds are present in the soil and are not restricted to the leaves provides support for the hypothesis that *C. ladanifer* has allelopathic potential.

Key Words: Allelochemicals, allelopathic effect, phenolic compounds.

INTRODUCTION

C. ladanifer is a species that secretes an exudate rich in phenolics. The flavonoids show a marked qualitative and quantitative seasonal variation depending on factors such as moisture stress, ultraviolet radiation, and high temperatures (Chaves et al., 1993, 1997). The exudate becomes enriched in flavonoids during summer, when the flavonols (kaempferols) are more prevalent than the flavones (apigenins). Given the phytotoxicity of *C. ladanifer* that was demonstrated in earlier studies (Chaves and Escudero, 1997) and that some of the compounds responsible for the effect form part of the mixture of aglycone flavonoids extracted from its leaf exudate, the aim of the present study was to gain deeper knowledge of

* To whom correspondence should be addressed. e-mail: natchalo@unex.es

0098-0331/00/0300-0623$19.50/0

the potential allelopathic activity presented by this species. We set two objectives: (1) to determine which of the aglycone flavonoids identified in *C. ladanifer* exudate might be responsible for the phytotoxic effect that was demonstrated in earlier studies, by using *R. crispus* (an herb that is not found in the presence of *C. ladanifer*) as a test bed; and (2) to check for the presence of these compounds in the soil and determine at which times of ear they are present, to see whether the compounds may be able to exert their effect in their natural setting.

METHODS AND MATERIALS

Sample Collection. *C. ladanifer* leaves for flavonoid extraction were gathered in spring and summer (young leaves). Samples were taken in these two seasons because of the qualitative and quantitative seasonal variation in leaf flavonoid content (Chaves et al., 1993, 1997); a greater amount of flavones are extracted in spring, and flavonols in summer.

The *Rumex crispus* seeds used for the germination trials were collected at the end of the summer from meadows in its natural habitat. This species was chosen because its seed and seedling growth are affected negatively when they are watered with the fraction of exudate that contains flavonoids. (Chaves and Escudero, 1997).

After first having cleared the existing herb vegetation, soil samples were collected from the surface (less than 5 cm), this being the depth to which herb seeds are usually found. Each month, three soil samples were taken from beneath *C. ladanifer* plants, and another two samples as controls away from the influence of this plant. The sampling months were from April (when the secretion of flavonoids begins in the leaves) to November (when the secretion stabilizes).

Extraction of Flavonoids. The flavonoids identified in *C. ladanifer* (Chaves et al., 1998) are not commercially available and therefore were extracted from the exudate. The exudate was extracted with chloroform. For separation of the flavonoids, the extract was dissolved in hot methanol (10 ml/mg extract) (Vogt and Gülz, 1991), and the solution stored at −20°C for 12 hr, provoking the precipitation of methanol-insoluble compounds, mainly hydrocarbons and waxes. The flavonoid-containing supernatant was directly chromatographed on Sephadex LH-20 (12.5 g Sephadex LH-20) on a 25-cm-long × 1.5-cm-diam. column) with methanol as the eluent. With this procedure, the flaonoids are separated from the terpenoids and other phenolics that elute first. Three extracts were obtained, and these were subsequently analyzed by HPLC to determine the fraction containing the flavonoids.

Separation and Purification of Exudate Flavonoids by HPLC. The flavonoids were separated by using a semipreparative Nucleosil 5μ C-18 (250 × 10 mm) column and a water–methanol–acetonitrile–tetrahydrofurane

(56 : 16 : 6 : 22) solution at a flow rate of 1.75 ml/min. They were detected with a diode array at 350 nm wavelength. As the peak of each flavonoid was detected, it was collected in a separate tube. To eliminate any possible contamination from other compounds eluting close to the flavonoid being purified, the fraction was again separated by HPLC with a methanol–water (80 : 20) solvent at a 2.5 ml/min flow rate.

The flavonoids purified from the extract were: kaempferol-3-(*O*)-methyl (K3), kaempferol-3,7-di-(*O*)-methyl (K3,7), apigenin-4′-(*O*)-methyl (Ap4′) and apigenin-7-(*O*)-methyl (Ap7). Apigenin (Ap) was commercially available and was purchased from Aldrich-Quimica.

Extraction of Flavonoids from Soil. The soil samples were air-dried and stored in bags in darkness until analysis. Twenty-five grams of soil of each sample were extrated with 40 ml of methanol (Inderjit and Dakshini 1996b). The mixture was vortexed for 2 hr and then filtered through Whatman filter paper. The filtrate was evaporated and redissolved in 1 ml methanol.

HPLC Identification of Soil Flavonoids. The solution of soil extract was assayed by HPLC. Aliquots of 200 μl were injected into a Nucleosil 5μ C-18 (15 $\times$ 4.5 mm) analytical column with a water–methanol–acetonitle–tetrahydrofurane (56 : 16 : 6 : 22) solution at a flow rate of 0.7 ml/min. Flavonoids were detected with a diode array at a wavelength of 350 nm. The templates used to quantify the soil flavonoids were the purified flavonoids from the exudate.

Seed Germination. Rumex crispus seeds were watered with different concentrations of each of the flavonoids (Ap, Ap7, Ap4′, K3 and K3,7). The highest concentration was 1 mM and the lowest 0.1 mM (recommended effective concentration) (Williams and Hoagland, 1982; Li et al., 1993; Inderjit and Dakshini, 1995). We also performed experiments with the two major flavonoids, K3 and K3,7, together in order to analyze the interaction between these two flavonols. Measurements of pH were made to eliminate the possibility that these might influence observed trends. The pH of the extracts was 6.8 $\pm$ 0.2.

For each trial, 50 seeds (four replicates, 200 seeds in total) were placed in Petri dishes on Whatman No. 118 filter paper and to each dish, 5 ml of the corresponding extract was added. Control dishes were watered with Milli Q water. Dishes were placed in a culture chamber with controlled temperature and lighting: 16L : 8D at a constant temperature of 25°C. The corresponding solutions (extract or distilled water) were resupplied every two days. Germinated seeds were counted daily (germination being taken to have occurred when the testa had ruptured with the consequent appearance of the radicle). The experiment was continued for 10 days. Cotyledon emergence was also recorded, and cotyledon size (length) and root length were determined at the end of the experiment by measuring 10 seedlings (at random) for each dish (Jaderlund et al., 1996).

The results for root length inhibition and cotyledon size inhibition were expressed as percentages with respect to the controls:

$$\text{Root length inhibition (\%)} = \left(\frac{\text{root length with treatment}}{\text{root length control}} \right) \times 100$$

the lag in germination and cotyledon emergence was quantified by using the Weibull function (Brown and Mayer, 1988). The form of the fitted Weibull function is: $y = M\{1-\exp[-K(t-z)]^c\}$; where y is the cumulative germination at time t, and M, k, z, and c are empirically derived constants: M is the asymptote, k the rate of increase, z the lag in germination, and c is a shape parameter. The value of k, which represents the germination rate for each treatment, was estimated with this function. The significance level of comparisons between treatments was estimated by using the Mann-Whitney U test. Differences were taken as significant with $P < 0.05$.

RESULTS

Seedling Germination and Growth. The germination and cotyledon emergence percentages were not affected by any of the compounds ($P > 0.05$). The root length (Table 1), was significantly inhibited ($P < 0.05$) only by the 1 and 0.5 mM concentrations of Ap4′ and K3,7 and the K3/K3,7 mixture. In the case of K3,7, inhibition was concentration dependent ($r^2 = 0.99$). No concentration dependence was detected for Ap4′ or for the K3/K3,7 mixture. The K3,7 activity at 0.5 mM was comparable to that of the flavone.

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF FLAVONOIDS ISOLATED FROM *C. ladanifer* EXUDATE ON *R. crispus* ROOT LENGTH AND COTYLEDON SIZE[a]

	Concentration					
	Root length			Cotyledon size		
	1 mM	0.5 mM	0.1 mM	1 mM	0.5 mM	0.1 mM
Apigenin	94.57	93.47	95.62	101.05	100.9	103.4
Apigenin 4′	76.28b	71.37b	97.07	103.7	110.38	103.4
Apigenin 7	101.19	91.21	90.69	81.2	88.4	113.5
Kaempferol 3	98.9	99.04	88.39	97.26	96.75	113.5
Kaempferol 3.7	19.26b	72.39b	103.94	95.04	94.13	105.9
Kaempferol 3–3.7	69.54b	75.8b		78.57b	78.85b	

[a] Expressed as percentage relative to the control. Apigenin 4′: apigenin-4-(*O*)-methyl; apigenin 7: apigenin-7-(*O*)-methyl; kaempferol 3: kaempferol-3-(*O*)-methyl; kaempferol 3.7: kaempferol-3,7-di-(*O*)-methyl; kaempferol 3–3.7: kaempferol-3-(*O*)-methyl and kaempferol-3,7-di-(*O*)-methyl.
[b] Significantly different $P < 0.05$ from controls (Mann-Whitney U test).

TABLE 2. EFFECT OF DIFFERENT CONCENTRATIONS OF FLAVONOIDS ISOLATED FROM *C. ladanifer* EXUDATE ON RATE OF GERMINATION AND COTYLEDON EMERGENCE OF *Rumex crispus*[a]

	Concentration					
	Root of germination			Rate of cotyledon emergence		
	1 mM	0.5 mM	0.1 mM	1 mM	0.5 mM	0.1 mM
Control	0.72			0.35		
Apigenin	0.71	0.73	0.69	0.34	0.33	0.34
Apigenin 4′	0.03b	0.72	0.68	0.0008b	0.3	0.32
Apigenin 7	0.75	0.65	0.71	0.33	0.52	0.36
Kaempferol 3	0.78	0.94	0.75	0.37	0.45	0.39
Kaempferol 3.7	0.98	0.86	0.76	0.51	0.5	0.42
Kaempferol 3–3.7	0.43b	0.83		0.0002b	0.0005b	

[a] The value expressed as germination rate is the estimated value of the parameter k in the Weibull function. (see Methods and Materials). Apigenin 4′: apigenin-4-(*O*)-methyl; apigenin 7: apigenin-7-(*O*)-methyl; kaempferol 3: kaempferol-3-(*O*)-methyl; kaempferol 3.7: kaempferol-3,7-di-(*O*)-methyl; kaempferol 3–3.7: kaempferol-3-(*O*)-methyl and kaempferol-3,7-di-(*O*)-methyl.
[b] Significantly different $P < 0.05$ from controls (Mann-Whitney U test).

With respect to cotyledon size (Table 1), there was no effect of any of the flavonoids alone, but the cotyledons were smaller when K3 and K3,7 acted together. The interaction between these two flavonols (which are the major flavonoids in *C. ladanifer* exudate) had different effects on root length and cotyledon size: their, joint action had a smaller effect on root length than did K3,7 alone, but was more effective in inhibiting cotyledon size ($P < 0.05$) than either flavonoid alone, where no inhibition was seen.

Delays in Germination and Cotyledon Emergence. Table 2 gives the rates of germination and cotyledon emergence for each flavonoid treatment. These indices quantify the effect on delaying germination and cotyledon emergence and are useful tools in understanding the degree to which development and the competitive possibilities of seedlings were affected. At 1 mM concentration, Ap4′ delayed germination and cotyledon emergence. The mixture of the two flavonols induced delay in germination rate at the 1 mM concentration and a notable delay in cotyledon emergence at 1 and 0.5 mM. Although the total germination percentage was unaffected, Ap4′ and K3/K3,7 had a clear effect on lengthening the time required for germination and cotyledon emergence.

Flavonoids in Soil. The flavonoids K3, K3,7, and Ap4′ were detected in the extract of soils associated with *C. ladanifer*. Table 3 lists the amounts of each flavonoid extracted from both the jaral soils and the control soils for each month of the sampling period.

One can see that in the controls (soils which were adjacent to the jaral) no

TABLE 3. LEVELS OF FLAVONOIDS DETECTED IN SOILS IN PRESENCE OF *C. ladanifer* AND IN ADJACENT CONTROL SOILS IN SAMPLING MONTHS (APRIL TO NOVEMBER)[a]

	Spring		Summer			Autumn		
	April	May	June	July	August	September	October	November
Control 1								
K3	ND	ND	ND	ND	ND	ND	ND	ND
K3,7	ND	ND	ND	ND	ND	ND	ND	ND
Ap4′	ND	ND	ND	ND	ND	ND	ND	ND
Control 2								
K3	ND	ND	ND	ND	ND	ND	ND	ND
K3,7	ND	ND	ND	ND	ND	ND	ND	ND
Ap4′	ND	ND	ND	ND	ND	ND	ND	ND
Soil jara 1								
K3	ND	ND	2.9	2.6	0.2	5.3	4.8	ND
K3,7	ND	ND	5	ND	0.9	12	10	ND
Ap4′	ND	ND	ND	ND	ND	ND	ND	ND
Soil jara 2								
K3	ND	ND	1.3	3.7	4.8	24	ND	0.26
K3,7	ND	ND	4	ND	8	60	18	0.8
Ap4′	ND	ND	ND	ND	ND	6	ND	ND
Soil jara 3								
K3	ND	ND	9.6	3.5	1.3	8	4.8	0.26
K3,7	ND	ND	7.9	ND	2	22	10	6
Ap4′	ND	ND	8.5	ND	ND	ND	ND	ND
Average soils jaras								
K3			4.6	3.15	2.1	12.4	3.2	0.26
K3,7			29	ND	3.6	31.3	10	3.4
Ap4′			8.5	ND	ND	6	ND	ND

[a] Values are expressed in mg/g dw $\times 10^{-5}$. ND: not detected. K3: kaempferol-3-(*O*)-methyl; K3,7: kaempferol-3,7-di-(*O*)-methyl; Ap4′: apigenin-4′-(*O*)-methyl.

flavonoids were detected in any of the sampling months and also that the aglycone flavonoids secreted by the *C. ladanifer* leaves were the only three detected in the soil (Ap4′, K3, and K3,7).

No flavonoids were found in the *C. ladanifer* soils in the spring months (April and May), their presence began to be detectable in the summer (June). The greatest amounts were obtained from the September soil samples. One observes that there is great variability among sampling points (different soil samples) in a given month, and an even greater temporal variability: e.g., in June, K3 ranged between 1.3 and 9.6 mg/g dry wt $\times 10^{-5}$ and K3,7 between 4 and 79 mg/g dry wt $\times 10^{-5}$, and in September the ranges were from 5.3 to 24 mg/g dry wt $\times 10^{-5}$ for K3 and from 12 to 60 mg/g dry wt $\times 10^{-5}$ for K3,7. While this variability is patent, a common pattern is observed in the greater amount

of K3.7 for all sampling points in all months except July, when there was no K3,7 detected in any of the samples. It is noteworthy that Ap4′ was detected in only June and September, and then only in one sample, that which contained the greatest quantity of flavonoids. The amount of flavonoids declined considerably in October and November, being undetectable at some sampling sites.

The variability among sampling points and the temporal variation highlight the lack of homogeneity in the distribution of these compounds in the soil and the need for further work to determine their degradation and residence times. The results show that the presence of flavonoids with phytotoxic activity is not restricted to the leaves, but that they can also be detected in the soils, especially those with the greatest phytotoxic activity.

DISCUSSION

The present results provide evidence that the two flavonols (K3 and K3,7) and the flavone (Ap4′), the three aglycone flavonoids identified in the *C. ladanifer* exudate (Chaves et al., 1998), are those with the greatest phytotoxic activity. The activity is not directly related to inhibition of germination but is reflected in the effect on root length, cotyledon size, and the delay in rate of germination and of cotyledon emergence. A delay in germination rate and radicle growth will be important in the establishment of any species under natural conditions. The plant will have less opportunity to establish itself since it will be unable to take advantage of favorable conditions such as the first rains, and the roots may find it more difficult to penetrate the soil. This process is even more important in nutrient-poor ecosystems, such as those in which *C. ladanifer* grows, where competition is greater and the lack of nutrients may increase susceptibility to water deficit. The results are consistent with those of earlier studies, which have demonstrated the influence of other flavonoids on the size and development of roots and cotyledons (Inderjit and Dakshini, 1991, 1992, 1995) and the concentrations that are effective (1, 0.5, and 0.1 mM). The effect of the flavonoids on different physiological parameters is unclear and, therefore, so is the mode of action.

Trials performed by Jaderlund et al. (1996) suggest that the compounds that negatively affect germination and seedling development are water soluble and pass into the soil with the rain or the first stages of litter decomposition. The absorption and retention of allelochemicals in the soil has been documented (Huang et al., 1999), and therefore one must check for presence of these compounds there. The present work has demonstrated the presence of the flavonoids K3,7, K3, and Ap4′ in the soils of a jaral, mainly during the months when many species establish themselves. While it is essential to have data on the persistence and residence in soils in order to determine with precision the allelopathic activ-

ity of any given species, the presence and availability of these flavonoids in the soil, detected here for the first time, reaffirms the allelopathic likelihood of *C. ladanifer*.

In previous work, we have quantified the variation of these flavonoids in *C. ladanifer* leaves, as well as the factors that induce their synthesis (Chaves and Escudero, 1999; Chaves et al., 1993, 1997). We found a marked seasonal variation. The greatest flavonoid concentration in the leaf is in summer (specifically, in August). The route by which they enter the soil has not been established, but it could well be by rain (Inderjit and Dakshini, 1996a). This would explain the peak presence of K3 and K3,7 in September, since in the exudate, the greatest concentration is found in August: in this month, there may be up to 25 mg/g dry wt of flavonoids (Chaves et al., 1997) with these two flavonols representing about 70% of the total flavonoids. Although the summer is dry, often at the end of the season there are storms that may be sufficient to wash the flavonoids into the soil.

It is necessary to emphasize the variability in the amounts detected at different sampling points and different months. Studies by other authors (Inderjit and Dakshini, 1996b; Inderjit, 1998) have shown the variation in phenolic content of soils with respect to season and sampling site. For instance, phloroglucinol was found to vary from one site to another from 5×10^{-3} M to 10^{-6} M in the field (Inderjit and Dakshini, 1996b). It is no easy matter to determine the precise bioactive concentration of these compounds in soils under natural conditions, since they may decompose, be absorbed by seeds and roots, be leached, be fixed by other soil compounds, and be exposed to microbial activity (Dalton et al., 1983; Rice 1984; Blum and Shafer, 1988; Cheng, 1995). The concentration at which flavonoids are demonstrated to be active may possibly be greater than the concentration at which they are detected in the soil. A very high concentration in the soil is probably not needed for them to exert their allelopathic effect, however, since the compounds are continually being incorporated into the soil whenever it rains or leaves fall, an effect which may be even more important when the plant is a perennial such as *C. ladanifer*.

Acknowledgments—This research was supported by the Ministerio de Educación y Cultura, project of the CICYT number: PB97-0372. We are grateful to the anonymous reviewers for contributing to the improvement of our manuscript.

REFERENCES

BLUM, U., and SHAFER, S. R. 1988. Microbial populations and phenolic acids in soil. *Soil Biol. Biochem.* 20:793–800.

BROWN, R. F., and MAYER, D. G. 1988. Representing cumulative germination. 2. The use of the Weibull function and other empirically derived curves. *Ann. Bot.* 61:127–138.

CHAVES, N., and ESCUDERO, J. C. 1997. Allelopathic effect of *Cistus ladanifer* on seed germination. *Funct. Ecol.* 11:432–440.

CHAVES, N., and ESCUDERO, J. C. 1999. Variation of flavonoid synthesis induced by ecological factors, pp. 267–285, *in* Inderjit, K. M. N. Dakshini, and F. L. Chester (eds.). Principles and Practices in Plant Ecology, Allelochemical Interactions. CRC Press, Boca Raton, Florida.

CHAVES, N., ESCUDERO, J. C., and GUTIÉRREZ-MERINO, C. 1993. Seasonal variation of exudate of *Cistus ladanifer. J. Chem. Ecol.* 19(11):2577–2591.

CHAVES, N., ESCUDERO, J. C., and GUTIÉRREZ-MERINO, C. 1997. Role of ecological variables in the seasonal variation of flavonoid content of *Cistus ladanifer* exudate. *J. Chem. Ecol.* 23(3):579–603.

CHAVES, N., RÍOS, J. J., GUTIÉRREZ, C., ESCUDERO, J. C., and OLÍAS, J. M. 1998. Analysis of secreted flavonoids of *Cistus ladanifer* L. by high-performance liquid chromatography-particle beam mass spectrometry. *J. Chromatogr. A.* 799:111–115.

CHENG, H. H. 1995. Characterization of the mechanisms of allelopathy: Modeling and experimental approaches, pp. 132–141, *in* Inderjit, K. M. N. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications. American Chemical Society, Washington, D.C.

DALTON, B. R., BLUM, U., and WEED, S. B. 1983. Allelopathic substances in ecosystems: Effectiveness of sterile soil components in altering recovery of ferulic acid. *J. Chem. Ecol.* 9:1185–1201.

HUANG, P. M., WANG, M. C., and WANG, M. K. 1999. Catalytic transformation of phenolic compounds in the soils, pp. 287–306, *in* Inderjit, K. M. N. Dakshini, and F. L. Chester (eds.). Principles and Practices in Plant Ecology, Allelochemical Interactions. CRC Press, Boca Raton, Florida.

INDERJIT 1998. Influence of *Pluchea lanceolata* (Asteraceae) on selected soil properties. *Am. J. Bot.* 85(1):64–69.

INDERJIT, and DAKSHINI, K. M. M. 1991. Hesperetin 7-rutinoside (hesperidin) and taxifolin 3-arabinoside as germination and growth inhibitors in the soils associated with the weed, *Pluchea lanceolata* (DC) C.B. Clarke (Asteraceae). *J. Chem. Ecol.* 17:1585–1591.

INDERJIT, and DAKSHINI, K. M. M. 1992. Formononetin 7-*O*-glucoside, an additional inhibitor from the soil associated with the weed, *Pluchea lanceolata* (DC) C.B. Clarke (Asteraceae). *J. Chem. Ecol.* 18:713–718.

INDERJIT, and DAKSHINI, K. M. M. 1995. Quercetin and quercetrin from *:luchea lanceolata* and their effect on growth of asparagus bean, *in* pp. 86–95, Inderjit, K. M. N. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications. American Chemical Society, Washington, D.C.

INDERJIT, and DAKSHINI, K. M. M. 1996a. Allelopathic potential of well water from *Pluchea lanceolata*-infested cultivated fields. *J. Chem. Ecol.* 22(6):1123–1131.

INDERJIT, and DAKSHINI, K. M. M. 1996b. Allelopathic potential of *Pluchea lanceolata:* Comparative study of cultivated fields. *Weed Sci.* 44:393–396.

JADERLUND, A., ZACKRISSON, O., and NILSSON, M. C. 1996. Effects of bilberry (*Vaccinium myrtillus* L.) litter on seed germination and early seedling growth of four boreal tree species. *J. Chem. Ecol.* 22(5):973–986.

LI, J., INOUE, M., NISHIMURA, H., MIZUTANI, J., and TSUZUKI, E. 1993. Interactions of trans-cinnamic acid, its related phenolic allelochemicals, and abscisic acid in seedling growth and seed germination of lettuce. *J. Chem. Ecol.* 19(8):1775–1787.

RICE, E. L. 1984. Allelopathy. Academic Press, Orlando, Florida.

VOGT, T., and GÜLZ, P. G. 1991. Isocratic column liquid chromatographic separation of a complex mixture of epicuticular flavonoid aglycones and intracellular flavonol glycosides from *Cistus laurifolius* L. *J. Chromatogr.* 537:453–459.

WILLIAMS, R. D., and HOAGLAND, R. E. 1982. The effects of naturally occurring phenolic compounds on seed germination. *Weed Sci.* 30:206–212.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

KAIROMONAL RESPONSE BY FOUR *Monochamus* SPECIES (COLEOPTERA: CERAMBYCIDAE) TO BARK BEETLE PHEROMONES

JEREMY D. ALLISON,[1,*] JOHN H. BORDEN,[1] RORY L. MCINTOSH,[1,3] PETER DE GROOT,[2] and REGINE GRIES[1]

[1]*Centre for Environmental Biology*
Department of Biological Sciences
Simon Fraser University
Burnaby, British Columbia, V5A 1S6, Canada

[2]*Great Lakes Forestry Centre*
Canadian Forest Service
Sault Ste. Marie, Ontario, P6A 5M7, Canada

(Received August 16, 2000; accepted December 13, 2000)

Abstract—We investigated the hypothesis that wood-boring beetles in the genus *Monochamus* (Cerambycidae) utilize pheromones of sympatric bark beetles as host-finding kairomones. All nine bark beetle pheromones tested electrophysiologically were antenally active for both sexes of *M. scutellatus, M. clamator*, and *M. obtusus* from British Columbia. When field-tested with multiple-funnel traps (British Columbia) or cross-vane traps (Ontario), a blend composed of frontalin, ipsdienol, ipsenol, and MCH, in combination with a blend of host volatiles attracted significant numbers of *M. clamator, M. obtusus, M. notatus*, and *M. scutellatus* to baited traps. Traps baited with host volatiles in combination with a second blend composed of *endo*-brevicomin, *exo*-brevicomin, *cis*-verbenol, *trans*-verbenol, and verbenone caught no more beetles than unbaited traps or traps baited with the host blend alone. In British Columbia, traps baited with the first blend alone or both blends together captured more *M. scutellatus* and *M. clamator* than unbaited traps, demonstrating a response to bark beetle pheromones in the absence of host volatiles. These results suggest that *Monochamus* spp. are minimizing foraging costs by using the pheromones of sympatric bark beetles as kairomones.

Key Words—Coleoptera, Scolytidae, Cerambycidae, Buprestidae, *Monochamus*, chemical ecology, allelochemics, kairomones, coevolution.

*To whom correspondence should be addressed.
[3]Current address: Forest Ecosystems Branch, Saskatchewan Environment & Resource Management, Box 3003, Prince Albert, Saskatchewan S6V 6G1, Canada.

0098-0331/01/0400-0633$19.50/0

INTRODUCTION

Most wood-boring insects reproduce in stressed, dying, or dead trees that are usually randomly distributed in space and time (Schroeder, 1992). The larvae feed under the bark in the phloem tissue, in the sapwood, and sometimes deep into the heartwood (Linsley, 1961), often boring long tunnels that weaken and degrade the wood and provide infection courts for wood-rotting fungi. Wood-boring beetles have caused economic losses as high as 30% in British Columbia log yards (Safranyik and Raske, 1970). A southern interior British Columbia mill, which converts 700,000 m^3 of coniferous timber into lumber annually, could expect annual degrade losses from all wood-borers to total US $1.8–4.8 million (Phero Tech Inc. 7572 Progress Way, RR#5, Delta, British Columbia. V4G 1E9, Canada. Damage Assessment of Woodborers in the Interior of British Columbia. Unpublished report. 1997). If these values were extrapolated to encompass all interior mills, annual losses would be US $293 million, $43.6 million of which would be attributable to large wood-borers. Wood-borers in the genus *Monochamus* are of particular economic significance, because in addition to causing significant physical damage (Parmelee, 1941; Gardiner, 1957, 1975), they are vectors of the pinewood nematode, *Bursaphelenchus xylophilus* (Vallentgoed, 1991).

Coniferophagous wood-borers use olfactory stimuli to locate hosts (Linsley, 1961), and many are attracted to host monoterpenes and ethanol (Chénier and Philogène, 1989). Consequently, commercial wood-borer baits consist of host monoterpenes (usually α-pinene) and ethanol to simulate the odor of a stressed or dying tree. Current understanding of the cues involved in host selection by wood-borers is incomplete.

There is considerable overlap in pheromone components among sympatric scolytid beetles (Borden, 1982), facilitating kairomonal responses by entomophagous insects to trees or logs infested by bark beetles of numerous species (Vité and Williamson, 1970; Dixon and Payne, 1979; Bedard et al., 1980; Bakke and Kvamme, 1981; Raffa and Klepzig, 1989). The association between bark beetles and wood-boring beetles has long been recognized but has only been formally tested twice (Billings and Cameron, 1984; Billings, 1985). A kairomonal response to bark beetle pheromones would be adaptive to host-seeking wood-boring beetles because it could aid them in locating suitable host material. Coniferophagous wood-borers in the families Cerambycidae and Buprestidae often attack the same hosts at the same time as conifer-infesting bark beetles (Dahlsten and Stephen, 1974; Stephen and Dahlsten, 1976; Dixon and Payne, 1979; Coulson et al., 1976, 1980).

Subcortical interactions between bark beetles and wood-boring beetles have been classified as competitive (Coulson et al., 1976, 1980; Schroeder and Weslein, 1994a,b) or commensal (Flamm et al., 1989). A decline in larval density or emergence of adult bark beetles was attributed primarily to competition between

cerambycid and bark beetle larvae. A kairomonal response to bark beetle pheromones would be even more adaptive to host-seeking wood-borers if bark beetle larvae represent a food resource for wood-borer larvae. Dodds et al. (2001) found that 74% of the six-spined ips, *Ips calligraphus*, larvae encountered by *Monochamus carolinensis* larvae in phloem sandwiches were attacked; 85% were killed, suggesting that cerambycid larvae may be facultative predators of bark beetle larvae. This result supports the earlier observation by Schenk and Benjamin (1969) that in Wisconsin "up to 50% of a brood in the egg and 1st instar was destroyed by cerambycid larvae; a single cerambycid larvae could reduce the available food supply by 3%." However, Flamm et al. (1989) found that foraging by *M. titillator* results in low mortality of the southern pine beetle *Dendroctonus frontalis*, because the larvae migrate to the outer bark before *M. titillator* foraging becomes significant. One possible explanation for the observed behavior is coevolution between *M. titillator* and *D. frontalis*, with larval *D. frontalis* migration to the outer bark being an adaptive response that would reduce mortality due to *M. titillator* predation.

Billings and Cameron (1984) and Billings (1985) found a kairomonal response by the southern pine sawyer, *Monochamus titillator*, to a blend of *Ips* spp. pheromones (ipsenol, ipsdienol, and *cis*-verbenol). In one study, there was a synergistic interaction between this stimulus and blends of *endo*-brevicomin and verbenone, or *endo*- plus *exo*-brevicomin with verbenone, all of which are pheromones of *D. frontalis* (Billings and Cameron, 1984). In the other study, this stimulus was synergized by loblolly pine, *Pinus taeda*, turpentine (Billings, 1985). Raffa (1991) reported an undisclosed number of *M. carolinensis* captured in ipsdienol-baited traps. Miller and Borden (1990) captured *Monochamus clamator* in increasing numbers as the combined release rates of ipsdienol and (−)-β-phellandrene increased.

There is considerable potential in exploiting bark beetle pheromones in wood-borer management, if they prove to be attractive to pest species. We report the results of experiments in British Columbia and Ontario demonstrating that certain bark beetle pheromones are attractive to *M. clamator, M. obtusus*, the white-spotted sawyer *Monochamus scutellatus*, and the northeastern sawyer *M. notatus.*

METHODS AND MATERIALS

Adult *M. clamator, M. scutellatus*, and *M. obtusus* were collected on emergence from caged bolts of lodgepole pine, *Pinus contorta* var. *latifolia*, and ponderosa pine, *P. ponderosa.* Coupled gas chromatographic–electroantenno graphic detection (GC-EAD) analyses were performed on the antennae of both male and female *M. clamator, M. scutellatus*, and *M. obtusus* with an HP 5890 gas chromatograph equipped with a fused silica column (DB-5, 30 m × 0.32 mm ID,

J&W Scientific, Folsom, California 95630-4714) (Gries, 1995). Authentic samples of nine candidate bark beetle pheromones (ipsenol, ipsdienol, MCH, frontalin, *cis*-verbenol, *trans*-verbenol, *exo*-brevicomin, *endo*-brevicomin, and verbenone) (Borden, 1985) were mixed in hexane solutions at concentrations of both 10 ng/μl and 100 ng/μl. These were analysed in 1-μl amounts by GC-EAD under splitless conditions with a temperature program of 60°C (1 min) then 10°C/min to 220°C. The analyses were repeated with a temperature program of 50°C for (1 min), then 10°C/min to 90°C and 4°C/min to 240°C to separate ispdienol and *trans*-verbenol.

Synthetic antennally active pheromones were arbitrarily partitioned into two blends: blend 1 consisting of ipsenol, ipsdienol, MCH, and frontalin and blend 2 consisting of *cis*- and *trans*-verbenol, *exo*- and *endo*-brevicomin, and verbenone (Table 1). These were tested in the field for behavioral activity with and without a host blend composed of ethanol and α-pinene (experiment 1) or ethanol with a synthetic host blend (Table 1) composed of 10.7% (−)-α-pinene, 0.4% (+)-α-pinene, 13.7% (−)-β-pinene, 7.3% myrcene, 1.5% 3-carene, 0.1% α-phellandrene, 63.7% (+)-β-phellandrene, 0.3% γ-terpinene, and 2.4% terpinolene (experiments 2 and 3) (Table 1). These proportions represent an average of those found in subalpine firs, *Abies lasiocarpa*, and Engelmann spruce, *Picea engelmannii.* Experiment 1 was run July 7–28, 1999 and had 24 replicates of five traps per replicate, with nine replicates set up in the Gorman Bros. Ltd. mill yard in Westbank, British Columbia, and 15 in the Weyerhaeuser Canada Ltd. mill yard in Okanagan Falls, British Columbia. Experiment 2 was run June 23–August 11, 1999 and had 10 replicates of five traps per replicate set up in a recently thinned white pine stand north of Thessalon, Ontario. The surrounding forest was predominately jack pine, *Pinus banksiana.* Experiment 3 was run July 19–August 14, 1999 and had eight replicates of five traps per replicate in the Slocan Forest Products Ltd., Tackama Division mill yard in Ft. Nelson, British Columbia. Experiment 4 was run June 29–July 31, 1999 and had 20 replicates with 10 each in Ainsworth Forest Products Ltd. mill yards in 100-Mile House and Chasm, British Columbia.

Experiments 1, 3, and 4 used 12-unit multiple-funnel traps (Lindgren, 1983) deployed in randomized complete blocks with traps $\geq$15 m apart. A small block of Vapona No-Pest Strip (Green Cross, Fisons Horticulture Inc., Mississauga, Ontario) was placed in each collecting cup to minimize predation and cannibalism. Traps were hung from aluminum poles such that the top funnel was ca. 1.5 m above ground. Experiment 2 used cross-vane traps with collection bins containing soapy water, deployed as above (de Groot and Nott, 2001).

The treatments for experiments 1–3 were: (1) unbaited control, (2) host blend alone, (3) host blend and pheromone blend 1, (4) host blend and pheromone blend 2, and (5) host blend and both pheromone blends. Treatments for experiment 4 were: (1) unbaited control, (2) pheromone blend 1, (3) pheromone blend 2, and (4)

both pheromone blends. Release devices and rates for all compounds are given in Table 1.

Captured beetles were collected weekly and frozen until they could be identified and sexed. *Monochamus* spp. were identified and sexed by using elytral, antennal, and sternite characters (Linsley and Chemsak, 1984). Voucher specimens for all species reported have been deposited at the Pacific Forestry Centre, Canadian Forest Service, Victoria, British Columbia.

Data for each sex were transformed by $\log_{10}(x + 1)$ to correct for nonnormality and heteroscedasticity (Zar, 1984), and analyzed by ANOVA (GLM) and the Ryan-Einot-Gabriel-Welsch (REGW) multiple-range test (Day and Quinn, 1989) with SAS Institute Inc. software (SAS Institute Inc., 1988). In all cases $\alpha = 0.05$.

RESULTS

As shown in a representative example from a female *M. scutellatus* antenna (Figure 1), all nine pheromones were detected by the antennae of both male and female *M. scutellatus, M. clamator*, and *M. obtusus*, with no differences by species or sex in the level of response.

In experiment 1, one or both sexes of *M. clamator* and *M. obtusus* were captured in higher numbers in traps baited with the host blend plus pheromone blend 1 than in traps baited with the host blend alone (Figure 2). Fewer *M. obtusus* of both sexes were captured in traps baited with the host blend plus both bark beetle pheromone blends. In no case were there significantly more beetles of either sex captured in traps baited with the host blend plus pheromone blend 2 than in either unbaited traps or traps baited with the host blend alone. Only male *M. obtusus* were more attracted to traps baited with the host blend than to the unbaited control traps.

In experiment 2, both sexes of *M. scutellatus* and *M. notatus* were captured in higher numbers in traps baited with the host blend plus pheromone blend 1. Both sexes of *M. scutellatus* and female *M. notatus* were captured in higher numbers in traps baited with the host blend plus both pheromone blends than in other traps (Figure 2). Similarly, both sexes of *M. scutellatus* and female *M. notatus* were captured in higher numbers in traps baited with the host blend plus pheromone blend 2 or the host blend alone than in unbaited traps. In experiment 3, both sexes of *M. scutellatus* were caught in higher numbers in all baited traps than in unbaited control traps.

In experiment 4, male and female *M. clamator* and *M. scutellatus* were captured in higher numbers in traps baited with pheromone blend 1 alone or combined with pheromone blend 2 than in unbaited traps or traps baited with pheromone blend 2 alone (Figure 3).

For experiments 1, 3, and 4 other wood-borer species were counted and sexed if $\geq$50 individuals were caught. Significant responses, all by beetles in the family

TABLE 1. COMPOUNDS AND THEIR SOURCE, CHEMICAL PURITY, RELEASE DEVICES, ENANTIOMERIC COMPOSITION, AND RELEASE CHARACTERISTICS OF SEMIOCHEMICALS TESTED IN FIELD EXPERIMENTS AS ATTRACTANTS FOR *Monochamus* SPP

Blend and compounds	Source[a]	Chemical purity (%)	Release device[b]	Enantiomeric composition (+:−)	Release rate (mg/24 hr)[c]
Pheromone blend 1					
Ipsenol	Phero Tech	98	bubble cap	50:50	0.24
Ipsdienol	Phero Tech	95.4	bubble cap	50:50	0.11
MCH	Phero Tech	98.8	bubble cap	50:50	4.5
Frontalin	Phero Tech	99.9	closed 400-μl Eppendorf tube	50:50	2.6
Pheromone blend 2					
endo-Brevicomin	Phero Tech	99.1	closed 250-μl Eppendorf tube	50:50	0.28
exo-Brevicomin	Phero Tech	99	closed 250-μl Eppendorf tube	50:50	0.28
cis-Verbenol	Phero Tech	92.7 (5% *trans*-)	bubble cap	22:78	0.35
trans-Verbenol	Phero Tech	75 (15% *cis*-)	bubble cap	18:82	1.5
Verbenone	Phero Tech	98	bubble cap	18:82	5
Synthetic host monoterpene blend					
(−)-α-Pinene	Aldrich	98	plastic sleeve	81:19	47.5
(+)-α-Pinene	Aldrich	98	plastic sleeve	91:9	1.8
(−)-β-Pinene	Aldrich	99	plastic sleeve	1:99	77.4
Myrcene	Phero Tech	92.6	plastic sleeve	Not Chiral	88.1

3-Carene	Aldrich	90	plastic sleeve	NA[e]	5.4
(−)-α-Phellandrene	Fluka	59.6	plastic sleeve	NA[e]	15.8
(+)-β-Phellandrene[d]	Liberty Natural Products	70	plastic sleeve	99:1	409.9
γ-Terpinene	Aldrich	97	plastic sleeve	Not Chiral	16.9
Terpinolene	Fluka	92.6	plastic sleeve	Not Chiral	36.3
Host compounds released separately					
α-Pinene	Phero Tech Inc.	>99	plastic sleeve	90-95:5-10	2187
Ethanol	Phero Tech Inc.	95	plastic sleeve	Not Chiral	1200

[a] Phero Tech Inc., 7572 Progress Way, Delta, British Columbia V4G 1E9, Canada; Aldrich Chemical Company, Sigma-Aldrich Canada Ltd., 2149 Winston Park Drive, Oakville, Ontario L6H 6J8, Canada; Fluka, Sigma-Aldrich Canada Ltd., 2149 Winston Park Drive, Oakville, Ontario L6H 6J8, Canada; Liberty Natural Products, 8120 SE Stark Street, Portland, Oregon 97215.

[b] All release devices fom Phero Tech Inc. For the host blend, ethanol and all other compounds were released from separate sleeves. All other compounds were released fom separate devices.

[c] Release rates for ipsenol, ipsdienol, MCH, frontalin, *cis*- and *trans*-verbenol, *endo*- and *exo*-brevicomin, and verbenone were determined at 20–23°C by Phero Tech Inc. Rates for (−)-α-pinene, (+)-α-pinene, (−)-β-pinene, myrcene, 3-carene, (−) α-phellandrene, (−)-β-phellandrene, γ-terpinene, terpinolene, α-pinene, and ethanol were determined at 28–30°C at Simon Fraser University.

[d] Obtained from angelica seed oil (Liberty Natural Products). Enantiomeric determination after the field season revealed almost pure (+) enantiomer, the antipode of naturally occuring β-phellandrene in conifers.

[e] Standards not available. Unable to determine.

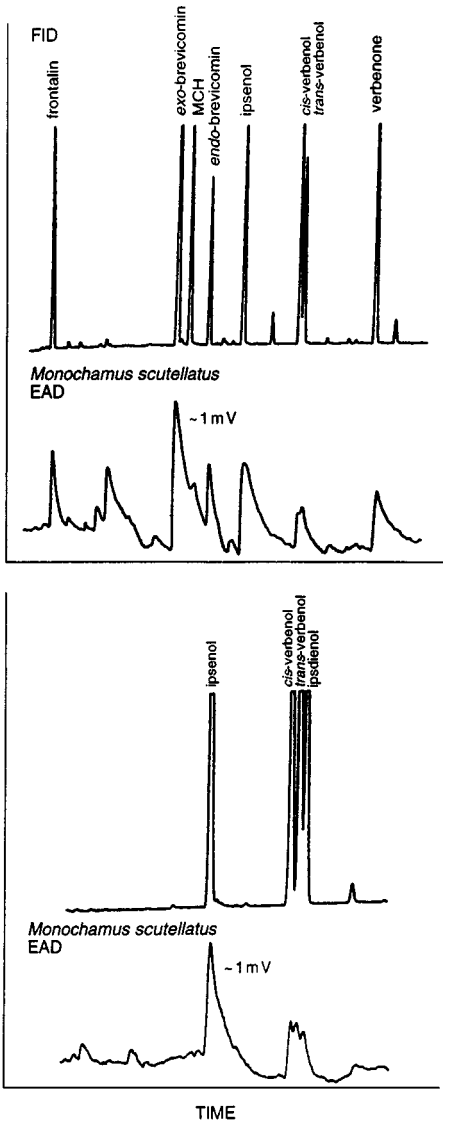


FIG. 1. Responses in GC-EAD analysis of female *M. scatellatas* antenna to nine bark beetle pheromones with a DB-5 column with a temperature program of 60°C (1 min), 10°C/min to 220°C (top), and a DB-5 column with a temperature program of 50°C (1 min), 10°C/min to 90°C, then 4°C/min to 240°C (bottom) to separate ipsdienol from *trans*-verbenol.

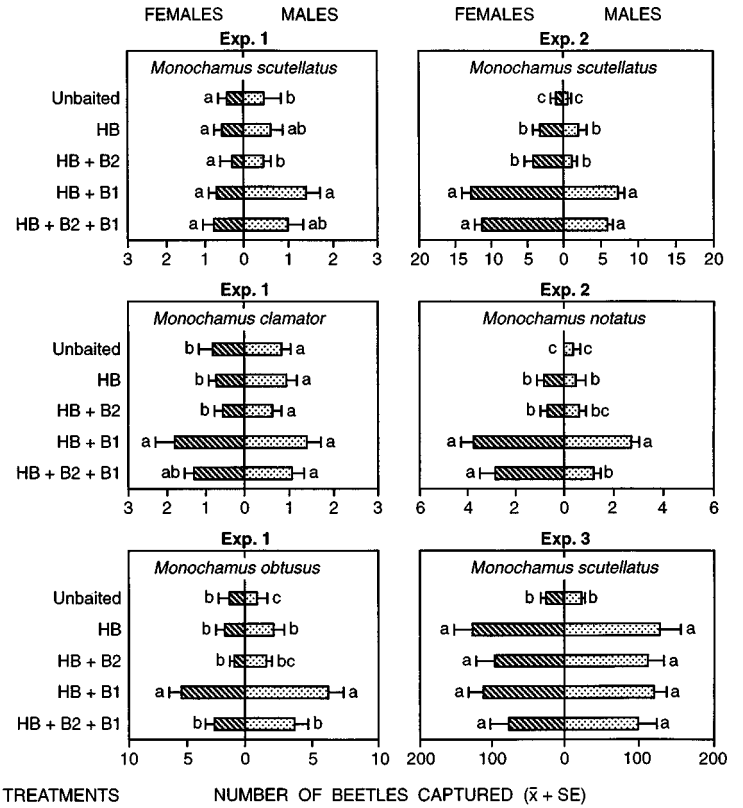


FIG. 2. Catches of four *Monochamus* spp. in experiments 1–3 in unbaited traps or in traps baited with a blend of host compounds alone (HB = host blend) or with pheromone blend 1 (B1), pheromone blend 2 (B2), or both blends together (Table 1). The host blend was composed of ethanol with α-pinene (experiment 1) or a complex blend of monoterpenes (experiments 2 and 3) (Table 1). Means within a species, experiment, and sex followed by the same letter are not significantly different, REGW test, $\alpha = 0.05$.

Buprestidae, were as follows: female *B. subornata* ($N = 89$) in experiment 1 captured in greater numbers in unbaited traps than in traps baited with the host blend and pheromone blend 2, with an intermediate level attracted to the host blend and host blend with pheromone blend 1 or pheromone blends 1 and 2; female *Chalcophora virginiensis* ($N = 158$) in experiment 4 captured in greater numbers in traps baited with pheromone blend 1 alone or with pheromone blend 2 than in other traps; male *Dicerca tenebrica* ($N = 482$) in experiment 4 captured in higher numbers in traps baited with either pheromone blend than in unbaited control traps, with an intermediate level attracted to both pheromone blends combined; female

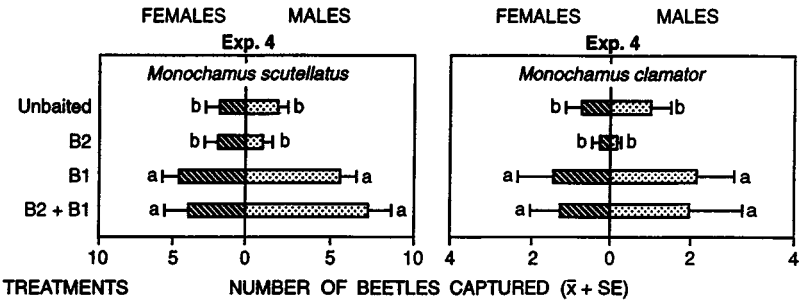


FIG. 3. Catches of *M. scutellatus* and *M. clamator* in experiment 4 in unbaited multiple-funnel traps or in traps baited with pheromone blend 1 (B1), pheromone blend 2 (B2), or both blends together. Means within a species and sex followed by the same letter are not significantly different, REGW test, $\alpha = 0.05$.

D. tenebrica ($N = 1288$) in experiment 4 captured in higher numbers in traps baited with blend 1 than in traps baited with both blends combined and unbaited traps, with an intermediate level attracted to blend 2; and female *Buprestis lyrata* ($N = 46$) in experiment 4 captured in higher numbers in traps baited with phero mone blend 2 than pheromone blend 1, with other treatments intermediate. Despite $\geq$50 beetles being captured, no significantly greater responses to baited over unbaited traps in any experiment were found for the following: *Xylotrechus longitarsus, X. undulatus*, *Asemum* spp., and *Arhopalus* spp. (all Cermabycidae), *Dicerca tenebrosa, B. nutalli, B. laeviventris, B. maculativentris,* and *Chrysobothris* spp. (all Buprestidae).

DISCUSSION

Our results demonstrate that *M. clamator, M. notatus, M. obtusus*, and *M. scutellatus* can perceive and are attracted by pheromones of sympatric bark beetles in combination with host volatiles. For two species, *M. scutellatus* and *M. clamator*, they also demonstrate that a response can occur to bark beetle pheromones in the absence of host volatiles, supporting the results of Billings and Cameron (1984), but not Billings (1985) for *M. titillator.*

These findings suggest that these *Monochamus* spp. use heterospecific pheromones as kairomones during host selection. The ability to respond to both sympatric bark beetle pheromones and host volatiles would minimize the energy spent in foraging to locate hosts, the exposure to predation or environmental extremes (Dahlsten, 1982), and the opportunity cost of time lost for other biologically important activities (Campbell, 1996) in both the presence and absence of mass-attacking primary and secondary bark beetles.

The occurrence of intraguild predation is well documented in many terrestrial and aquatic communities (Polis et al., 1989). Dodds et al. (2001) recently found that *M. carolinensis* is a facultative, intraguild predator of bark beetle larvae. We hypothesize that *M. clamator, M. notatus, M. obtusus,* and *M. scutellatus* are intraguild predators, and, thus, gain an additional adaptive advantage from being able to orient to bark beetle pheromones. Evolution of a kairomonal response by *Monochamus* spp. to the pheromones of numerous species of sympatric bark beetles would be facilitated by overlapping host ranges and similar larval requirements. The absence of one or both of these may explain why the majority of other wood-borers captured, mainly buprestids, did not respond to either the host or pheromone blends. If *Monochamus* spp. gain a significant adaptive advantage by preying on the larvae of bark beetles, they would inevitably be in competition with entomophagous insects that use the same compounds as kairomones (Borden, 1982).

In Ft. Nelson, population levels during experiment 3 were high, and it is possible that the high numbers of responding beetles obscured responses by *M. scutellatus* to different stimuli. Alternatively, the Ft. Nelson population of *M. scutellatus* may represent a behavioral ecotype that is associated with different bark beetles than those in southern British Columbia or Ontario.

The blends tested contained two antiaggregation pheromones: MCH and verbenone. Positive orientation toward sources of both aggregation and antiaggregation pheromones should be adaptive for *Monochamus* spp., as long as they indicate the presence of a suitable host. However, the lack of a response to pheromone blend 2 alone or combined with the host blend, as well as reduced responses by *M. obtusus* when it was combined with pheromone blend 1 in experiment 1, suggest that one or more of its components, possibly verbenone, could actually be repellent. The production of verbenone by microorganisms (Leufven et al., 1984; Hunt and Borden, 1990) after a host has been overcome by aggressive bark beetles may provide a signal to *Monochamus* spp. of hosts that are no longer acceptable. Verbenone might also be produced by microorganisms associated with larval wood-borers.

Kairomonal responses by *Monochamus* spp. may have practical application in pest management. If further research results in simplified and, thus, inexpensive kairomonal blends composed of both host volatiles and bark beetle pheromones, an improved trap (McIntosh et al., 2001; de Groot and Nott, 2001) baited with a more potent attractive lure might be used effectively in operational monitoring and mass-trapping programs. These in turn could lead to reduced degrade losses and (where pine-wilt disease occurs) curtailment of the spread or infection rate of the pine wood nematode.

Acknowledgments—We thank Peter Katinic, Dave Boyle, Bob Setter, Ian Wilson, Sarah Butler, and James Burns for assistance; Dave Moore and Dean Morewood for critical reviews; and Ken Raffa for helpful comments. This research was supported by the Natural Sciences and Engineering Research

Council of Canada, the Science Council of British Columbia, Forest Renewal British Columbia, The Canadian Forest Service, Ainsworth Lumber Co. Ltd., British Columbia, Hydro and Power Authority, Bugbusters Pest Management Inc., Canadian Forest Products Ltd., Crestbrook Forest Industries Ltd., Donohue Forest Products Inc., Gorman Bros. Ltd., International Forest Products Ltd., Lignum Ltd., Manning Diversified Forest Products Ltd., Millar Western Forest Products Ltd., Phero Tech Inc., Riverside Forest Products Ltd., Slocan Forest Products Ltd., TimberWest Ltd., Tolko Industries Ltd., Weldwood of Canada Ltd., West Fraser Mills Ltd., Western Forest Products Ltd., and Weyerhaeuser Canada Ltd.

REFERENCES

BAKKE, A., and KVAMME, T. 1981. Kairomone response in *Thanisimus* predators to pheromone components of *Ips typographus. J. Chem. Ecol.* 7:305–312.

BEDARD, W. D., WOOD, D. L., TILDEN, P. E., LINDAHL, K. Q., JR., SILVERSTEIN, R. M., and RODIN, J. O. 1980. Field responses of the western pine beetle and one of its predators to host- and beetle-produced compounds. *J. Chem. Ecol.* 6:625–641.

BILLINGS, R. F. 1985. Southern pine bark beetles and associated insects. Effects of rapidly released host volatiles on response to aggregation pheromones. *Z. Angew. Entomol.* 99:483–491.

BILLINGS, R. F., and CAMERON, R. S. 1984. Kairomonal responses of Coleoptera, *Monochamus titillator* (Cerambycidae), *Thanisimus dubius* (Cleridae), and *Temnochila virescens* (Trogossitidae), to behavioural chemicals of southern pine beetles (Coleoptera: Scolytidae). *Environ. Entomol.* 13:1542–1548.

BORDEN, J. H. 1982. Aggregation pheromones, pp. 74–139, *in* J. B. Mitton and K. B. Sturgeon (eds.). Bark Beetles in North American Conifers: A system for the Study of Evolutionary Biology. University of Texas Press, Austin.

BORDEN, J. H. 1985. Aggregation pheromones, pp. 257–285, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Pergamon Press, New York.

CAMPBELL, N. A. 1996. Biology. Benjamin/Cummings, New York.

CHÉNIER, J. V. R., and PHILOGÈNE, B. J. R. 1989. Evaluation of three trap designs for the capture of conifer feeding beetles and other forest Coleoptera. *Can. Entomol.* 121:159–167.

COULSON, R. N., MAYYASI, A. M., FOLTZ, J. L., and HAIN, F. P. 1976. Interspecific competition between *Monochamus titillator* and *Dendroctonus frontalis. Environ. Entomol.* 5:235–247.

COULSON, R. N., POPE, D. N., GAGNE, J. A., FARGO, W. S., PULLEY, P. E., EDSON, L. J., and WAGNER, T. L. 1980. Impact of foraging by *Monochamus titillator* (Col.: Cerambycidae) on within-tree populations of *Dendroctonus frontalis* (Col.: Scolytidae). *Entomophaga* 25:155–170.

DAHLSTEN, D. C. 1982. Relationship between bark beetles and their natural enemies, pp. 140–182, *in* J. B. Mitton and K. B. Sturgeon (eds.). Bark Beetles in North American Conifers: A System for the Study of Evolutionary Biology. University of Texas Press, Austin.

DAHLSTEN, D. L., and STEPHEN, F. M. 1974. Natural enemies and insect associates of the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae), in sugar pine. *Can. Entomol.* 106:1211–1217.

DAY, R. W., and QUINN, G. P. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecol. Monogr.* 59:433–463.

DE GROOT, P., and NOTT, R. 2001. Evaluation of traps of six different designs to capture pine sawyer beetles (Coleoptera: Cerambycidae). *Agric. For. Entomol.* In press.

DIXON, W. N., and PAYNE, T. L. 1979. Sequence of arrival and spatial distribution of entomophagous and associate insects on southern pine beetle-infested trees. Texas Agriculture Experiment Station Miscellaneous Publication No. 1432.

DODDS, K. J., GRABER, C., and STEPHEN, F. M. 2001. Facultative and intraguild predation by larval Cerambycidae (Coleoptera) on bark beetle larvae (Coleoptera: Scolytidae). *Environ. Entomol.* 30:17–22.

ERBILGIN, N., and RAFFA, K. F. 2000. Opposing effects of host monoterpenes on responses by two sympatric species of bark beetles to their aggregation pheromones. *J. Chem. Ecol*. 26:2527–2548.

FLAMM, R. O., COULSON, R. N., BECKLEY, P., PULLEY, P. E., and WAGNER, T. L. 1989. Maintenance of a phloem-inhabiting guild. *Environ. Entomol.* 18:381–387.

GARDINER, L. M. 1957. Deterioration of fire-killed pine in Ontario and the causal wood-boring beetles. *Can. Entomol.* 89:241–263.

GARDINER, L. M. 1975. Insect attack and value loss in wind-damaged spruce and jack pine stands in northern Ontario. *Can. J. For. Res.* 5:387–398.

GRIES, G. 1995. Prospects of new semiochemicals and technologies, pp. 44–47, *in* S. M. Salom and K. R. Hobson (eds.). Application of Semiochemicals for Management of Bark Beetle Infestations—Proceedings of an Informal Conference. US Department of Agriculture Forest Service General Technical Report INT-GTR-318.

HUNT, D. W. A., and BORDEN, J. H. 1990. Conversion of verbenols to verbenone by yeasts isolated from *Dendroctonus ponderosae* Coleoptera:Scolytidae). *J. Chem. Ecol.* 16:1385–1397.

LEUFVEN, A., BERGSTROM, G., and FALSEN, E. 1984. Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus. J. Chem. Ecol.* 10:1349–1361.

LINDGREN, B. S. 1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol.* 115:299–302.

LINSLEY, E. G. 1961. The Cerambycidae of North America. University of California Publications, 18.

LINSLEY, E. G., and CHEMSAK, J. A. 1984. The Cerambycidae of North America, Part VII, No. 1: Taxonomy and Classification of the Subfamily Lamiinae, Tribes Parmenini through Acanthoderini. University of California Press, Berkeley and Los Angeles.

MCINTOSH, R. L., KATINTIC, P. V., ALLISON, J. D., BORDEN, J. H., and DOWNEY, D. L. 2001. Comparative efficacy of five types of traps for trapping large woodborers in the Cerambycidae, Buprestidae and Siricidae. *Agric. For. Entomol.* In press.

MILLER, D. R., and BORDEN, J. H. 1990. β-Phellandrene: Kairomone for pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 16:2519–2531.

PARMELEE, F. T. 1941. Longhorned and flatheaded borers attacking fire-killed coniferous timber in Michigan. *J. Econ. Entomol.* 34:377–380.

POLIS, G. A., MYERS, C. A., and HOLT, R. D. 1989. The ecology of intraguild predation: Potential competitors that eat each other. *Annu. Rev. Ecol. Syst.* 20:297–330.

RAFFA, K. F. 1991. Temporal and spatial disparities among bark beetles, predators and associates responding to synthetic bark beetle pheromones: *Ips pini* (Coleoptera: Scolytidae) in Wisconsin. *Environ. Entomol.* 20(6):1665–1679.

RAFFA, K. F., and KLEPZING, K. D. 1989. Chiral escape of bark beetles from predators responding to a bark beetle pheromone. *Oecologia* 80:566–569.

SAFRANYIK, L., and RASKE, A. G. 1970. Sequential sampling plan for larvae of *Monochamus* in lodgepole pine logs. *J. Econ. Entomol.* 63:1903–1905.

SAS Institute Inc. 1988. SAS/STAT Users Guide, Release 6.03 Edition. Cary, North Carolina.

SCHENK, J. A., and BENJAMIN, D. M. 1969. Notes on the biology of *Ips pini* central Wisconsin jack pine forests. *Ann. Entomol. Soc. Am.* 62:480–485.

SCHROEDER, L. M. 1992. Olfactory recognition of nonhosts aspen and birch by conifer bark beetles *Tomicus piniperda* and *Hylupgops palliatus. J. Chem. Ecol.* 18:1583–1593.

SCHROEDER, L. M., and WESLEIN, J. 1994a. Reduced offspring production in bark beetle *Tomicus piniperda* in pine bolts baited with ethanol and α-pinene, which attract antagonistic insects. *J. Chem. Ecol.* 20:1429–1444.

SCHROEDER, L. M., and WESLEIN, J. 1994b. Interactions between the phloem-feeding *Tomicus piniperda* (Col.: Scolytidae) and *Acanthocinus aedilis* (Col.: Cerambycidae) and the predator *Thanasimus formicarius* (Col.: Cleridae) with special reference to brood production. *Entomophaga* 39:149–157.

STEPHEN, F. M., and DAHLSTEN, D. L. 1976. The arrival sequence of the arthropod complex following attack by *Dendroctonus brevicomis* (Coleoptera: Scolytidae) in ponderosa pine. *Can. Entomol.* 108:283–304.

VALLENTGOED, J. 1991. Some important woodborers related to export restrictions. Forestry Canada, Forest Pest Leaflet 74.

VITÉ, J. P., and WILLIAMSON, D. L. 1970. Thanasimus dubius: Prey perception. *J. Insect Physiol.* 16:233–239.

ZAR, J. H. 1984. Biostatistical Analysis., 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

ATTRACTION OF A LEAF BEETLE (*Oreina cacaliae*) TO DAMAGED HOST PLANTS

NICOLE M. KALBERER,*,[1] TED C. J. TURLINGS, and MARTINE RAHIER

Institute de Zoologie
Université de Neuchâtel
CH-2007 Neuchâtel, Switzerland

(Received September 1, 2000; accepted December 13, 2000)

Abstract—Early in spring, just after the snow melts, the leaf beetle *Oreina cacaliae* feeds on flowers of *Petasites paradoxus.* Later in spring they switch to their principle host plant *Adenostyles alliariae.* The attractiveness of short- and long-term damaged host plants was studied in a wind tunnel. The spring host *P. paradoxus* was more attractive to the beetles after it had been damaged overnight by conspecifics or artificially, but not when the plants were damaged half an hour before the wind-tunnel experiments. Contrary to *P. paradoxus,* the principle host plant, *A. alliariae* was more attractive shortly after an attack by conspecifics (half an hour before the experiment) compared to a undamaged plant, but lost its increased attractiveness when damaged overnight. The enhanced attraction of damaged plants was longer lasting in the spring host *P. paradoxus* than in the main host *A. alliariae.* Volatiles emitted by host plants were collected and gas chromatographic analyses of the odors collected showed qualitative and quantitative differences between damaged and undamaged plants. Among the volatiles recorded, green leaf volatiles and mono- and sesquiterpenes dominated. In overnight damaged *P. paradoxus* plants with an enhanced attractiveness, limonene was emitted in higher amounts. In freshly damaged *A. alliariae* leaves, more α-humulene and germacrene D were emitted compared to (*E,E*)-α-farnesene, whereas in the less attractive *A. alliariae* plants, more (*E,E*)-α-farnesene was emitted compared to α-humulene and germacrene D. In the field, the long lasting attraction of flowering *P. paradoxus* early in the season may facilitate mating in *O. cacaliae* after a successful overwintering.

Key Words—Olfaction, behavior, wind-tunnel, gaschromatography, Coleoptera, Chrysomelidae, *Oreina cacaliae.*

*To whom correspondence should be addressed.
[1]Current address: Arizona Research Laboratories, Division of Neurobiology, University of Arizona, 606 Gould-Simpson Bldg, Tucson, Arizona 85721.

0098-0331/01/0400-0647$19.50/0

INTRODUCTION

Herbivory leads to plant injury and thus to enhanced emission of plant volatiles, which may in turn increase the attractiveness of these plants to beetles (Harari et al., 1994; Loughrin et al., 1995; Bolter et al., 1997). Host-derived volatiles can also interact with aggregation pheromones to produce synergized attraction, like that described in bark beetles, or they can be converted into pheromones, as in mountain pine beetles (Borden, 1984; Jones, 1985).

The alpine *Oreina cacaliae* (Coleoptera: Chrysomelidae) feed on two host plants at a field site in La Fouly (Val Ferret: Valais) in the Swiss Alps. In spring, just after the snow melts, the beetles feed on *Petasites paradoxus* (Asteraceae) in its flowering state. This is one of the first annual plants to occur at sun-exposed sites. Wind-tunnel experiments confirmed the attractiveness of *P. paradoxus* over a nonhost (*Tussilago farfara*) in the laboratory (Kalberer, 2000). Up to 80 individual *O. cacaliae* beetles can be found on a single flowering *P. paradoxus* plant in the field (Kalberer, personal observation). This observation led to the hypothesis that an aggregation pheromone might exist, that attracts beetles to an individual host plant. *O. cacaliae* beetles live for up to three years and generations overlap. Females are ready to copulate immediately after emergence in summer, whereas males do not mate until the following spring and summer (Rahier, unpublished results).

A second host plant, *Adenostyles alliariae* (Asteraceae), emerges three to four weeks after *P. paradoxus* and serves as summer host for the beetles. The leaves of *A. alliariae* contain pyrrolizidine alkaloids that are sequestered by *O. cacaliae* larvae and adults for their own defense (Ehmke et al., 1991; Dobler and Rowell-Rahier, 1994). *A. alliariae* has one single large leaf (0.2×0.3 m) in its vegetative state and occurs in patches. The leaves of adjacent plants are often overlapping, making it easy for *O. cacaliae* to walk from one plant to another. In the field, the beetles are much less aggregated on the *A. alliariae* leaves than on their spring host *P. paradoxus* (Kalberer, unpublished data). Quite often two and sometimes three beetles can be found on a single *A. alliariae* leaf, but their number rarely exceeds more than four (Kalberer, personal observation).

In relation to observations of aggregation in this beetle we tested (1) if an aggregation pheromone exists, (2) if plant injury changes the attractiveness of the host plants, and (3) if *O. cacaliae* reacts differently to short versus prolonged injury to *P. paradoxus* and *A. alliariae* plants. Different responses to already damaged *P. paradoxus* and *A. alliariae* may explain the observed differences in aggregation on these plants. Experiments to test ambulatory responses of beetles towards plant odors were conducted in a wind-tunnel. In addition, the volatiles emitted by undamaged and damaged host plants were collected, identified, and compared.

METHODS AND MATERIALS

The Insects. Adults of *O. cacaliae* were collected weekly from *P. paradoxus* in spring and from *A. alliariae* in summer, near La Fouly (Val Ferret: Valais) in the Swiss Alps. To prevent stress, beetles were transported to the laboratory in cooled containers. The beetles were maintained in glass jars in an incubator at a day temperature of 16°C and a night temperature of 12°C and a 16L: 8D light regime. Beetles were used within three days of collection in experiments and were sexed using sexual dimorphism of the tarsi (Lohse and Lucht, 1994).

The Plants. Plants used in the experiments were dug up weekly at or near the sites where beetles were collected and immediately transplanted into 15-cm-diameter ceramic pots. Potted plants were transported to the laboratory, where they were placed near a window and kept at room temperature until use. To obtain herbivore-damaged plants, 10 adult beetles (5 males and 5 females) were placed on *P. paradoxus* and *A. alliariae* plants and allowed to feed, either for half an hour before the experiment (short-term damage) or overnight beginning at 2000 hr, and the experiments were conducted during the next day (overnight damage). *A. alliariae* was also subject to another treatment in which five last-instar *O. cacaliae* larvae were allowed to feed on a plant for half an hour before the experiment (larval short-term damage). Both host plants were also subjected to an artificial injury treatment. Artificial injury to *P. paradoxus* was inflicted by a razor blade, with which two out of several dozen flowerheads were removed together with the edge of a basal leaf surrounding the floral stalk. In *A. alliariae*, artificial injury was administered by cutting two pieces (35 × 10 mm) from the edge of the leaf with a razor blade and punching a hole (14 mm diameter) in the middle of the leaf with a cork borer. Each plant was used once as an undamaged plant and once after being damaged in wind-tunnel experiments and for odor collection.

Wind-Tunnel. The Plexiglas wind-tunnel (0.6 × 0.6 × 1.8 m) was surrounded by a white tent to eliminate visual distraction for the beetles. Air was pulled through the tunnel at 0.1 m/sec with the use of a tubular duct fan (RR 125 C, Melios Ventilatoren AG, Urdorf, Switzerland) and was exhausted outside the room. A charcoal impregnated fabric cleaned the air at the tunnel entrance. Room temperature in the wind-tunnel facility was 22–23°C and humidity around 55% (for details see Kalberer, 2000).

Experimental Procedure. The assay was designed as a dual-choice test for walking beetles. Two differently treated potted plants were placed 0.3 m apart at the upwind end of the wind-tunnel. A glass plate (1.5 × 0.5 m) was placed horizontally in the wind-tunnel at a height adjusted such that air from the volatile-emitting, upper plant parts would pass over it. The distance between the point of beetle release on the glass plate and the plants was 1 m. The glass plate was cleaned with 70% ethanol after testing 10 beetles. Each plant combination was replicated four times with

20 beetles (10 females and 10 males) per replicate. Replication took place at intervals of at least one week with different plants and different beetles. The insects were deprived of food for at least 24 hr before being tested to promote their anemotactic response (Visser and Nielsen, 1977; Miller and Strickler, 1984). Glass jars with beetles were taken out of the incubator 30 min before an experiment and left near the wind tunnel to let the beetles acclimatize to the experimental conditions. Details on the behavior of the beetles in the wind tunnel are described in Kalberer (2000).

Wind-Tunnel Experiments Excluding Visual Cues. To exclude visual cues, the plants were not put in the wind-tunnel but placed outside the white curtain surrounding the wind tunnel. Compressed air, humidified and purified by a charcoal filter, entered a heat-sealed Nalophan cooking bag (Kalle Nalo, Wiesbaden, Germany) containing the potted plant. Teflon tubes led the air enriched with plant odors out of the cooking bag and into the wind tunnel through two holes at the upwind end of the wind-tunnel. The ends of the tubes were taped vertically to the upwind edge of the glass plate.

Pheromone Test. To test for pheromonal attraction, we placed either 10 overwintered males or 10 freshly emerged females in a large mesh metallic cage and released beetles of the opposite sex. To establish a dual-choice test, we had two cages, one with beetles and one empty. The experiments were conducted with 20 beetles and replicated four times.

Collection of Plant Volatiles. Volatiles were collected from potted plants inside a climate chamber at 15°C, 50–60% relative humidity, and 35,000 lux. The plants were placed inside odorless polyethylene terephthalate (PET) Nalophan cooking bags. One bag opening was sealed before use with a heat sealer (TEW Electric Heating Equipment Co. Ltd., type TISH-400), the other was tied around the plant stem with a plastic tie provided with the bags. Humidified and charcoal-filtered air entered the bag via a Teflon tube at a rate of 1000 ml/min. The Teflon tube was connected to the bags by a glass tube. The glass tube entered the PET bag and was introduced through a screw cap containing a Teflon-coated O-ring of the same diameter [6 mm; for details see Turlings et al. (1998)].

A volatile collection trap, consisting of a 10-cm-long × 6-mm-diameter glass tube containing 25 mg Super Q adsorbent (as described by Heath and Manukian, 1992), was attached to the PET bag in the same way as the inlet glass tube. Air was pulled through the collection trap in the bag via Tygon tubing at a rate of 800 ml/min (Turlings et al., 1998). Volatiles were collected for 4 hr, after which each trap was rinsed with 150 μl methylene chloride, and internal standards (400 ng *n*-octane and nonylacetate) were added before injection to a gas chromatograph (GC). Six collections per treatment were made.

Plant Volatile Analysis. Volatiles were analyzed with a Hewlett Packard 6890 GC equipped with an on-column injector and a flame ionization detector. Aliquots of 3 μl were injected onto a 30 m × 0.25 mm nonpolar column (EC1, film thickness) 0.25 μm, Alltech Associates), preceded by a 10-m × 0.25-mm deactivated

retention gap and a deactivated precolumn (30 cm × 0.53 mm, both Connex). Helium was used as the carrier gas at a flow rate of 24 cm/sec. The following temperature program was used: after a hold time of 3 min at 50°C, temperature was linearly increased at a rate of 8°C/min to 230°C and held for 9.5 min.

Data were collected and processed with ChemStation software (Hewlett-Packard). The main compounds were identified by GC-MS analysis on a Hewlett-Packard 5973 quadrupole-type mass selective detector (transfer line temp: 230°C, source temp: 230°C, quadrupole temp: 150°C, ionization potential: 70 eV, and scan range: 50 – 400 amu) coupled to the gas chromatograph described above. The compounds were identified by comparison of retention times to those of commercially available standards [nonene, α-phellandrene, limonene, linalool and *(E,E)*-α-farnesene] and by comparison of spectra to those of the Wiley and NIST libraries.

Behavioral Response to Collected Volatiles. This experiment was conducted to test whether the collected volatiles elicit a behavioral response in the beetles. Samples (50 μl each) containing the volatiles collected from healthy and artificially damaged *A. alliariae* plants, identical to the ones used for injection in the gas chromatograph, were offered on folded filter paper discs (90 mm diameter; Schleicher and Schuell, Dassel, Germany) placed on the glass plate at the upwind end of the wind-tunnel. No visual cues could help the beetles approach the odor source in the wind-tunnel.

Statistical Analysis. Odor preferences in the wind-tunnel were tested with a two-tailed binomial test, using the total number of beetles that made a choice for a particular odor ($\alpha = 0.05$) and pooling the data of the four replicates. To analyze the quantitative differences in the plant odors emitted by undamaged and damaged plants, we used an ANOVA followed by a Bonferroni-Dunn versus control (undamaged plant) post hoc test. Collected odor data were log (X + 1) transformed before analysis.

RESULTS

Choice Experiments

Damaged vs. Undamaged Petasites paradoxus: *Flowering State.* In choice experiments with flowering *P. paradoxus,* beetles showed a significant preference for plants damaged by overnight feeding beetles (Figure 1A, $P < 0.001$) and by artificial damage the previous evening (Figure 1B, $P < 0.01$) over undamaged plants. There was no difference in attractiveness between overnight damage caused by adult beetles and overnight artificial damage (Figure 1C, $P = 0.43$). Beetles were not more attracted to *P. paradoxus* plants that had been damaged only half an hour before the experiment (short term attack) than they were to undamaged plants (Figure 1D, $P = 0.43$).

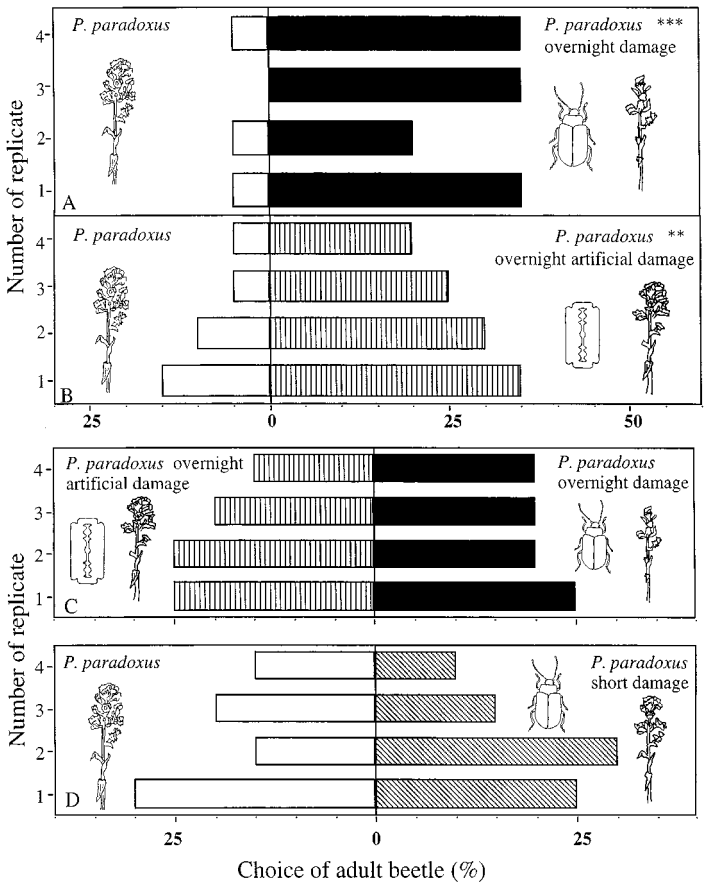


FIG. 1. Choices of *Oreina cacaliae* in wind-tunnel experiments with *Petasites paradoxus.* The bars represent the percentage of tested beetles that made a particular choice. The asterisks indicate a significant preference for a treatment (two-tailed binomial test; $^{**}P < 0.01$, $^{***}P < 0.001$).

Damaged vs. Undamaged. Adenostyles alliariae, *Vegetative State.* Freshly damaged leaves of *A. alliariae* were more attractive to beetles than undamaged leaves, regardless whether leaf damage had been inflicted by adult or larval feeding (Figure 2A, $P < 0.01$ and Figure 2B, $P < 0.05$). Freshly damaged *A. alliariae* leaves were significantly more attractive than overnight damaged leaves (Figure 2C, $P < 0.001$). Artificial fresh attack was as attractive as fresh damage caused by adult beetles feeding on the plant (Figure 2D, $P = 0.42$). Plants damaged over night by adult beetles were not more attractive than undamaged plants (Figure 2E, $P = 0.42$).

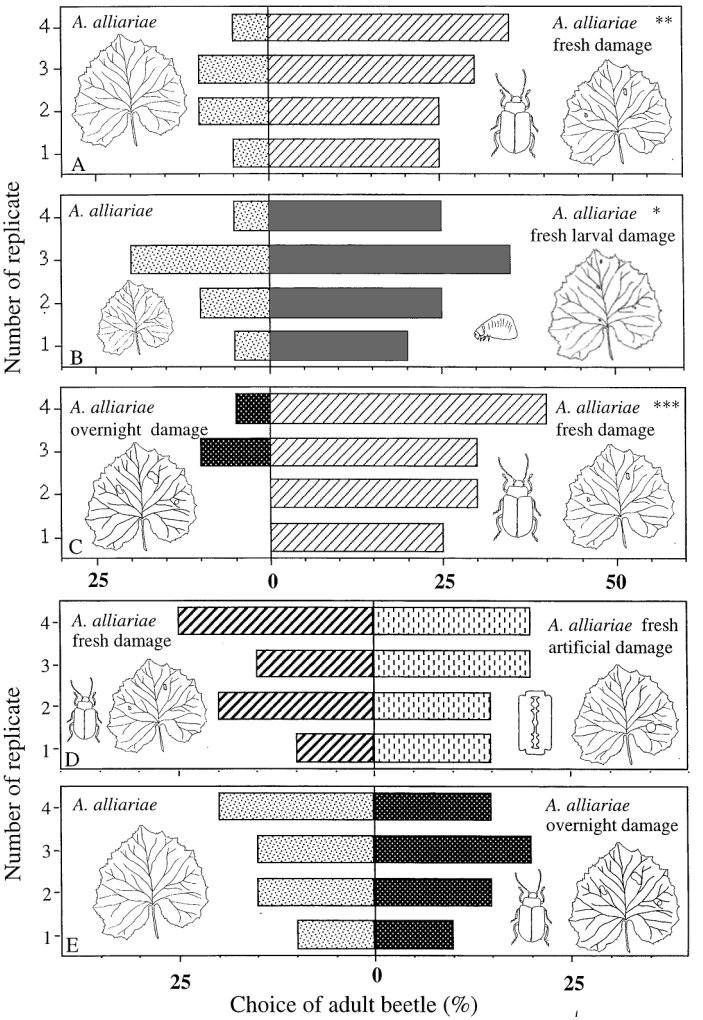


FIG. 2. Choices of *Oreina cacaliae* in wind-tunnel experiments with *Adenostyles alliariae.* The bars represent the percentage of tested beetles that made a particular choice. The asterisks indicate a significant preference for a treatment (two-tailed binomial test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Host Odor without Visual Cues. The tube releasing odor collected from an undamaged *A. alliariae* plant attracted significantly more beetles than the tube releasing clean air (Figure 3A, $P < 0.05$). Collected headspace volatiles of a freshly damaged *A. alliariae* leaf presented on a filter paper, attracted more

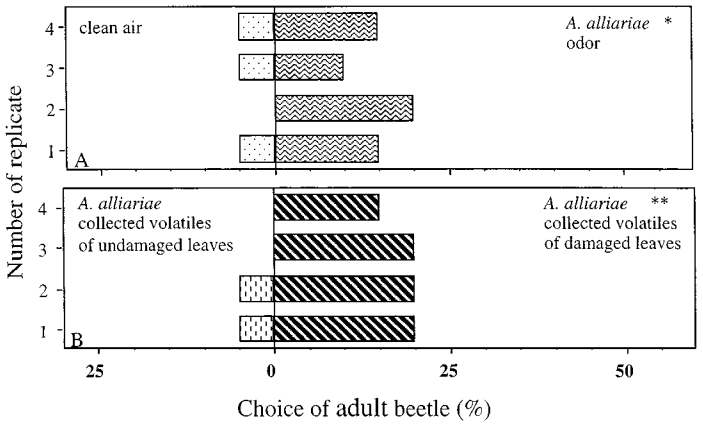


FIG. 3. (A) Choice experiments without visual cues, where the host odor entered the wind-tunnel from plants standing outside the wind-tunnel. Odor from *A. alliariae* plants attracted more beetles than pure air. (B) Collected headspace volatiles from damaged *A. alliariae* leaf put on a filter paper attracted more beetles than the collected odor of a undamaged *A. alliariae* leaf. The asterisks indicate a significant preference for a treatment (two-tailed binomial test; $*P < 0.05$, $**P < 0.01$.

beetle than did collected headspace volatiles of an undamaged leaf (Figure 3B, $P < 0.01$).

Attractiveness of Conspecifics. O. cacaliae beetles of one sex were not attracted by conspecifics of the other sex. Neither overwintered males nor new generation females attracted more adults of the opposite sex than did an empty cage (Figure 4, $P = 0.34$, both sexes). When two potted *A. alliariae* plants were added at the upwind end of the wind-tunnel, beetles still did not discriminate between the cage with conspecifics and the empty cage ($P = 0.4$, data not shown).

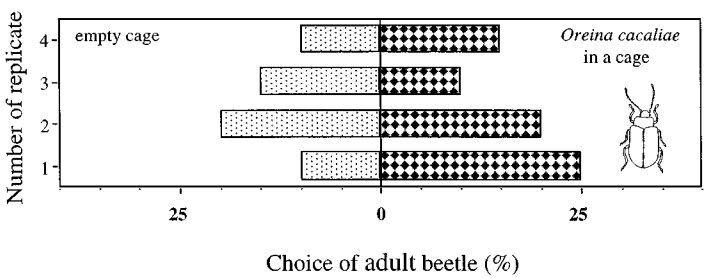


FIG. 4. *O. cacaliae* was not attracted to the smell of the opposite sex and did not approach the conspecifics hidden in a metallic cage more often than an empty cage ($P = 0.34$).

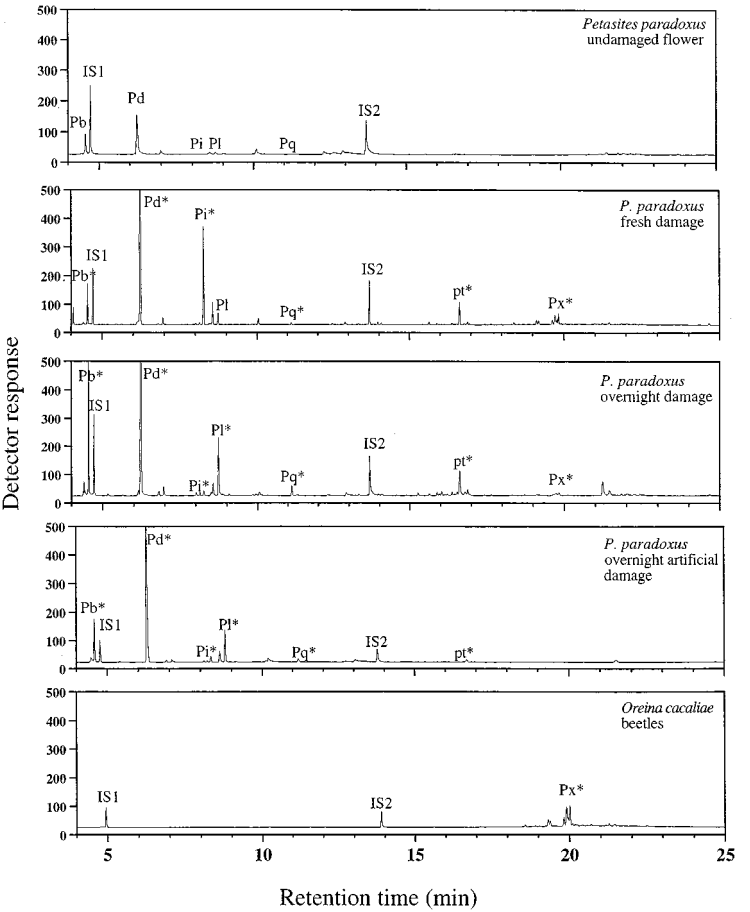


FIG. 5. Representative gas chromatographic profiles of headspace volatiles from undamaged, freshly damaged, overnight damaged and artificially overnight damaged *Petasites paradoxus* plants. The bottom chromatogram shows the odor from *Oreina cacaliae* beetles. Peak identities: (P for *Petasites*) Pb, unknown; Pd, nonene; Pi, α-phelandrene; Pl, limonene; Pq, unknown; Pt, eremophylene; Px, chromolaenin. IS1 and IS2 represent the internal standards *n*-octane and nonyl acetate, respectively. Asterisks indicate significant differences in the amount of an emitted substance as compared to a undamaged *P. paradoxus* flower.

Analysis of Headspace Volatiles

Petasites paradoxus *Flowering State*. Representative gas chromatographic profiles of headspace volatiles collected from *P. paradoxus* plants illustrate the differences in volatile blends between undamaged and damaged plants (Figure 5).

TABLE 1. DATA OF ANOVA TEST FOR COLLECTED PLANT ODOR COMPOUNDS OF DAMAGED PLANTS COMPARED WITH UNDAMAGED CONTROL PLANTS[a]

Compound	MS	*F*	*P*
Petasites paradoxus flowering			
Unknown (Pb)	0.49	7.69	0.0013
Nonene (Pd)	1.16	7.8	0.0012
(*E,E*)-α-phellandrene (Pi)	5.81	11.7	0.0001
Limonene (Pl)	1.15	4.94	0.01
Unknown (Pq)	4.33	12.63	0.0001
Eremophylene (Pt)	4.29	23.59	0.0001
Chromolaenin (Px)	7.64	69.48	0.0001
Adenostyles alliariae vegetative			
(*Z*)-3-Hexenol (a)	2.69	3.34	0.025
Unknown (b)	1.75	3.27	0.028
(*Z*)-3-Hexenyl acetate (c)	1.9	3.84	0.015
Linalool (e)	0.94	7.38	0.0005
Dodecanol (h)	2.78	6.14	0.0014
α-Humulene (k)	0.87	7.51	0.0004
Germacrene D (n)	0.48	3.61	0.019
(E,E)-α-fannesene (p)	0.25	2.42	0.075

[a] Significant differences between treatments are indicated with an asterisk in the Figures 4 and 5.

Control collections from empty PET bags showed no background odor. The sesquiterpene α-phellandrene was emitted in large amounts by freshly damaged *P. paradoxus* plants (Figure 5, peak Pi). Overnight damaged plants (adult and artificial attack) with enhanced attractiveness emitted significantly more limonene (peak Pl) than both undamaged and freshly damaged plants. The other volatile compounds—nonene, eremophylene, and two unknown substances were released in significantly larger amounts by damaged plants (either fresh or overnight damage) than by undamaged plants (Table 1, Figure 5).

Adenostyles alliariae *Vegetative State*. Representative gas chromatograms of headspace volatiles collected from *A. alliariae* leaves following different treatments are shown in Figure 6. Freshly damaged leaves with enhanced attractiveness, damaged either by adult beetles, larvae, or artificially, contained significantly larger amounts of linalool (e), dodecanol (h), α-humulene (k), and germacrene D (n) than did undamaged leaves (Table 1, Figure 6). When *A. alliariae* leaves were damaged overnight by adults, they emitted additional volatiles such as (*Z*)-3 hexenol (a), (*Z*)-3-hexenyl acetate (c), (*E,E*)-α-farnesene (p), and an unknown substance (b). In undamaged and overnight damaged *A. alliariae* more (*E,E*)-α-farnesene (p) is produced compared to α-humulene (k) and germacrene D (n), whereas in freshly damaged leaves (by adults, larvae, or artificially) more α-humulene (k) and germacrene D (n) are emitted compared to (*E,E*)-α-farnesene (p).

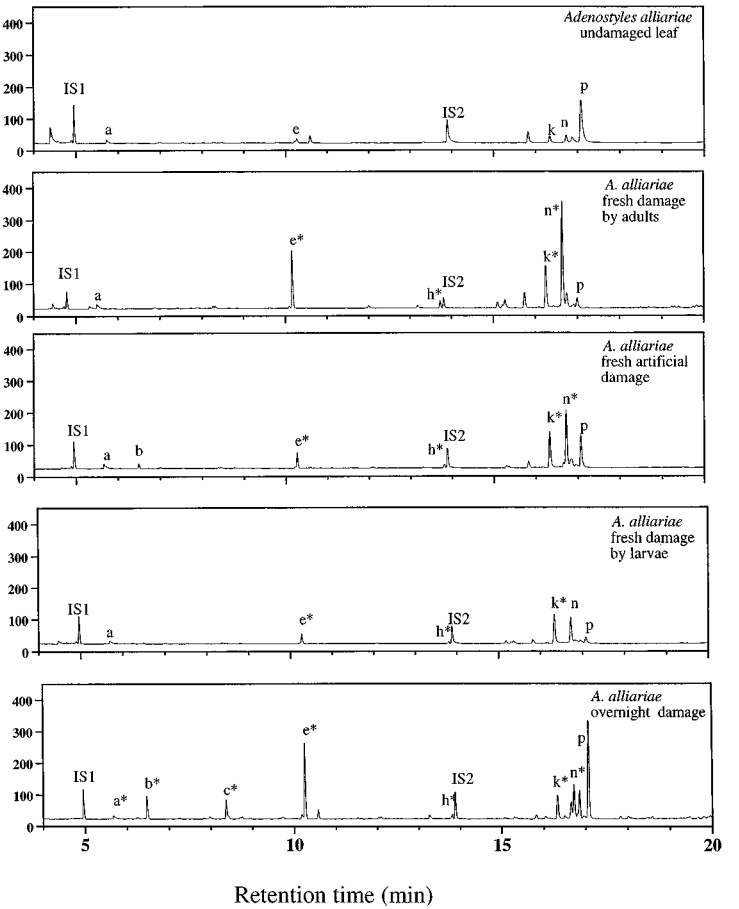


FIG. 6. Representative gas chromatographic profiles of *Adenostyles allliariae* headspace volatiles collected from leaves after various treatments. Treatments from top to bottom were: undamaged *A. alliariae* leaf, a leaf freshly damage by adult *O. cacaliae*, a leaf artificially damaged with a razor blade, a leaf freshly damaged by *O. cacaliae* larvae, and a leaf damaged overnight by *O. cacaliae* adults. Peak identities: a, (*Z*)-3-hexenol; b, unknown; c, (*Z*)-3 hexenylacetate; e, linalool; h, dodecanol; k, α-humulene; n, germacrene D; and p, (*E*,*E*)-α-farnesene. IS1 and IS2 represent the internal standards *n*-octane and nonyl acetate, respectively. Asterisks indicate significant differences in the amount of an emitted substance as compared to a undamaged *A. alliariae* leaf.

Results of Beetle Odor Collections

Oreina cacaliae. Chromolaenin was the only identified substance emitted from *O. cacaliae* beetles and was also present in plant odor collections from damaged plants, where beetles had fed on the plant during collection (Figure 5).

DISCUSSION

Wind-tunnel experiments with *O. cacaliae* and the host plants *P. paradoxus* and *A. alliariae* provide evidence that this alpine leaf beetle is more attracted to volatiles released by damaged host plants than it is to undamaged hosts. Domek and Johnson (1988) found that green June beetles feeding on ripe peach attract significantly more conspecifics than did ripe sliced fruit or beetles alone, demonstrating that beetles feeding on a host attract more conspecifics than the presence of host plants or conspecifics alone. The attraction to a general odor of wounded plants could be excluded in an experiment with *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae), where beetles were attracted to conspecifics feeding on the host plant oilseed rape, but not to conspecifics feeding on the nonhost crambe (Peng and Weiss, 1992). *O. cacaliae* beetles responded similarly to insect (larvae or adult) or artificially damaged host plants, showing that the volatile blend emitted is solely a result of plant injury and not part of the odors emitted by the insect while feeding.

Even in the absence of any visual cues characterizing its host plant in the wind tunnel *O. cacaliae* approached the host plant odor inlet more often than the clean air inlet. Nevertheless, the fact that fewer beetles responded in experiments without visual cues than in trials in which plants were placed in the wind tunnel indicates that some visual cues might help the beetles to approach a plant. In the field, the role of visual cues in host plant finding behavior is clearly less important than the role of odor cues (Kalberer, 2000).

Pasteels et al. (1994) speculated on the existence of a sexual pheromone in *O. gloriosa*, based on differences in the composition of defensive secretion, between the sexes (Eggenberger and Rowell-Rahier, 1991, 1993). We found no evidence for a sex pheromone in *O. cacaliae.* Our beetles were transported together in a pot from the field to the lab, and they may have released their defensive secretion during transport, which could have affected their subsequent responses. Keeping the beetles separate may yield different results.

Short- or long-term attack, respectively, lead to an enhanced attractiveness in one host plant of *O. cacaliae* but not in the other. The spring host *P. paradoxus* was more attractive after overnight damage, whereas *A. alliariae* had an enhanced attractiveness only after a fresh attack, but lost this enhanced attractiveness hours later. An enhanced attractiveness for overnight damaged plants similar for the one on *P. paradoxus* had been described for the Japanese beetle on crabapple leaves where overnight damaged leaves attract more beetles than undamaged leaves or leaves with fresh damage (Loughrin et al., 1995).

Collected headspace volatiles from damaged *A. alliariae* leaves were more attractive to the beetles than collected volatiles from undamaged *A. alliariae* leaves, showing that the compounds triggering a response in *O. cacaliae* had been collected. The compounds collected were analyzed in order to determine how the

composition of the odor blend changed as a result of herbivore attack. Visser et al. (1979) showed that fresh attack by Colorado potato beetles results in the emission of green leaf volatiles from injured plant cells. These volatiles are emitted by most plant species after damage as a result of the oxidative degradation of plant lipids (Visser and Avé, 1978). The compounds (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate that were released from damaged *A. alliariae* leaves after a short attack belong to this category, nevertheless they were also produced after overnight attack. Several studies have shown that the production of other volatiles is induced in the plant following the attack by a herbivore (Turlings et al., 1990, Takabayashi et al., 1994) and is the result of active chemical processes in the plant (Paré and Tumlinson, 1997). Mono- and sesquiterpenes, built via the isoprenoid pathway, belong to this category and are the most common volatiles induced by herbivore attack (Paré and Tumlinson, 1997). Nonene, α-phelandrene, limonene, and eremophylene, were the compounds emitted in higher amounts by damaged *P. paradoxus* plants compared to undamaged plants. After an overnight infestation by beetles as well as after artificial damage the previous evening, *P. paradoxus* flowers released significantly more limonene than plants that were less attractive for the beetles. It is tempting to speculate that the enhanced attractiveness of overnight damaged *P. paradoxus* is due to an increased amount of limonene in the emitted plant volatiles. Nevertheless it is risky to speculate that a single compound evokes the observed response in the beetle, because several studies have shown that the blend of odor components is crucial to provoke a certain behavioral response (Visser and Avé, 1978; Saxena and Goyal, 1978).

Linalool, dodecanol, α-humulene, and germacrene D were the compounds emitted in higher amounts by damaged *A. alliariae* leaves compared to undamaged leaves. In freshly damaged *A. alliariae* leaves with enhanced attractiveness, more α-humulene and germacrene D were emitted compared to (*E,E*)-α-farnesene whereas in the less attractive *A. alliariae* undamaged and overnight-damaged plants, more (*E,E*)-α-farnesene was emitted compared to α-humulene and germacrene D. This reverse relationship between those sesquiterpenes might be responsible for the enhanced attractiveness of freshly damaged *A. alliariae* leaves.

Most of the compounds that we found after an attack of *O. cacaliae* in its host plants have been described for damaged host plants of other systems. For instance the terpene (*E,E*)-α-farnesene is released by damaged crabapple and by *Psylla*-infested pear trees (Loughrin et al., 1995; Scutareanu et al., 1997), (*Z*)-3-hexenyl acetate was found to be associated with herbivory in maize (Turlings et al., 1990), and the sesquiterpene (-)-germacrene D appears to mask the attractiveness of undamaged pine trees to *Monochamus alternatus* (Coleoptera: Cerambycidae) (Yamasaki et al., 1997).

The fact that damaged host plants are more attractive than undamaged plants may promote aggregation of *O. cacaliae* on plants suitable for feeding and larval

development. The difference in the attractiveness of the two host plants with respect to time since attack corresponds nicely with field observations of beetle densities on plants. Dozens of beetles often feed on a single flowering *P. paradoxus* plant in spring, while only a few beetles (2–4) were ever observed on *A. alliariae* plants. The long-lasting attraction of flowering *P. paradoxus* early in the season may facilitate mating in *O. cacaliae.* It might be especially rewarding for the females to remate in spring with a male who successfully overwintered, to increase the fitness of their offspring (Stevens and Cauley, 1989).

Acknowledgments—We thank Sandrine Gouingueuné for the GC-MS analyses and Jonathan Gershenzon at the Max-Planck-Institut für Chemische Ökologie in Jena, Germany, for access to equipment. Ann Fraser, Bernd Hägele, and Håkan Häggström made helpful comments on the manuscript.

REFERENCES

BOLTER, C. J., DICKE, M. V. LOON, J. J. A. VISSER J. H., and POSTHUMUS, M. A. 1997. Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. *J. Chem. Ecol.* 23:1003–1023.

BORDEN, J. H. 1984. Semiochemical-mediated aggregation and dispersion in the Coleoptera, pp. 123–149, *in* T. Lewis (ed.). *Insect Communication.* Academic Press, Orlando, Florida.

DOBLER, S., and ROWELL-RAHIER, M. 1994. Production of cardenolides versus sequestration of pyrrolizidine alkaloids in larvae of *Oreina* species (Coleoptera, Chrysomelidae). *J. Chem. Ecol.* 20:555–568.

DOMEK, J. M., and JOHNSON, D. T. 1988. Demonstration of semiochemically induced aggregation in the green June beetle, *Cotinis nitida* (L.) (Coleoptera, Scarabaeidae). *Environ. Entomol.* 17:147–149.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1991. Chemical defence and genetic variation. *Naturwissenschaften* 78:317–320.

EGGENBERGER, F., and ROWELL-RAHIER, M. 1993. Physiological sources of variation in chemical defense of *Oreina gloriosa* (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 19:395–410.

EHMKE, A., ROWELL-RAHIER, M. PASTEELS J. M., and HARTMANN, T. 1991. Sequestration of ingested [^{14}C]senecionine *N*-oxide in the exocrine defensive secretions of chrysomelid beetles. *J. Chem. Ecol.* 17:2367-2379.

HARARI, A. R., BEN-YAKIR D., and ROSEN, D. 1994. Mechanism of aggregation behavior in *Maladera matrida* Argaman (Coleoptera: Scarabidae). *J. Chem. Ecol.* 20:361–371.

HEATH, R. R., and MANUKIAN, A. 1992. Development and evaluation of systems to collect volatile semiochemicals from insects and plants using a charcoal-infused medium for air purification. *J. Chem. Ecol.* 18:1209–1126.

JONES, O. T. 1985. Chemical mediation of insect behavior, pp. 311–373, in D. H. Hutson and T. R. Roberts (eds.). Progress in Pesticide Biochemistry and Toxicology, Vol. 5, Insecticides. Wiley, New York.

KALBERER, N. M. 2000. Host-plant recognition, dispersal and its influence on reproduction in the alpine leaf beetle *Oreina cacaliae.* PhD thesis. University of Neuchâtel, Neuchâtel, Switzerland.

LOHSE, G. A., and LUCHT, W. H. 1994. page 73, *in* Goecke and Evers (ed.). *Die Käfer Mitteleuropas* 14. Krefeld.

LOUGHRIN, J. H., POTTER D. A., and HAMILTON-KEMP, T. R. 1995. Volatile Compounds induced by Herbivory act as Aggregation Kairomones for the Japanese beetle (*Popillia japonica* Newman). *J. Chem. Ecol.* 21:1457–1467.

MILLER, J. R., and STRICKLER, K. L. 1984. Finding and accepting host plants, *in* W. J. Bell and R. T. Cardé (eds.). *Chemical Ecology of Insects.* Chapman and Hall, New York. pp. 127–155.

PARE, P. W., and TUMLINSON, J. H. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114:1161–1167.

PASTEELS, J. M., ROWELL-RAHIER, M., BRAEKMAN, J.-C., and DALOZE, D. 1994. Chemical defense of adult leaf beetles updated, pp. 298–301, *in* P. Jolivet, M. Cox, and E. Petitpierre (eds.). *Novel Aspects of the Biology of Chrysomelidae,* Kluwer, Dordrecht.

PENG, C., and WEISS, M. J. 1992. Evidence of an aggregation pheromone in the flea beetle, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 18:875–884.

SAXENA, K., and GOYAL, N. S. 1978. Host-plant relations of the citrus butterfly *Papilio demolus* L.: Orientation and ovipositional responses *Entomol. Exp. Appli.* 24:1–10.

SCUTAREANU, P., DRUKKER, B., BRUIN, J., POSTHUMUS, M. A., and SABELIS, M. W. 1997. Volatiles from *Psylla*-infested pear trees and their possible involvement in attracting of anthocorid predators. *J. Chem. Ecol.* 23:2241–2260.

STEVENS, L., and CAULEY, D. E. M. 1989. Mating prior to overwintering in the important willow leaf beetle, *Plagiodera versicolora* (Coleoptera: Chrysomelidae). *Ecol. Entomol.* 14:219–223.

TAKABAYASHI, J., DICKE M., and POSTHUMUS, M. A. 1994. Volatile herbivore-induced terpenoids in plant-mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol.* 20:1329–1354.

TURLINGS, T. C. J., TUMLINSON J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odours by host-seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., BERNASCONI, M., BERTOSSA, R., BIGLER, F., CALOZ, G., and DORN, S. 1998. The induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. *Biol. Control* 11:122–129.

VISSER, J. H., and AVÉ, D. A. 1978. General green leaf volatiles in the olfactory orientation of the Colorado beetle, *Leptinotarsa decemlineata. Entomol. Exp. Appl.* 24:538–549.

VISSER, J. H., and NIELSON, J. K. 1977. Specificity in the olfactory orientation of the CPB. *Entomol. Exp. Appl.* 21:14–22.

VISSER, J. H., STRATEN S. V., and MAARSE, H. 1979. Isolation and identification of volatiles in the foliage of potato, *Solanum tuberosum,* a host plant of the Colorado beetle, *Leptinotarsa decemlineata. J. Chem. Ecol.* 5:13–25.

YAMASAKI, T., SATO, M., and SAKOGUCHI, H. 1997. Germacrene D: Masking substance of attractants for the cerambycid beetle, *Monochamus alternatus* (Hope). *Appl. Entomol. Zool.* 32:423–429.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

HOST LOCATION AND HOST DISCRIMINATION BEHAVIOR OF *Telenomus isis*, AN EGG PARASITOID OF THE AFRICAN CEREAL STEM BORER *Sesamia calamistis*

A. CHABI-OLAYE,[1,2] F. SCHULTHESS,[1] H.-M. POEHLING,[2] and C. BORGEMEISTER[2,*]

[1]*International Institute of Tropical Agriculture (IITA)*
Plant Health Management Division (PHMD)
08 BP 0932 Tripostal
Cotonou, Republic of Benin, West Africa

[2]*University of Hanover*
Institute of Plant Diseases and Plant Protection
Herrenhäuser Str. 2, 30419 Hanover, Germany

(Received August 8, 2000; accepted December 15, 2000)

Abstract—In the Republic of Benin, the scelionid egg parasitoid *Telenomus isis* (Polaszek) is one of the most important control factors of the noctuid maize stem borer *Sesamia calamistis.* In the present study, the role of various sources of contact kairomones (male or virgin or mated female moths) and of the moth's oviposition substrate (leaf sheath versus filter paper; host plant species) in host location and oviposition behavior of *T. isis* was investigated in Munger cells, open arenas, and/or Petri dish assays. Furthermore, its ability to distinguish between unparasitized eggs and eggs parasitized by a conspecific female or by the trichogrammatid *Lathromeris ovicida* was studied. In the Munger cell experiment, *T. isis* spent more time in moths' odor fields than in the control. There was no difference between virgin and mated females. In the open arena assay, traces left by both the male and female moths acted as contact cues, which elicited an arrestment response in the parasitoid. The residence and patch retention time in the arena with virgin or mated females of *S. calamistis* was about 4.8 times as long as that with males. The presence of maize leaf sheaths stimulated the oviposition behavior of *T. isis* when compared to eggs offered on filter paper. During the first 6 hr, more eggs were parasitized on maize leaves, although there was no difference in the final number of offspring between the two substrates. In addition, if eggs of *S. calamistis* were offered together with different host plant species or alone, maize and sorghum were both more attractive than millet or the egg alone and equally attractive between themselves, indicating that the plant tissue influences host finding of *T. isis.* Both *T. isis* and *L. ovicida* recognized

*To whom correspondence should be addressed. e-mail: borgemeister@ipp.uni-hannover.de

0098-0331/01/0400-0663$19.50/0

markings of conspecific females, and intraspecific superparasitism was therefore low. Interspecific superparasitism was more than three times higher for *L. ovicida* than for *T. isis*, indicating that only *T. isis* was able to recognize the marking of the other species and tried to avoid superparasitism. Emergence of parasitoids from multiparasitized eggs generally was in favor of *L. ovicida* regardless of species order.

Key Words—*Telenomus isis*, *Lathromeris ovicida*, *Sesamia calamistis*, host location, host discrimination, superparasitism, West Africa.

INTRODUCTION

In southern Benin, the solitary scelionid egg parasitoids *Telenomus busseolae* (Gahan) (Hymenoptera: Scelionidae) and *T. isis* (Polaszek) (Hymenoptera: Scelionidae) are the most important natural control factors of the maize stem borer *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Sétamou and Schulthess, 1995; Schulthess et al., 1997). A third species commonly found on *S. calamistis* eggs, but of low importance, is the gregarious trichogrammatid *Lathromeris ovicida* Risbec (Hymenoptera: Trichogrammatidae). *T. busseolae* is a relatively well-described species known as an important biological control agent of several noctuid pests throughout Africa as well as Greece and the Middle East (Alexandri and Tsitsipis, 1990; Kornosor et al., 1994; Polaszek, 1998). In contrast, *T. isis* was described only in 1993 (Polaszek et al., 1993) and, so far, has been reported only from West Africa, including Cameroon (Polaszek, 1998; Ndemah, 1999). As a result, there is little information about the biology and ecology of this species, although, as shown for southern Benin, it can contribute significantly to reducing noctuid stem borer populations on maize (Schulthess et al., 1997).

Southern Benin is characterized by derived savanna vegetation, which forms a continuum of wild and cultivated host plants of *S. calamistis*, most of them belonging to the family Poacea. Both the generally low egg batch densities, ranging between 0.01 and 0.1 per tiller (Schulthess et al., 2000) and their regular dispersion pattern (Sétamou and Schulthess, 1995) make host finding difficult. Nevertheless, in region-wide surveys in southern Benin in 1995–1996, up to 62% of the egg batches were parasitized by *T. isis*, indicating good host-finding ability (Schulthess et al., 2000). It is not clear how *T. isis* detects its host. The importance of chemical stimuli orienting females to their host has been documented for several egg parasitoids species (Noldus and van Lenteren, 1985; Aldrich et al., 1984; Mattiaci et al., 1993). These stimuli vary in their information content and in their detectability. Stimuli from the host itself are more reliable but generally difficult to detect by the parasitoid, whereas stimuli from the host microhabitat or food plants can be detected easily, but generally carry less host-specific information (Vet et al., 1991; Vet and Dicke, 1992; Wäckers and Lewis, 1994). The first objective of this

study was to investigate the potential sources of infochemicals used by *T. isis* in its host location process by testing virgin or mated females moths and male moths, as well as several host plants of *S. calamistis.*

Surveys showed that ca. 15% of the *S. calamistis* egg batches were detected and parasitized by up to three species (Schulthess et al., 2000), and it was theorized that this may have been a result of superparasitism. Agboka (1998) demonstrated, however, that both *T. isis* and *T. busseolae* were able to detect eggs already parasitized by a conspecific female or another *Telenomus* species and, thus, tended to avoid superparasitism. If superparasitism occurred, *T. busseolae* won over *T. isis,* irrespective of species order. During the surveys, *L. ovicida* was more often found together with *T. isis* than *T. busseolae.* Thus, a second objective of this study was to assess the ability of the two species to discriminate between parasitized and nonparasitized eggs and the outcome in the case of interspecies superparasitism. This study is part of a larger project to assess the chances of *T. isis* to becoming established in East and southern Africa.

METHODS AND MATERIALS

Insect Colonies. Eggs of *S. calamistis* were obtained from a colony maintained on an artificial diet at the International Institute of Tropical Agriculture (IITA), Calavi, Benin, following the protocol developed by Bosque-Pérez and Dabrowski (1989). Adults of both *T. isis* and *L. ovicida* were reared on *S. calamistis* eggs according to the method of Chabi-Olaye et al. (1997). The parasitoids originated from parasitized eggs of *S. calamistis* collected in the derived savanna in southern Benin. For the behavioral assays, female and male pupae of *S. calamistis* were isolated in plastic containers (5 cm high $\times$ 18 cm diameter), and kept at $27 \pm 1^{\circ}$C, $75 \pm 5\%$ relative humidity, and 10D:14L photoperiod. After emergence, three groups of moths were tested: newly emerged males, virgin females, and mated females.

Munger Cells Behavioral Assays. Three-chamber Munger cells were used to investigate the response of *T. isis* females to moths of *S. calamistis.* The device is a transparent glass plate (10 cm long $\times$ 5 cm wide $\times$ 1 cm thick) composed of three cells (2 cm diameter $\times$ 1 cm deep), i.e., a central cell, and two side cells. Each side cell is situated 0.5 cm from the central cell and 1.5 cm from the edge of the device. The device is provided with a longitudinal ventilation system (0.4 cm diameter), consisting of two interior air ducts connecting each side cell to the central cell, and two side air ducts from the side cells to the outside. The top and the bottom of the device were covered with two transparent glass plates (10 cm long $\times$ 5 cm wide $\times$ 0.3 cm thick).

The experiment consists of four tests (virgin female versus control field, mated female versus control field, male versus control field, and virgin female

versus mated female of *S. calamistis*). For the first three tests, the bait, i.e., a male, a virgin female, or a mated female moth was placed in one of the side cells, while the second cell served as the control (control field). In the last test, the mated and virgin females of *S. calamistis* were placed separately in each side cell. One ca. 0.5-day-old, naïve, mated female parasitoid was introduced into the central cell and was allowed 10 min to choose one of the side cells. Thereafter, the wasp was discarded whether or not it had made a choice. A choice was defined when a wasp had crossed any interior air duct, penetrated into a side cell, and remained there for at least 2 sec. After every five observations, the device was rotated to prevent bias due to positional factors. A minimum of 30 wasps was observed for each tested bait type. After each experiment, the whole system was cleaned with chloroform and neutral soap. All tests were conducted at 26 $\pm$ 1°C and 65 $\pm$ 5% relative humidity under permanent light conditions. Data were gathered on the proportion of wasps that made a choice for each side cell and the time spent by wasps in each side cell.

Open Arena Behavioral Assays. Arena experiments were conducted on filter paper (18.6 cm diameter). The parasitoids could easily move within this area or fly away. In the center of the filter paper, a circular area 9.3 cm diameter was either left untreated or treated in the following way: one adult *S. calamistis* was exposed under a plastic cover (9.3 cm diameter $\times$ 1 cm high) to the circular area for 1 hr. All bioassays were performed with naïve, mated, female parasitoids ca. 0.5 days old collected from the stock culture. One individual was placed in the middle of the treated area with a camel-hair brush. The response of the parasitoid was determined by monitoring the retention of the wasp on the treated surface (patch retention time) and in the experimental arena (arena residence time) by using a stopwatch. The measurement began when the wasp touched the treated surface and was terminated when it left the arena. Each observation lasted 15 min. Each treatment (i.e., filter paper treated with a male, virgin female, or mated female *S. calamistis*) was replicated 42 times. All experiments were conducted at 27 $\pm$ 1°C and 65 $\pm$ 5% relative humidity regime.

The arena residence time was defined as the time from when a female parasitoid entered the arena until it flew away or walked out of the arena. The patch retention time is the total time the female parasitoid spent in the treated surface.

Response of T. isis *to* S. calamistis *Oviposition Substrate.* Prior to this experiment, maize stems collected in a nearby field were exposed overnight to *S. calamistis* adults for oviposition. Leaf sheaths containing one egg mass with approximately 100 eggs were selected for subsequent testing. One naïve, mated female parasitoid ca. 0.5 days old was placed with a camel-hair brush in a vial (11 cm long $\times$ 4.5 cm diameter) containing 100 eggs of *S. calamistis* either on maize leaf sheaths or on filter paper. After 6 hr, eggs were renewed, and then provided ad libitum until the death of the female. Removed eggs were transferred to another vial of similar dimensions and incubated at 28 $\pm$ 1°C and 70 $\pm$ 5%. Each

treatment was replicated 20 times for each substrate. Six traits were observed, i.e., percent parasitism after 6 hr, time to first oviposition (in seconds), total number of offspring, percent emergence, oviposition time, and sex ratio of F_1 progeny.

Relative Attractiveness of Different Host Plants of Stem Borers to Female T. isis. A four-arm olfactometer, similar to that described by Scholz et al. (1997), was used to investigate the attractiveness of odors from different host plants of cereal stem borers for *T. isis* females. The experiment was designed to test the following assumptions: (1) that *S. calamistis* eggs with leaf sheaths of different host plants of stem borers are more attractive than eggs without leaf sheaths, and (2) that attraction varies among the three different host plants tested. The device has been used successfully in several studies on the host-finding behavior of Coleoptera (e.g., Scholz et al., 1998; Borgemeister et al., 1999). Females were released into the central arena through a hole, which was drilled into the bottom of the olfactometer. Five minutes before the females were released into the olfactometer, the odor sources were introduced into the device to ensure a gradient of volatiles. Each bait was introduced into a 500-ml jar. Four different baits were tested simultaneously. Initially, the different baits were distributed at random among the four jars. After 25, 50, and 75% of the replications, the positions of the odor sources were permutated to reduce bias due to environmental factors such as light. The following odor sources were tested: (1) eggs of *S. calamistis*, (2) maize leaf sheaths with eggs of *S. calamistis*, (3) sorghum leaf sheaths with eggs of *S. calamistis*, and (4) millet leaf sheaths with eggs of *S. calamistis*. Eggs of *S. calamistis* were artificially released in the leaf sheath of each host plant, and the control jar contained only one egg of *S. calamistis*. Before and after use, the olfactometer and the jars containing the different odor sources were cleaned with methanol, rinsed in hot water, and dried in an oven at 45°C. Experiments were conducted in a climatic chamber (25°C, $70 \pm 5\%$ relative humidity) with a light source above the olfactometer. In total, 20 female *T. isis* were tested (i.e., 20 replicates). Each observation lasted 15 min, and the total time spent in each odor field was recorded.

Host Discrimination by T. isis *and* L. ovicida. Both intraspecific and interspecific host discrimination was tested. The objective of the study on intraspecific competition was to examine the response of a *T. isis* and a *L. ovicida* female to eggs previously parasitized by a conspecific female. Only experienced females were used. Parasitoids were considered experienced after they had oviposited in and had marked at least one host egg. According to Bosque and Rabinovich (1979), oviposition experience improves the ability of egg parasitoids to discriminate between parasitized and nonparasitized hosts. A 12 to 24-hr-old egg mass of *S. calamistis*, containing ca 20 eggs was exposed to a female parasitoid in a glass vial (28 mm long $\times$ 23 mm diameter). All eggs within an egg mass were numbered in order to observe the oviposition sequence of a particular parasitoid. The first female was allowed to probe and mark half of the egg mass and was then removed. After 1 hr, a second female was introduced to the egg mass, and the ovipositional

behavior was observed. The interspecific host discrimination study was carried out to examine the response of a *T. isis* female to egg masses previously parasitized by *L. ovicida* and vice versa.

Parasitized eggs were removed and incubated in a glass vial at $27 \pm 1°C$ and $75 \pm 5\%$ relative humidity. Emergence of parasitoids from eggs parasitized by the two females was recorded for both conspecific and interspecific pairs. Upon emergence, all individuals were identified and sexed. *Telenomus isis* adults were isolated from *L. ovicida* according to Polaszek et al. (1993). The time between removal of the first parasitoid and introduction of the second parasitoid was fixed at 1 hr. Both experiments were replicated 10 times.

Statistical Analysis. In the Munger cells bioassay, differences in wasp choices were analyzed by means of a χ^2 test, and the mean percent time spent in tested fields with Wilcoxon signed ranks test. Differences in patch retention time, residence time, percent parasitism, time to first oviposition, total number of offspring, percent eggs with parasitoid emergence, the oviposition time, and the sex ratio of the F_1 progeny for different ovipositional substrates were analyzed by analysis of variance (ANOVA), by using the general linear model (GLM) procedure of SAS (SAS Institute, 1992). In case of significant *F* values, means were compared by using the Student-Newman-Keul test. Friedman two-way analysis of variance by ranks, based on time spent by female *T. isis* per odor field, was used to test for field preferences in the four-chamber olfactometer experiment. Mean time spent per odor field was compared by using Wilcoxon signed ranks test. The significant level was set at $P = 0.05$.

For host discrimination behavior, the numbers of ovipositions per female, expressed as probing and marking (see Agboka, 1998), in parasitized and nonparasitized eggs (eggs acceptance) were calculated for each treatment and compared by means of a χ^2 test. Emerged parasitoids from multiparasitized host eggs were compared by using a *t* test with a Bonferroni probability adjustment procedure at $P = 0.05$ (SAS Institute, 1992).

RESULTS

Responses of T. isis *to Adult* S. calamistis *Exposed in Munger Cells.* In a control position (empty cells), *T. isis* females did not show any preference for either side cell and spent about equal percentage of their time in each (12.4 ± 3.7 right vs. 12.1 ± 3.3 left, $N = 20$, $Z = 0.112$, $P = 0.911$). Similarly, no differences in the percentage of first choices of the parasitoids were recorded between the control field and test fields containing a male, virgin female, or mated female *S. calamistis* (Figure 1). However, *T. isis* females spent more time in the side cell containing any of the tested type of *S. calamistis* adults than in the control field (Figure 2). Yet,

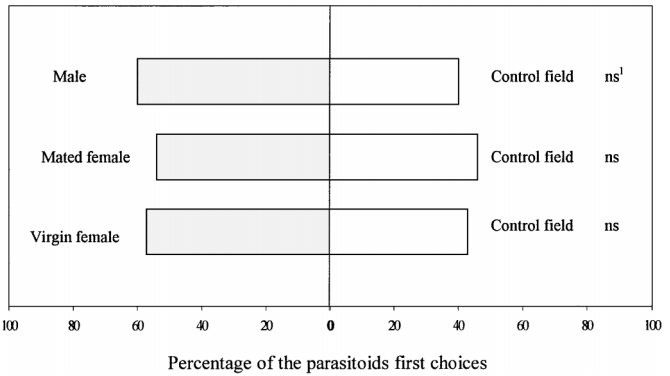


FIG. 1. Percentage of *T. isis* females' first choices in each side cells of the Mugen cell to males, virgin females and mated females of *S. calamistis.*[1] not significant; parasitoid choices were analyzed with χ^2 test.

no differences in the time spent were observed between cells containing a virgin and a mated female (Figure 2).

Responses of T. isis *to Contact Kairomones of* S. calamistis *in an Open Arena.* In a control situation, i.e., without the presence of contact kairomones of *S. calamistis* adults, the patch area did not elicit any response from *T. isis.* The parasitoids spent little time in the patch area (mean 18.1 sec.), walking fast, and left the arena after an average of 44.4 sec (Table 1). In presence of contact kairomones of *S. calamistis* adults, however, female parasitoids were arrested in the patch area

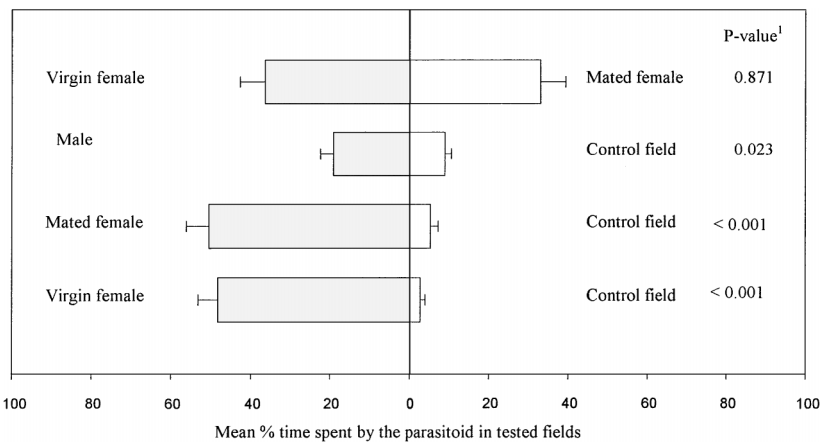


FIG. 2. Response of *T. isis* to males, virgin females and mated females of *S. calamistis.* [1]Wilcoxon signed ranks test for pairwise comparison.

TABLE 1. ARENA RESIDENCE TIME, PATCH RETENTION TIME, AND DURATION OF STOP OF *T. isis* IN PATCH TREATED WITH MALE, VIRGIN FEMALE, OR MATED FEMALE OF *S. calamistis*

	Type of baits			
	Control	Virgin female	Mated female	Male
Residence time (sec)	44.4 ± 3.25 a[a]	599.1 ± 0.72 b	598.2 ± 0.89 b	124.0 ± 8.50 c
Patch retention time (sec)	18.1 ± 1.45 a	577.3 ± 3.42 b	577.1 ± 3.61 b	57.6 ± 5.33 c
Duration of stops (sec)		124.1 ± 7.40 a	121.6 ± 7.30 a	7.1 ± 0.86 b

[a]Within row, means followed by different letters are significantly different at $P = 0.05$ (Student-Newman-Keul test).

and stayed longer (Table 1). The response of the wasp began with a stop. After a few seconds, it began to walk very slowly within the treated patch area, intensively searched the treated and eventually the surrounding area, and then returned to the patch area. Wasps interrupted their searching behavior with stops while searching intensively the treated patch areas. Each parasitoid female displayed 0–11 stops. All parasitoid females made stops when the patch area had been previously exposed to a virgin female or a mated female *S. calamistis.* In contrast, only 16.7% of the wasps tested made a stop in the patch area previously exposed to a male *S. calamistis.* Each stop lasted on average of 124.1, 121.6, and 7.1 sec. after prior exposure of the patch area to a virgin female, mated female, or male, respectively, and was significantly higher for the two female treatments compared to the male (Table 1). The residence time in the arenas treated with virgin or mated females was about 4.8 times longer than that of arenas treated with a males (Table 1). The patch retention time followed the same pattern.

Response of T. isis *to* S. calamistis *Oviposition Substrate.* The presence of maize leaf sheaths stimulated the oviposition behavior of *T. isis* on *S. calamistis* eggs. Within the first 6 hr of exposure, oviposition on eggs on leaf sheaths commenced earlier than on filter paper, and consequently more eggs were attacked on maize leaf sheaths than on filter paper (Table 2). However, no differences in the total number of offspring, the percentage of emergence, or the sex ratio of the F_1 progeny were recorded between the two treatments, but mean oviposition time was longer for females on filter paper than on maize leaf sheaths (Table 2).

Relative Attractiveness of Different Host Plants of Stem Borers for Female T. isis. Female parasitoids preferred odors from *S. calamistis* eggs accompanied by certain host plants of stem borers (Figure 3). The wasps spent more time in the maize and *S. calamistis* eggs and in the sorghum/eggs compared to the millet/eggs and the *S. calamistis* eggs only fields.

Host Discrimination by T. isis *and* L. ovicida. Both females of *L. ovicida* and *T. isis* were able to discriminate between host eggs that had been previously

TABLE 2. RESPONSE OF *T. isis* TO *S. calamistis* EGGS EXPOSED ON TWO DIFFERENT OVIPOSITIONAL SUBSTRATES

	Ovipositional substrates	
	Maize leaf sheaths	Filter paper
Eggs attacked after 6 hr (*N*)	63.1 ± 1.19 a[b]	47.2 ± 2.06 b
Time to first oviposition (sec)	23.7 ± 1.53 a	123.2 ± 12.9 b
Total offspring (*N*)	85.9 ± 1.85	82.7 ± 2.32
Emergence (%)	99.8 ± 0.10	99.7 ± 0.20
Sex ratio[a]	89.1 ± 0.67	88.9 ± 0.32
Oviposition time (days)	2.5 ± 0.14 a	4.6 ± 0.27 b

[a]Percentage of females in total progeny.
[b]Within row, means followed by different letters are significantly different at $P = 0.05$ (Student-Newman-Keul test).

parasitized by a conspecific female. *T. isis* accepted 79.9% and 8.8% of the unparasitized and parasitized eggs, respectively, vs. 68.3% and 27.4% for *L. ovicida* (Table 3). Hence, superparasitism in *T. isis* was lower than in *L. ovicida* ($F = 25.47$, $df = 18$, $P < 0.001$).

When female *L. ovicida* were exposed to egg masses partially parasitized by *T. isis*, they displayed no discrimination behavior, as indicated by a high acceptance of already parasitized and unparasitized eggs (74.7 and 68.7%, respectively; Table 4). In contrast, *T. isis* displayed high discrimination behavior towards eggs already parasitized by *L. ovicida*. *T. isis* females rejected up to 77.6% of the eggs already parasitized by *L. ovicida*, whereas 57.2% of the unparasitized eggs were accepted (Table 4).

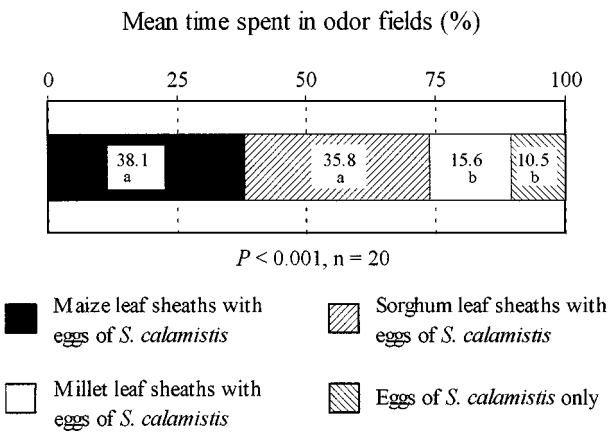


FIG. 3. Response of *T. isis* to different host plants with eggs and eggs alone. Percentages followed by different letters are significantly different.

TABLE 3. RESPONSE OF *L. ovicida* AND *T. isis* TO UNPARASITIZED EGGS AND EGGS PARASITIZED BY CONSPECIFIC FEMALE

Species	Host eggs[a]	Egg acceptance (mean %)
L. ovicida	Unparasitized	68.3 ± 3.23
	Parasitized	27.4 ± 2.50
		($\chi^2 = 31.81$; $P < 0.001$)
T. isis	Unparasitized	79.9 ± 3.77
	Parasitized	8.8 ± 2.69
		($\chi^2 = 100.1$; $P < 0.001$)

[a]For each species a total of 10 egg batches were observed.

The percentage of emergence did not vary with the sequence of introduction of the two parasitoid species (Table 5). Irrespective of which parasitoid species was first introduced, a higher proportion of *L. ovicida* offspring emerged from the multiparasitized eggs (Table 5). Moreover, in 80% of the cases, two *L. ovicida* emerged from a single *S. calamistis* egg. In *T. isis*, in all cases, only one adult parasitoid emerged per host egg.

DISCUSSION

In the Munger cell experiments, no significant differences were found in first choice between control and the different moth types. Host-finding behavior of egg parasitoids is often governed by long-range chemical cues such as sex pheromones (Lewis et al., 1982; Colazza et al., 1997). However, as shown by Babilis and Mazomenos (1992) for the noctuid *Sesamia nonagrioides* (Lefebvre), the production of sex pheromones is often influenced by the photoperiod. Female moths started calling about 4–6 hr after onset of scotophase, while our bioassays were carried out during the photophase. The wasps spent significantly more time in

TABLE 4. RESPONSE OF *L. ovicida* AND *T. isis* TO UNPARASITIZED EGGS AND EGG PARASITIZED BY THE OTHER SPECIES

Species	Host eggs[a]	Egg acceptance (mean %)
L. ovicida	Unparasitized	68.7 ± 4.98
	Parasitized by *T. isis*	74.7 ± 3.77
		($\chi^2 = 0.7909$; $P = 0.3738$)
T. isis	Unparasitized	57.2 ± 6.72
	Parasitized by *L. ovicida*	22.4 ± 4.66
		($\chi^2 = 58.40$; $P < 0.001$)

[a]For each species a total of 10 egg batches were observed.

TABLE 5. EMERGENCE OF MULTIPARASITIZED EGGS OF *S. calamistis* BY *T. isis* AND *L. ovicida* AND PROPORTION OF PROGENY WITH REGARD TO SEQUENCE OF INTRODUCTION OF TWO PARASITOIDS (Ti = *T. isis* AND Lo = *L. ovicida*)

	Order of species	
	Ti/Lo	Lo/Ti
% Emergence	63.8 ± 3.84	70.4 ± 11.38
% Progeny		
% *T. isis*	37.3 ± 5.72 a[a]	29.2 ± 12.9 a
% *L. ovicida*	62.7 ± 5.72 b	70.8 ± 12.9 b

[a]Within column means followed by different letter are significantly different at $P = 0.05$ (t-test followed by a Bonferroni probability adjustment).

the cells containing adult *S. calamistis* than in the empty cells, however, suggesting that the response pattern of the parasitoids was induced through physical contact with or through kairomones produced by the moth (Noldus et al., 1990; Gazit et al., 1996). Often *T. isis* females mounted on the wings of *S. calamistis* females, searched actively, or stopped in front of their abdomen for a long time. Such a behavioral pattern suggests that the moth scales and the abdomen of adult female may be sources of chemical cues that elicit arrestment behavior by *T. isis.* Scales of various moth species contain contact kairomones that arrest egg parasitoids (Jones et al., 1973; Shu and Jones, 1989). This is also corroborated by the results from the open arena experiments, which showed that traces of *S. calamistis* moths acted as contact kairomones, eliciting an arresting response and induced the wasps to remain longer in the arena. An arrestment response elicited by cues from host stages other than eggs is common in *Telenomus* spp. (Gazit et al., 1996; Arakaki et al., 1996). The role of contact kairomones for guiding parasitoids to their hosts eggs has been investigated by various authors (e.g., Lewis et al., 1972; Jones et al., 1973; Beevers et al., 1981; Noldus and van Lenteren, 1985; Zaborski et al., 1987).

Host eggs are not easily detected because they do not emit long-range volatiles (Noldus, 1989; Kaiser et al., 1989) and in the case of *S. calamistis* are hidden within the leaf sheath. The present finding suggests that the parasitoid is attracted by unspecific chemical clues, volatiles emanating from the plants rather than the host. Volatiles play an important role in the long-distance orientation and host location of parasitoids (e.g., Vinson 1976; Tumlinson et al., 1992; Vet and Dicke, 1992; Turlings et al., 1995). The presence of the host plant also stimulated the oviposition behavior of *T. isis* and oviposition happened significantly faster. In addition, the parasitoid distinguished between host plant species. The nonpreference for millet makes sense. This crop is prevalent in the Sudan savanna and Sahel, which is outside the geographic range of *S. calamistis,* and the major stem-boring pest in these zones, the crambid *Coniesta ignefusalis* Hampson, is not attacked by *T. isis*

(Chabi-Olaye et al., 2000). Both millet and sorghum are indigenous to Africa, but maize originated in Meso-America, and in most of West Africa became important only in the 20th century. Thus, the equal preference for maize and sorghum appears not to be logical. However, *S. calamistis* is highly polyphagous and is found on many gramineous species (Schulthess et al., 1997) that may have similar volatile profiles. The host plant preference indicates that *T. isis* females increase their foraging activity in areas or on plants where host egg masses are more likely to be present.

Avoidance of superparasitism is only possible when a parasitized host egg can be recognized by a parasitoid female. Such recognition is often based on the marking of host eggs after oviposition (Strand and Vinson, 1983; Okuda and Yeargan, 1988; Higuchi and Suzuki, 1996; Agboka, 1998). Our results show that both *T. isis* and *L. ovicida* were highly efficient in recognizing marks of conspecific females. The ability of parasitoids to recognize host eggs already parasitized by conspecifics prevents females from wasting their eggs (van Lenteren, 1981). Consequently, parasitoids will disperse in areas where suitable eggs are available. However, in situations with a low probability of finding unparasitized host stages and a certain likelihood of survival of offspring of the second female in superparasitized hosts, intraspecific superparasitism may be an adaptive strategy (Bakker et al., 1985; van Alphen and Visser, 1990; Visser et al., 1990; Visser, 1993).

Interspecific mark recognition is rare in nature and often occurs among closely related species (Rosen and DeBach, 1979). Our results show that, in contrast to *L. ovicida*, *T. isis* females tend to avoid eggs already parasitized by an interspecific female. Similarly, in a study by Agboka (1998), *T. isis* rejected up to 82.8% of eggs parasitized by *T. busseolae.* Bakker et al. (1985) argued that interspecific host discrimination is not an evolutionarily stable strategy. Hence, species that start to discriminate interspecifically would face a disadvantage. This implies that the behavior of *T. isis* may be an adaptation strategy for its oviposition. A significantly greater proportion of *L. ovicida* emerged from the offspring of multiparasitized eggs of *S. calamistis*, regardless of the sequence of introduction of the two parasitoid species. Similar results were obtained by Agboka (1998) when the same eggs were parasitized by *T. isis* and *T. busseolae.* Thus, efforts by *T. isis* to superparasitize eggs already parasitized by another sympatric species are not likely to be productive and, thus, interspecific mark recognition is an advantage.

In contrast to *T. isis*, which only parasitizes noctuid eggs laid within leaf sheaths of grasses, *L. ovicida* has been reported from the pyralids *Chilo zacconius* Bleszynski, *Scircophaga subumbrosa* Meyrick, and *Maliarpha separatella* Ragonot that oviposit on the lower or upper surfaces of leaves (Polaszek, 1998). In Benin, parasitism by *L. ovicida* was low, ranging between 1.6 and 5.3%, and parasitism within parasitized egg batches was, on average, only 27% vs. >70 for *T. isis* and *T. busseolae* (Schulthess et al., 2000). In 93% of the cases on maize and 88% on grasses, *L. ovicida* was obtained from egg batches also parasitized by

another *Telenomus* species and mainly by *T. isis.* It appears that the trichogrammatid is not only attracted by the female moth, but also by the other parasitoid species, and the question arises whether *L. ovicida* is a facultative hyperparasitoid.

We conclude that the intra- and interspecific mark recognition ability of *T. isis*, coupled with its ability to respond to volatiles of the host plant and the contact kairomones of its host *S. calamistis* for host location, could make it a promising candidate for future biological control programs outside its area of distribution.

Acknowledgments—We thank G. Goergen (IITA) for help in identifying *T. isis* and *L. ovicida.* The research was supported by IITA's Maize Health Management Project, funded by the International Fund of Agricultural Development (IFAD). A. Chabi-Olaye was partly supported by a grant of the German Academic Exchange Service (DAAD).

REFERENCES

AGBOKA, K. 1998. Etude du comportement compétitif de *Telenomus busseolae* Gahan et de *Telenomus isis* Polaszek (Hymenoptera: Scelionidae), deux parasitoides des oeufs de *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae). Mémoire d'Ingénieur Agronome, Université du Bénin, Lomé, Togo, pp. 72.

ALDRICH, J. R., KOCHANSKY, J. P., and ABRAMS, C. B. 1984. Attractant for a beneficial insect and its parasitoids: Pheromone of the predatory spined soldier bug, *Podisus maculiventris* (Hemiptera: Pentatomidae). *Environ. Entomol.* 13:1031–1036.

ALEXANDRI, M. P., and TSITSIPIS, J. A. 1990. Influence of the egg parasitoid *Platytelenomus busseolae* (Gahan) (Hym.: Scelionidae) on the populations of *Sesamia nonagrioides* Lef. (Lep.: Noctuidae) in Central Greece. *Entomophaga* 35:16–25.

ARAKAKI, N., WAKAMURA, S., and YASUDA, T. 1996. Phoretic egg parasitoid, *Telenomus euproctidis* (Hymenoptera: Scelionidae), uses sex pheromone of tussock moth *Euproctis taiwana* (Lepidoptera: Lymantriidae) as a kairomone. *J. Chem. Ecol.* 22:1079–1085.

BABILIS, N. A., and MAZOMENOS, B. E. 1992. Pheromone production in *Sesamia nonagrioides.* Diel periodicity and effect of age and mating. *J. Insect Physiol.* 8:561–564.

BAKKER, K., VAN ALPHEN, J. J. M., VAN BATENBURG, F. H. D., VAN DER HOEVEN, N., NELL, H. W., VAN STRIEN-VAN LIEMPT, W. T. F. H., and TURLINGS, T. C. J. 1985. The function of host discrimination and superparasitization in parasitoids. *Oecologia* 67:572–576.

BEEVERS, M., LEWIS, W. J., GROSS, H. R., JR., and NORDLUND, D. A. 1981. Kairomones and their use for management of entomophagous insects: X. Laboratory studies on manipulation of hostfinding behaviour of *Trichogramma pretiosum* Riley with a kairomone extracted from *Heliothis zea* (Boddie) moth scales. *J. Chem. Ecol.* 7:635–648.

BORGEMEISTER, C., SCHÄFER, K., GEORGEN, G., AWANDE, S., SÉTAMOU, M., POEHLING, H.-M., and SCHOLZ, D. 1999. Host-finding behavior of *Dinoderus bifoveolatus* (Coleoptera: Bostrichidae), an important pest of stored cassava: The role of plant volatiles and odors of conspecifics. *Ann. Entomol. Soc. Am.* 92(5):766–771.

BOSQUE, C., and RABINVOICH, J. E. 1979. Population dynamics of *Telenomus fairai* (Hymenoptera: Scelionidae), a parasite of Chagas's disease vectors. VII. Oviposition behavior and host discrimination. *Can. Entomol.* 3:171–180.

BOSQUE-PÉREZ, N. A., and DABROWSKI, Z. T. 1989. Mass rearing of maize stem borers *Sesamia calamistis* and *Eldana saccharina* at IITA. pp. 22–26, *in* Towards Insect-Resistant Maize for the Third World. Proceedings of the International Symposium on Methodologies for Developing

Host Plant Resistance of Maize Insects. Centro Internacional de Mejoramiento de Maizy Trigo (CIMMYT), Texcoco, Mexico.

CHABI-OLAYE, A., SCHULTHESS, F., SHANOWER, T. G., and BOSQUE-PÉREZ, N. A. 1997. Factors influencing the developmental rates and reproductive potentials of *Telenomus busseolae* (Gahan) (Hymenoptera: Scelionidae), an egg parasitoid of *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae). *Biol. Control* 8:15–21.

CHABI-OLAYE, A., SCHULTHESS, F., POEHLING, H.-M., and BORGEMEISTER, C. 2000. Factors affecting the biology of *Telenomus isis* (Polaszek) (Hymenoptera: Scelionidae), an egg parasitoid of cereal stem borer, in Benin. *Biol. Control* In press.

COLAZZA, S., ROSI, M. C., and CLEMENTE, A. 1997. Response of the egg parasitoid *Telenomus busseolae* (Gahan) to sex pheromone of *Sesamia nonagrioides* (Lefebvre). *J. Chem. Eco.* 23:2437–2444.

GAZIT, Y., LEWIS, W. J., and TUMLISON, J. H. 1996. Arrestment of *Telenomus remus* (Hymenoptera: Scelionidae) by a kairomone associated with eggs of its host, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Biol. Control* 6:283–290.

HIGUCHI, H., and SUZUKI, Y. 1996. Host handling behaviour of the egg parasitoid *Telenomus triptus* to the egg mass of sting bug *Piezodorus hybneri. Entomol. Exp. Appli.* 80:475–479.

JONES, R. L., LEWIS, W. J., BEROZA, M., BIERL, B. A., and SPARKS, A. N. 1973. Host-seeking stimulants (kairomones) for the egg parasite, *Trichogramma evanescens. Environ. Entomol.* 2:593–596.

KAISER, L., PHAM-DELEGUE, M. H., BAKCHINE, E., and MASSON, C. 1989. Olfactory responses of *Trichogramma maidis* Pint. et Voeg.: Effect of chemical cues and behavioral plasticity. *J. Insect Behav.* 2:701–712.

KORNOSOR, S., SERTKAYA, E., and ÖZPINAR, A. 1994. Distribution of the egg parasitoid *Platytelenomus busseolae* (Gahan) (Hym.: Scelionidae) and its effect on the population of *Sesamia nonagrioides* Lef. (Lep.: Noctuidae) in the Mediterranean region of Turkey, pp. 193–199, *in* E. Wajnberg (ed.). *Trichogramma* and Other Egg Parasitoids. 4th International Symposium, Cairo, Egypt, October 4–7, 1994. INRA, Paris 1995. (Les Colloques, no. 73).

LEWIS, W. J., JONES, R. L., and SPARKS, A. N. 1972. A host seeking stimulant for the egg parasite *Trichogramma evanescens* : Its source and demonstration of its laboratory and field activity. *Ann. Entomol. Soc. Am.* 65:1087–1089.

LEWIS, W. J., NORDLUND, D. A., GUELDNER, R. C., TEAL, P. E. A., and TUMLINSON, J. H. 1982. Kairomones and their use for management of entomophagous insects. XIII. Kairomonal activity for *Trichogramma* spp. of abdominal tips, excretion, and a synthetic sex pheromone blend of *Heliothis zea* (Boddie) moths. *J. Chem. Ecol.* 8:1323–1331.

MATTIACCI, L., VINSON, S. B., WILLIAMS, H. J., ALDRICH, J. R., and BIN, F. 1993. A long-range attractant kairomone for egg parasitoid *Trissolcus basalis* (Woll.), isolated from defensive secretion of its host, *Nezara viridula. J. Chem. Ecol.* 19:1067–1181.

NDEMAH, N. R. 1999. Towards an integrated crop management strategy for the African stalk borer, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) in maize systems in Cameroon. PhD thesis. University of Hannover, Hannover, Germany. 145 pp.

NOLDUS, L. P. J. J. 1989. Semiochemicals, foraging behavior and quality of entomophagous insects for biological control. *J. Appl. Entomol.* 108:425–451.

NOLDUS, L. P. J. J., and NAN LENTEREN, J. C. 1985. Kairomones for the egg parasite *Trichogramma evanescens* Westwood. II. Effect of contact chemicals produced by two of its hosts, *Pieris brassicae* L. and *Pieris rapae* L. J. *J. Chem. Ecol.* 11:793–800.

NOLDUS, L. P. J. J., LEWIS, W. J., and TUMLINSON, J. H. 1990. Beneficial arthropod behavior mediated by airborne semiochemicals. IX. Differential response of *Trichogramma pretiosum*, an egg parasitoid of *Heliothis zea*, to various olfactory cues. *J. Chem. Ecol.* 16:3531–3544.

OKUDA, M. S., and YEARGAN, K. V. 1988. Intra and inter host discrimination in *Telenomus podisi* and *Trissolcus euschisti* (Hymenoptera: Scelionidae). *Ann. Entomol. Soc. Am.* 81(6):1017–1020.

POLASZEK, A. 1998. African cereal stem borers; economic importance, taxonomy, natural enemies and control. CAB International in association with the ACP-EU Technical Centre for Agricultural and Rural Co-operation (CTA) 530 pp.

POLASZEK, A., UBEKU, J. A., and BOSQUE-PÉREZ, N. A. 1993. Taxonomy of *Telenomus busseolae* species complex (Hymenoptera: Scelionidae) egg parasitoids of cereal stem borers (Lepidoptera: Noctuidae, Pyralidae). *Bull. Entomol. Res.* 83:221–226.

ROSEN, D., and DEBACH, P. 1979. Species of *Aphitis* of the World (Hymenoptera: Aphelinidae). Dr. W. Junk, The Hague, The Netherlands.

SAS Institute. 1992. SAS User's Guide: Statistics, Version 6th ed. SAS Institute, Cary, North Carolina.

SCHOLZ, D., TCHABI, A., BORGEMEISTER, C., MARKHAM, R. H., POEHLING, H.-M., and LAWSON, A. 1997. Host-finding behaviour of *Prostephanus truncatus*: Primary attraction or random attack? *J. App. Entomol.* 121:261–269.

SCHOLZ, D., TCHABI, A., MARKHAM, R. H., POEHLING, H.-M, and BORGEMEISTER, C. 1998. Factors affecting pheromone production and behavioral responses by *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae). *Ann. Entomol. Soc. Am.* 91(6):872–878.

SCHULTHESS, F., BOSQUE-PÉREZ, N. A., CHABI-OLAYE, A., GOUNOU, S., NDEMAH, R., and GOERGEN, G. 1997. Exchange of natural enemies of lepidopteran cereal stem borers between African regions. *Insect Sci. Appl.* 17(1):97–108.

SCHULTHESS, F., CHABI-OLAYE, A., and GOERGEN, G. 2000. Seasonal fluctuations of noctuid stem-borer egg parasitism in southern Benin with special reference to *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) and *Telenomus* spp. (Hymenoptera: Scelionidae) on maize. *Biocontrol Sci. Technol.* Submitted.

SÉTAMOU, M., and SCHULTHESS, F. 1995. The influence of egg parasitoids belonging to *Telenomus busseolae* (Hymenoptera: Scelionidae) species complex on *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) population in maize fields in southern Benin. *Biocontrol Sci. Technol.* 5:69–81.

SHU, S., and JONES, R. L. 1989. Kinetic effects of kairomone in moth scales of the European corn borer on *Trichogramma nubilale* Ertle & Davis (Hymenoptera: Trichogrammatidae). *J. Insect Behav.* 2:123–131.

STRAND, M. R., and VINSON, S. B. 1983. Host acceptance behavior of *Telenomus heliothidis* (Hymenoptera: Scelionidae) toward *Heliothis virescens* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 76:781–785.

TUMLINSON, J. H., TURLINGS, T. C. J., and LEWIS, W. J. 1992. The semiochemical complexes that mediate insect parasitoid foraging. *Agric. Zool. Rev.* 5:221–252.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attacking parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92:4169–4174.

VAN ALPHEN, J. J. M., and VISSER, M. E. 1990. Superparasitism as an adaptive strategy for insect parasitoids. *Annu. Rev. Entomo.* 35:59–79.

VAN LENTEREN, J. C. 1981. Host discrimination by parasitoids, pp. 25–79, *in* D. A. Nordlund, R. L. Jones, W. J. Lewis (eds.). Semiochemicals, Their Role in Pest Control, Wiley, New York.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VET, L. E. M., WÄCKERS, F. L., and DICKE, M. 1991. How to hunt for hiding hosts: the reliability–detectability problem in foraging parasitoids. *Neth. J. Zool.* 41:202–213.

VINSON, S. B. 1976. Host selection by insect parasitoids. *Annu. Rev. Entomol.* 21:109–133.

VISSER, M. E. 1993. Adaptive self-and conspecific superparasitism in the solitary parasitoid *Leptopilina heterotoma*. *Behav. Ecol.* 4:22–28.

VISSER, M. E., VAN ALPHEN, J. J. M., and NELL, H. W. 1990. Adaptive superparasitism and patch time allocation in solitary parasitoids: the influence of the number of parasitoids depleting a patch. *Behaviour* 114:21–36.

WÄCKERS, F. L., and LEWIS, W. J. 1994. Olfactory and visual learning and their combined influence on host site location by the parasitoid *Microplitis croceipes* (Cresson). *Biolo. Control* 4:105–112.

ZABORSKI, E., TEAL, P. E. A., and LAING, J. E. 1987. Kairomone-mediated host finding by spruce budworm egg parasites, *Trichogramma minutum. J. Chem. Ecol.* 13:113–122.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

EXOGENOUS METHYL JASMONATE INDUCES VOLATILE EMISSIONS IN COTTON PLANTS

CESAR RODRIGUEZ-SAONA,[1,*] STEVEN J. CRAFTS-BRANDNER,[1] PAUL W. PARÉ,[2] and THOMAS J. HENNEBERRY[1]

[1]*USDA-ARS, Western Cotton Research Lab.*
4135 E. Broadway, Phoenix, Arizona 85040

[2]*Department of Chemistry and Biochemistry*
Texas Tech University
Lubbock, Texas 79409

(Received June 20, 2000; accepted December 12, 2000)

Abstract—We investigated the effect of exogenous methyl jasmonate (MeJA) on the emission of herbivore-induced volatiles; these volatile chemicals can signal natural enemies of the herbivore to the damaged plant. Exogenous treatment of cotton cv. Deltapine 5415 plants with MeJA induced the emission of the same volatile compounds as observed for herbivore-damaged plants. Cotton plants treated with MeJA emitted elevated levels of the terpenes (*E*)-β-ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-α-farnesene, (*E*)-β-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene compared to untreated controls. Other induced components included (*Z*)-3-hexenyl acetate, methyl salicylate, and indole. Methyl jasmonate treatment did not cause the release of any of the stored terpenes such as α-pinene, β-pinene, α-humulene, and (*E*)-β-caryophyllene. In contrast, these compounds were emitted in relatively large amounts from cotton due to physical disruption of glands by the herbivores. The timing of volatile release from plants treated with MeJA or herbivores followed a diurnal pattern, with maximal volatile release during the middle of the photoperiod. Similar to herbivore-treated plants, MeJA treatment led to the systemic induction of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*,*E*)-α-farnesene, (*E*)-β-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Our results indicate that treatment of cotton with MeJA can directly and systemically induce the emission of volatiles that may serve as odor cues in the host-search behavior of natural enemies.

Key Words—Cotton, *Spodoptera exigua*, methyl jasmonate, induction, plant defense, volatile semiochemicals.

*To whom correspondence should be addressed. e-mail: crodriguez@wcrl.ars.usda.gov

0098-0331/01/0400-0679$19.50/0

INTRODUCTION

Plants protect themselves against herbivory by deploying a wide array of chemical defenses (Karban and Myers, 1989; Agrawal et al., 1999). Wounding-induced plant responses can directly target herbivores by stimulating the synthesis of toxic or antifeedant metabolites, as well as by activating antinutrient enzymes such as proteinase inhibitors or polyphenol oxidases (e.g., Felton et al., 1994; Stout et al., 1996). Plant volatiles can also serve as a chemical defense by recruiting beneficial insects that are natural enemies of the herbivore, thereby providing an indirect protection to the plant (Dicke and Vet, 1999; Paré and Tumlinson, 1999). These volatile organic compounds increase the foraging success of natural enemies by providing reliable odor cues for prey/host location (Dicke and Sabelis, 1988; Dicke et al., 1990; Turlings et al., 1995).

Currently, there is increasing evidence of a common biosynthetic pathway linking direct and indirect induced plant defenses. Wounding of leaf tissue activates the octadecanoid/lipoxygenase (LOX) pathway, a lipid-based signaling sequence, resulting in the accumulation of 12-oxyphytodienoic acid and 7-isojasmonic acid (JA) (Farmer and Ryan, 1990; Constabel and Ryan, 1998). The involvement of JA in regulating gene activation subsequent to wounding has been established in several plant species (Wasternack et al., 1998; Staswick and Lehman, 1999). The LOX pathway also plays an important role in regulation of indirect plant defenses. Volatile emissions can be stimulated by exogenous JA (see Boland et al., 1998, for review) and by a select group of amino acid conjugates of JA (Krumm et al., 1995) taken up through the stems of a plant. Another connection of the LOX signaling pathway with volatile synthesis and emissions was the identification of *N*-(17-hydroxylinolenoyl)-L-glutamine (volicitin), an herbivore-specific elicitor that is derived from linolenic acid and induces volatile production (Alborn et al., 1997). Incubation of amino acid conjugates of linolenic acid (e.g., linolenoyl-L-glutamine) mimics the action of 12-oxophytodienoic acid (PDA), an early intermediate on the LOX pathway, in inducing emission of a blend of volatiles in a Lima bean cut seedling bioassay (Koch et al., 1999). This elicitor response appears to be highly specific since *N*-(17-hydroxylinoleoyl)-L-glutamine, a compound closely related to volicitin, is ineffective in inducing volatile emissions (Alborn et al., 2000).

In several agricultural species that have been studied, volatile chemicals emitted from both damaged and undamaged portions of herbivore-injured leaves serve as essential host-location cues for parasitic insects (see review by Dicke and Vet, 1999). Chemical labeling studies have established that a subset of volatiles emitted from cotton (*Gossypium hirsutum* L.) damaged by insects is synthesized and rapidly emitted from damaged and undamaged leaves, while other chemical constituents, stored in specialized glands, are volatilized only from the leaves that are damaged by insects (Paré and Tumlinson, 1997a,b, 1998). In mint leaves, such

glands contain enzymes required for terpene synthesis (Gershenzon et al., 1989). By directly comparing volatile emission patterns of glanded cotton plants treated with jasmonates or damaged by herbivores, compounds induced by jasmonates can be distinguished from those that are emitted due to mechanical damage.

Our objectives were to compare the emission of volatiles from cotton plants treated with methyl jasmonate (MeJA), a volatile derivative of JA, or subjected to herbivore damage. Specifically, we determined the diurnal pattern of volatile emission induced by these treatments. Finally, we report that MeJA induces the systemic release of the same volatile compounds that are systemically induced by herbivore feeding.

METHODS AND MATERIALS

Plants and Insects

Four- to 6-week-old glanded cotton, *G. hirsutum* cv. Deltapine 5415, plants with four to six fully expanded true leaves were used in all experiments. Cotton was grown in air-conditioned greenhouses under natural light (2000 μmol/m^2/sec^{-1} max daily photosynthetically active radiation) and Arizona fall–spring conditions (11L:13D photoperiod, and 28°C day, 24°C night). Plants were grown in 15×15-cm pots containing a commercial potting mixture (Grow More, Gardena, California) and fertilized three times a week with 750 ml of a solution containing 2 g/liter Grow More 20-20-20 fertilizer. The nutrient solution was supplemented with 0.5 ml/l micronutrient solution containing 2 mM $MnCl_2$, 10 mM H_3BO_3, 0.4 mM $ZnSO_4$, 0.2 mM $CuSO_4$, 0.4 mM Na_2MoO_4, and 0.1 mM $NiCl_2$. All plants employed were free of insects and mites.

The generalist beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), was used in all experiments because cotton plants damaged by this herbivore emit a blend of volatiles important for parasitic insects in host location (Loughrin et al., 1994; Röse et al., 1996). Larvae were reared on an alfalfa-based diet (Henneberry and Kishaba, 1966) at 28°C and 50% relative humidity. The colony has been maintained in culture at the Western Cotton Research Lab., USDA-ARS, Phoenix, Arizona for more than 10 years, and had new genetic material added within 12 months prior to the study from the Southern Insect Management Research Unit, USDA-ARS, Stoneville, Mississippi.

Volatile Collection System

Volatile chemicals emitted by individual cotton plants were collected in a push/pull apparatus constructed after Heath and Manukian (1994). The system consisted of four independent glass chambers that allowed for simultaneous collections. Air entering each of the glass chambers initially was split into two

directions: in one, air was pushed through a flowmeter (to measure and regulate the amount of air) followed by a charcoal filter (to purify the air) and a humidifier bubbler (wet air). Air in the second direction was pushed through a flowmeter and a charcoal filter (dry air). Both moist and dry air entered a glass cylinder (42.5 cm high and 18 cm diameter; Analytical Research Systems, Inc., Gainesville, Florida) at 5 L/min (Loughrin et al., 1994; Röse et al., 1996). The air passed over a cotton plant placed inside the cylinder through the opened bottom (see Röse et al., 1996; Turlings et al., 1998, for details). The bottom of the chamber was then sealed by using a guillotine-like support base (Analytical Research Systems, Inc.) leaving a hole in the center around the stem of the plant. The system allows sampling volatiles from the aerial parts (all of its leaves) of a plant, while the pot remains outside. At 5 cm from the base of the cylinder, eight openings allowed for attachment of collection filter traps (Analytical Research Systems, Inc.). Collection traps consisted of 10 cm long and 6 mm diameter glass filters containing 30 mg of Super-Q adsorbent (Alltech Assoc. Inc., Deerfield, Illinois). Manual switching valves allowed the sample to be diverted to individual filter traps, thus facilitating volatile collection at various times of the day.

The tip of each collection trap was placed a few millimeters from the plant. During collection, air was pulled through one of the collection traps at a rate of 1 l/min (Loughrin et al., 1994; Röse et al., 1996). Thus, only 20% of the air passed through the collector trap, the remaining escaped through the opening at the bottom of the guillotine base, which was loosely closed with cotton to prevent abrasion (see Röse et al., 1996; Turlings et al., 1998).

Analysis of Headspace Volatiles

Volatiles were extracted from the collector traps by rinsing them with 180 μl of methylene chloride. An internal standard (600 ng of *n*-octane in 5 μl of methylene chloride) was added to the extract. From each sample, 1 μl was analyzed on a Hewlett-Packard model 6890 gas chromatograph (GC) equipped with a capillary injector system and flame ionization detector. Samples were injected by a Hewlett-Packard auto injector model 7683, programmed in a split mode (25:1). All samples were analyzed on a HP1 methyl siloxane column (30 m $\times$ 0.32 mm ID, 0.25 μm film). Helium at a linear flow velocity of 40 cm/sec was used as a carrier gas. Following injection, column temperature was maintained at 50°C for 3 min and then increased at 5°C/min to 190°C and maintained at 190°C for 5 min. Data were analyzed with Hewlett-Packard ChemStation software. For each sample, amounts of the detected volatiles were based on comparison of their peak areas with that of the internal standard.

Selected samples were analyzed by GC–mass spectroscopy (GC-MS) with a Hewlett-Packard model 5973 mass selective detector operated at an initial temperature of 40°C for 1 min, then programmed at 14°C/min to 180°C. The column

oven was maintained at 180°C for 2 min and then raised at 40°C/min to 200°C and held for 2 min. For GC-MS samples, a DB5-MS column (J&W Scientific, Folsom, California) 30 m × 0.25 mm ID, with a 0.1-μm-thick film of bonded methyl silicone with 5% phenyl, was used. Spectral data were compared with commercially available standards and spectra from the National Institute of Standards and Technology (NIST, 1995) database.

Comparison of Cotton Volatiles Induced by Herbivory versus MeJA Treatment

Volatiles were collected from cotton damaged by *S. exigua* larvae, treated with MeJA, and controls. Volatiles were collected as previously described beginning at 10:00 hr for a total of 22 hr. At least one control was run simultaneously with other treatments at a particular collection time. Treatments were replicated five times.

Herbivore-Damaged Plants. A cohort of recently molted third instar *S. exigua* from the colony was transferred from artificial diet cups to cotton plants (approx. 10 larvae/plant) 12 hr prior to the experiment to allow the larvae to habituate to the new diet. Larvae were allowed to feed on cotton overnight. The following morning larvae were removed from the plants, starved for 2 hr to encourage immediate feeding, and placed inside a collection chamber with a new cotton plant. At 10:00 hr, 10 larvae were placed inside a volatile collection chamber containing a plant and allowed to feed *ad libitum* over 22 hr.

Methyl Jasmonate-Treated Plants. Cotton was treated with MeJA (Aldrich, Milwaukee, Wisconsin) overnight, starting at 16:00 hr, in the greenhouse. Plants were treated by applying 20 μl of an ethanol–MeJA (9:1) solution (after Thaler et al., 1996; Constabel and Ryan, 1998) onto a 15-cm cotton tipped applicator (Fisher, Pittsburgh, Pennsylvania). Two treated cotton wicks (total of 18 μmol MeJA/plant) were placed underneath cotton leaves, without physically contacting the treated plants. Plants were treated inside a Plexiglas cylindrical chamber (26 cm diameter × 60 cm high with the top of the chamber and a 14-cm-diameter opening in the middle covered by a fine nylon mesh to allow air circulation). Control cotton plants were exposed to 40 μl ethanol but no MeJA and placed in separate chambers under the same conditions. Plants were kept in the chambers for 18 hr, after which cotton wicks from the treated plants were removed and plants were placed inside the chambers for volatile collection.

Timing of Induction

Volatiles released over time were collected from cotton damaged by *S. exigua* larvae, treated with exogenous MeJA, and controls. At least one control was run simultaneously with other treatments at a particular collection time. Volatiles were collected for 4-hr periods over two consecutive days: on day 1 at 10:00, 14:00, 18:00, and 22:00 hr; and on day 2 at 06:00, 10:00, 14:00, and 18:00 hr. Treatments

were replicated four times. In addition, daily fluctuation in leaf temperature was monitored for plants inside the collection chamber by using a thermocouple pressed to the bottom of a fully expanded leaf.

Herbivore-Damaged Plants. In the first series of experiments 10 *S. exigua* larvae were allowed to feed on cotton plants overnight, starting at 16:00 hr. Larvae were kept on the leaves by enclosing them inside a fine-mesh nylon bag sealed around the leaf petiole with Velcro. The following day (06:00 hr) larvae and plants were placed inside collection chambers (see above) for volatile collections. In these experiments, caterpillars were allowed to feed continuously. In a second set of experiments, larvae were also allowed to feed on cotton plants overnight, starting at 16:00 hr; however, herbivore feeding was stopped by removing larvae from the plants at the time when the plants were placed inside the collection chambers at 06:00 hr.

Methyl Jasmonate-Treated Plants. Starting at 16:00 hr, plants were placed inside Plexiglas cylinders and treated with MeJA as previously described. The following morning at 06:00 hr, cotton wicks were removed and plants were placed inside collection chambers and volatiles were collected. In addition, control plants were placed under the same conditions but with no exposure to MeJA.

Systemic Induction

In order to maximize treatment differences, systemically induced volatiles were collected in the same filter trap during the photoperiod for four consecutive days. Volatile collection was started at 10:00 hr each day and ended at 20:00 hr on days 1 to 3, and at 18:00 hr on day 4. At least one control was run simultaneously with other treatments at a particular collection time. Treatments were replicated 4 times.

Herbivore-Damaged Plants. Four beet armyworm larvae were enclosed in a fine-mesh nylon bag and allowed to feed on the lower two leaves of a cotton plant, while volatiles were collected from the undamaged upper leaves. Larvae started feeding on the lower leaves 18 hr before the beginning of the volatile collections.

Methyl Jasmonate-Treated Plants. Systemic response of cotton treated with MeJA was tested by treating the two lower leaves on a plant with 20 μl of an ethanol– MeJA (9:1) solution (total of 18 μmol MeJA/plant). Leaves and the cotton wicks treated with MeJA were enclosed in a perforated Ziploc plastic bag. Lower leaves were treated with MeJA at 16:00 hr the day prior to volatile collections and were treated with MeJA daily at 16:00 hr by reapplying 18 μmol of MeJA to the cotton wick. For controls, the lower two leaves were enclosed in plastic bags containing cotton wicks treated with 20 μl of ethanol each, but received no MeJA treatment.

Statistical Analyses

A completely randomized one-way ANOVA (SigmaStat, San Rafael, California) was used to determine differences in total volatile emissions among treatments (herbivore, MeJA, and controls). If significant differences were detected, an all pairwise multiple comparison procedure (Tukey test) was conducted. Differences in individual volatile components among treatments were determined by using the MULTTEST PROC (SAS Institute Inc., Cary, North Carolina).

RESULTS

Comparison of Cotton Volatiles Induced by Herbivory versus MeJA Treatment. Differences in volatile emissions were observed for cotton plants damaged by *S. exigua* larvae, treated with MeJA, or undamaged controls (Table 1). Control plants emitted much lower amounts of volatiles compared to herbivore damaged plants and plants treated with MeJA (Table 1).

Plants treated with herbivores or MeJA emitted a blend that consisted of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, (*E*,*E*)-α-farnesene, (*E*)-β-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Figure 1). These compounds are commonly referred to as inducible because they are synthesized *de novo* by herbivore-damaged cotton (Paré and Tumlinson, 1997a,b). Other detected components were methyl salicylate and an unidentified sesquiterpene (Figure 1). Control plants also emitted the inducible linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Figure 1; see also Röse et al., 1996).

The terpenes α-pinene, β-pinene, myrcene, limonene, (*E*)-β-caryophyllene, and α-humulene were detected only from herbivore-damaged cotton (MULTTEST;

TABLE 1. TOTAL AMOUNT OF VOLATILES EMITTED LOCALLY AND SYSTEMICALLY FROM COTTON PLANTS DAMAGED BY *S. exigua* LARVAE TREATED EXOGENOUSLY WITH METHYL JASMONATE AND CONTROL PLANTS

	Amount	
Treatment	Local[a] (ng/hr ± SE)	Systemic[b] (ng ± SE)
S. exigua damage	649.9 ± 153.3 a[c]	10317.2 ± 3005.8 a
Methyl jasmonate	573.4 ± 147.8 a	5173.6 ± 1388.3 a
Control	139.5 ± 50.2 b	376.3 ± 121.8 b

[a] Volatiles collected continuously for 22 hr; $N = 5$.
[b] Cumulative volatiles emitted during the Photoperiod of 4 consecutive days were collected; $N = 4$.
[c] Different letters within columns indicate statistical differences among treatments (Local: $F = 6.4; df = 2,14; P = 0.013$; systemic: $F = 6.8; df = 2,11; P = 0.016$; Tukey test; $P < 0.05$).

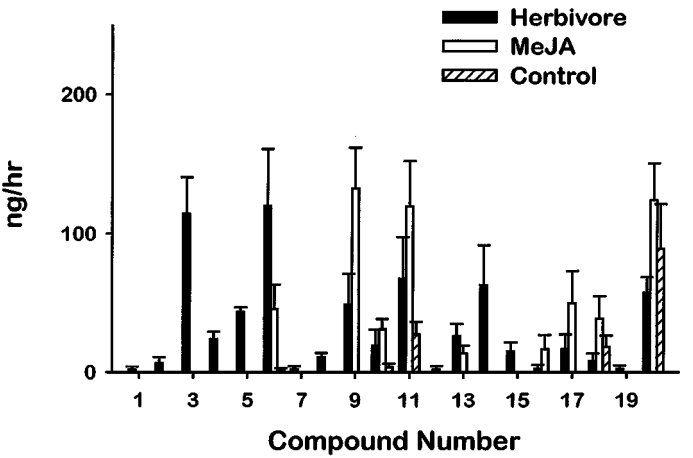


FIG. 1. Volatiles collected from the aerial portions of undamaged cotton plants (Control), plants damaged by *S. exigua*, and plants treated exogenously with methyl jasmonate (MeJA). Volatiles were collected continuously for 22 hr. Each bar represents the mean ± SE for five replicates. 1 = Hexanal; 2 = (*Z*)-3-hexenol; 3 = α-pinene; 4 = β-pinene; 5 = mycene; 6 = (*Z*)-3-hexenyl acetate; 7 = hexyl acetate; 8 = limonene; 9 = (*E*)-β-ocimene; 10 = linalool; 11 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene; 12 = methyl salicylate; 13 = indole; 14 = (*E*)-β-caryophyllene; 15 = α-humulene; 16 = (*E*)-β-farnesene; 17 = (*E*,*E*)-α-farnesene; 18 = tentatively identified as δ-cadinene [based on comparison of mass spectra with spectra published by Stenhagen et al. (1974) and the Eight Peak Index of Mass Spectra by the Mass Spectrometry Data Centre, Reading, UK]; 19 = nerolidol; 20 = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

$P < 0.05$). This result was expected, since these compounds, commonly referred to as constitutive, are predominantly stored in the glands of cotton leaves (Elzen et al., 1985; McAuslane and Alborn, 1998).

Hexanal, (*Z*)-3-hexenol, and hexyl acetate, all LOX-pathway compounds, were detected in response to herbivore damage, but not in response to MeJA treatment (Figure 1). These compounds result from the breakdown of stored lipids (Hatanaka et al., 1987) and are released during insect damage (McCall et al., 1994), but not systemically (Röse et al., 1996). (*Z*)-3-Hexenyl acetate was the only LOX pathway volatile detected from MeJA treatment (Figure 1).

Timing of Induction. The emission of inducible terpenes followed a diurnal cycle for herbivore-damaged, MeJA-treated, and control plants (Figures 2–5). In general, volatile emission was much higher for plants treated with herbivores and MeJA compared to controls.Volatile emissions were highest during the period of highest radiation (from 10:00 to 14:00 hr and from 14:00 to 18:00 hr), and emissions decreased markedly during the night. Periods of maximum volatile emission corresponded to times of the day when leaf temperatures were the highest

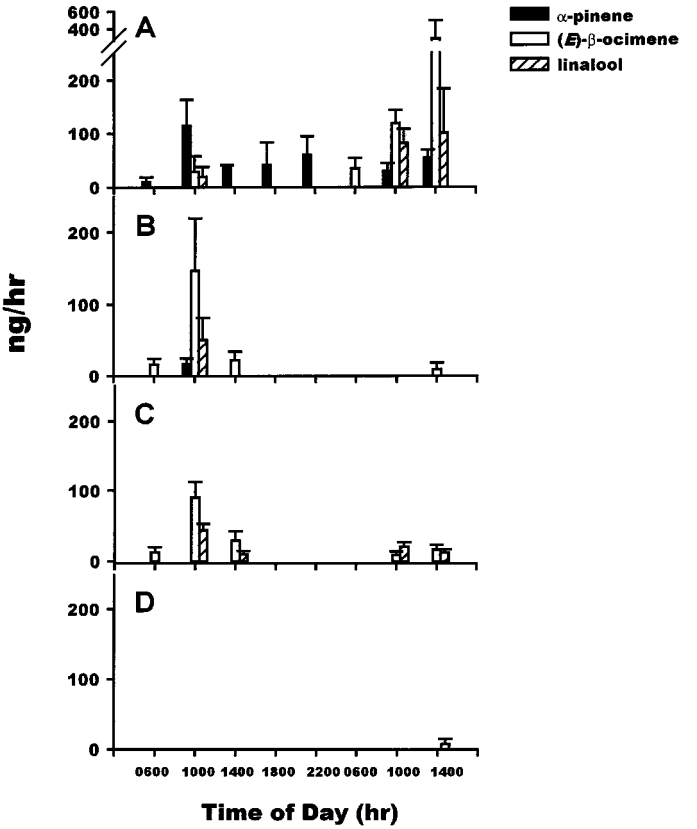


FIG. 2. Emissions of the monoterpenes α-pinene, (E)-β-ocimene, and linalool by aerial portions of cotton plants under continuous herbivore damage (A); with caterpillars removed from plants before being placed in collection chambers (B); treated overnight with exogenous methyl jasmonate prior to volatile collection (C); and undamaged controls (D). Each bar represents the mean $\pm$ SE for four replicates. Time of day indicates the time of initiation of each collection period and each collection represents a 4-hr interval.

[29.5 ± 0.3 (SE) and $35.5 \pm 0.5^{\circ}$C at 10:00 and 14:00 hr, respectively]. Lowest leaf temperatures were recorded at 06:00 hr ($22.9 \pm 0.4^{\circ}$C) and 22:00 hr ($22.6 \pm 0.5^{\circ}$C), which corresponded to the times of minimal volatile emission (Figures 2–5).

For plants subjected to continuous herbivore feeding, the emission of volatiles was highest during the second day of treatment (Figures 2A–5A). In contrast, when insect feeding was interrupted prior to collecting samples, volatile emission was much reduced on the second compared to the first day of the treatment (Figures 2B–5B). Additionally, plants treated with MeJA one time at the beginning of

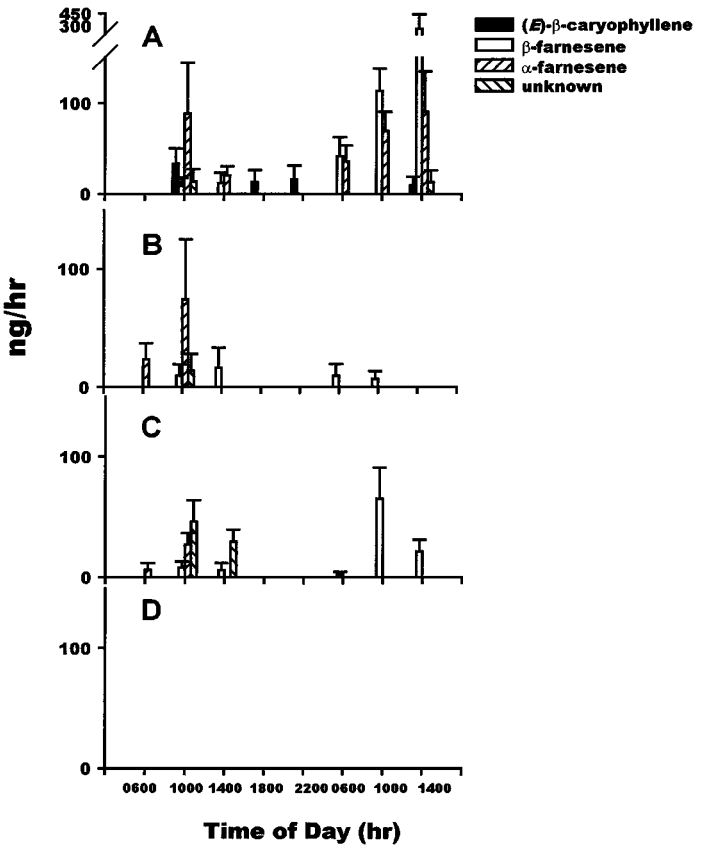


FIG. 3. Emissions of the sesquiterpenes (*E*)-β-caryophyllene, β-farnesene, α-farnesene, and an unknown by aerial portions of cotton plants under continuous herbivore damage (A); with caterpillars removed from plants before being placed in collection chambers (B); treated overnight with exogenous methyl jasmonate prior to volatile collection (C); and undamaged controls (D). Each bar represents the mean $\pm$ SE for four replicates. Time of day indicates the time of initiation of each collection period and each collection represents 4-hr interval.

the experiment had lower total volatile emission during the second, compared to the first, day of the experiment (Figures 2C–5C). In contrast to the inducible terpenes, the emission of the constitutive α-pinene and (*E*)-β-caryophyllene, released only from herbivore-damaged plants, did not follow a diurnal pattern (Figures 2A and 3A).

Systemic Induction. Both herbivore feeding and treatment with MeJA led to the systemic induction of volatile emission (Table 1; Figure 6). The blend of

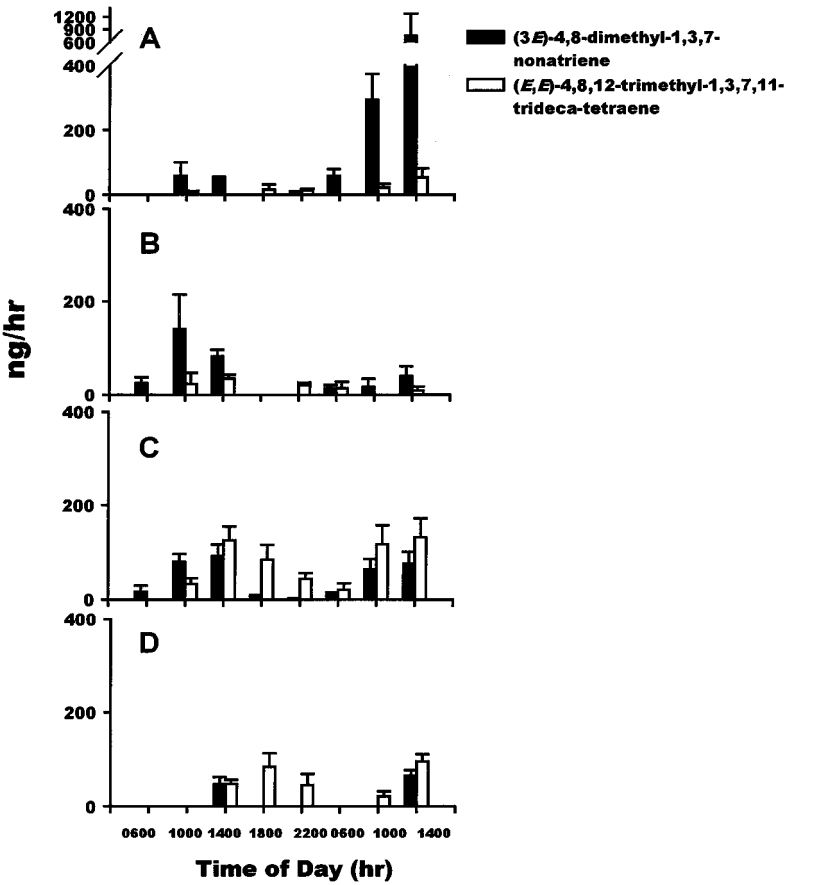


FIG. 4. Emissions of the homoterpenes (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene by aerial portions of cotton plants under continuous herbivore damage (A); with caterpillars removed from plants before being placed in collection chambers (B); treated overnight with exogenous methyl jasmonate prior to volatile collection (C); and undamaged controls (D). Each bar represents the mean ± SE for four replicates. Time of day indicates the time of initiation of each collection period and each collection represents a 4-hr interval.

volatiles emitted included (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E*,*E*)-α-farnesene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Figure 6). This systemically induced volatile blend was similar to the blend reported by Röse et al. (1996) for herbivore-damaged cotton.

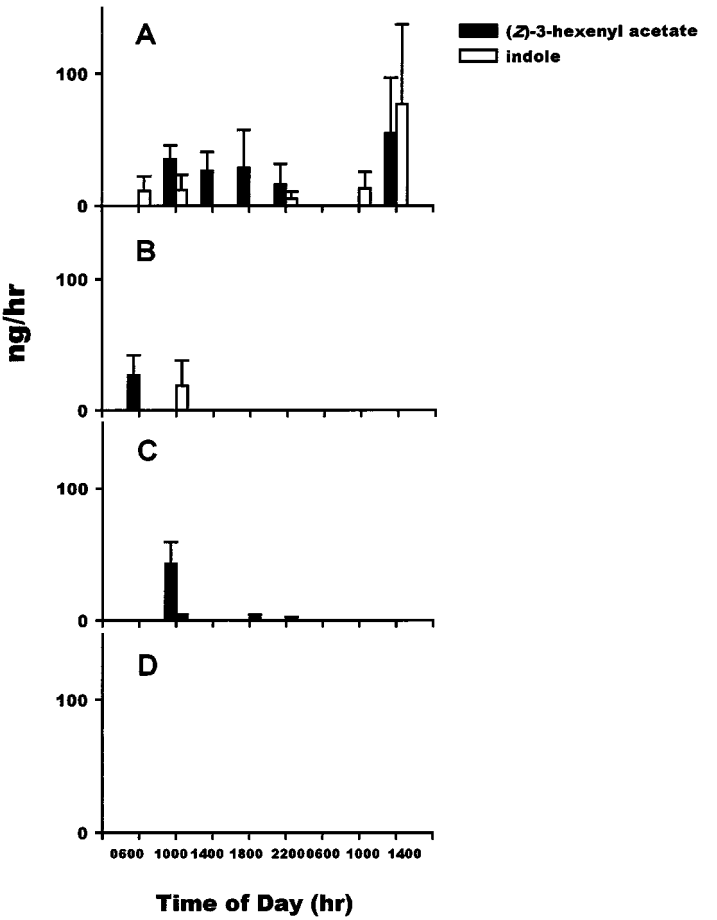


FIG. 5. Emissions of the LOX product (*Z*)-3-hexenyl acetate and indole by aerial portions of cotton plants under continuous herbivore damage (A); with caterpillars removed from plants before being placed in collection chambers (B); treated overnight with exogenous methyl jasmonate prior to volatile collection (C); and undamaged controls (D). Each bar represents the mean ± SE for four replicates. Time of day indicates the time of initiation of each collection period and each collection represents a 4-hr interval.

DISCUSSION

The induction of volatiles in cotton plants by herbivore damage has been documented (McCall et al., 1994), although the association of jasmonates in the induction of indirect defenses in cotton has not been previously reported. In cotton, MeJA mimicked the response of volatile emission induced by insects (Figure 1).

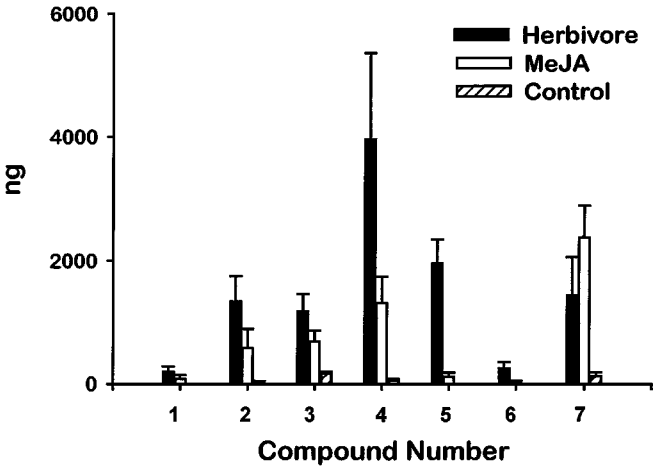


FIG. 6. Volatiles collected from the aerial portions of undamaged cotton plants (Control), undamaged leaves of cotton plants induced systemically by herbivore damage, and untreated leaves induced systemically by exogenous methyl jasmonate (MeJA). Cumulative volatiles emitted during the photoperiod of 4 consecutive days were collected. Each bar represents the mean ± SE for four replicates. 1 = (*Z*)-3-hexenyl acetate; 2 = (*E*)-β-ocimene; 3 = linalool; 4 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene; 5 = (*E*)-β-farnesene; 6 = (*E*,*E*)-α-farnesene; 7 = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

In fact, a blend of volatiles released by cotton plants treated exogenously with MeJA contained all compounds synthesized *de novo* following herbivore damage (Paré and Tumlinson, 1997b). Methyl jasmonate did not induce emissions of stored terpenes, a result that was expected since the release of stored terpenes is dependent on physical damage caused by herbivores.

These results also indicate that MeJA activates multiple biosynthetic pathways involved in the synthesis of cotton volatiles: the classic mevalonic acid pathway leading to sesquiterpenes, the alternate isopentenyl pyrophosphate pathway producing the monoterpenes, the homoterpene pathway producing the C_{11} and C_{16} unit terpenes, the shikimic acid/tryptophan pathway leading to the synthesis of methyl salicylate and indole, and the LOX pathway that leads to (*Z*)-3-hexenyl acetate (Paré and Tumlinson, 1996, 1999).

Similar to reports by Loughrin et al. (1994) and Turlings et al. (1998), herbivore-induced volatile emission was highest during the photoperiod (Figures 2–5). Our results indicated that MeJA-induced volatile emission followed a diurnal trend similar to herbivore treatment (Figures 2–5). Thus, volatile emission is highest during the time when natural enemies tend to forage (Turlings et al., 1995), when leaf temperature is highest, and when leaves are conducting photosynthesis (Paré and Tumlinson, 1997a,b).

The induction of volatiles was greatly diminished over time when herbivore feeding was interrupted or when MeJA was applied only one time (Figures 2B,C–5B,C). This indicates that the induction is dependent on a continuous supply of a chemical signal. Obviously, the reduced emission of constitutive volatiles after insect feeding was stopped due to the cessation of physical damage by the insects (Figures 2B–5B).

Systemically induced volatile emission for herbivore-damaged plants has been reported for cotton and other plant species (Turlings and Tumlinson, 1992; Röse et al., 1996). Additionally, it has been shown that jasmonates induce volatile emission by plants (Boland et al., 1998; Dicke et al., 1999; Gols et al., 1999). Here we show for the first time that jasmonates can systemically induce the emission of volatile compounds. The systemic emission of volatiles points to a mobile signal or signals that move from damaged leaf to the undamaged portions of the plant. Paré and Tumlinson (1998) in studies with $^{13}CO_2$, have established that the systemically released volatiles are synthesized at the site of release. However, to date no systemic signals specific for volatile induction have been identified. Our results indicate that MeJA, or an induced product, was transported to distal leaves leading to the systemic emission of the same cotton volatiles systemically induced by *S. exigua* (Figure 6).

In conclusion, our results indicate that indirect defenses in cotton can be activated by MeJA. Volatiles released from cotton after herbivore feeding and induced by MeJA treatment provide cues for natural enemies to locate hosts (McCall et al., 1993; De Moraes et al., 1998). Our results suggest that jasmonates may be used as an elicitor of volatiles to attract natural enemies in cotton cultivars. For instance, in other plant systems, indirect evidence has been presented that volatiles induced by jasmonates serve as important cues for natural enemies to locate potential host/prey sites. Although volatiles were not measured, the aggregation of beneficial parasitic wasps was greater in field plots of tomato treated exogenously with JA than for untreated control plots (Thaler, 1999).

Recently, Dicke et al. (1999) showed that Lima beans treated with JA or MeJA produce a volatile blend that is similar to plants infested with the two-spotted spider mite *Tetranychus urticae* Koch. Jasmonate-treated plants attracted more predatory mites, *Phytoseiulus persimilis* Athias-Henriot, than untreated plants. Comparable results were obtained when gerbera leaves were treated with exogenous JA (Gols et al., 1999). Thus, it appears that jasmonates are potential agents that may be used to improve biological control in agricultural crops.

Acknowledgments—The authors are grateful to Drs. Heather McAuslane (University of Florida), James Tumlinson (USDA-ARS, Gainesville, Florida), and Kenneth Korth (University of Arkansas) for comments on an earlier version of this manuscript. We thank Donald Brummett and Frances Arvallo who helped in keeping cotton plants available for the experiments. We also thank Anna Cervantes for providing us with the insects to conduct the experiments and Terry Steele for helping in the

construction of the volatile-collection apparatus. Statistical advice was provided by Bruce Mackey (Consulting Statistician at USDA-ARS, Albany, California).

REFERENCES

AGRAWAL, A. A., TUZUN, S., and BENT, E. 1999. Induced Plant Defenses Against Pathogens and Herbivores. APS Press, Minnesota, 390 pp.

ALBORN, H. T., TURLINGS, T. C. J., JONES, T. H., STENHAGEN, G., LOUGHRIN, J. H., and TUMLINSON, J. H. 1997. An elicitor of plant volatiles from beet armyworm oral secretions. *Science* 276:945–949.

ALBORN, H. T., JONES, T. H., STENHAGEN, G. S., and TUMLINSON, J. H. 2000. Identification and synthesis of volicitin and related components from beet armyworm oral secretions. *J. Chem. Ecol.* 26:203–220.

BOLAND, W., HOPKE, J., and PIEL, J. 1998. Induction of plant volatile biosynthesis by jasmonates, pp. 255–269, *in* P. Schreier, M. Herderich, H. Humpf, and W. Schwad (eds.). Natural Product Analysis: Chromatography, Spectroscopy, Biological Testing, Vieweg, Germany.

CONSTABEL, C. P., and RYAN, C. A. 1998. A survey of wound- and methyl jasmonate-induced leaf polyphenol oxidase in crop plants. *Phytochemistry* 47:507–511.

De MORAES, C. M., LEWIS, W. J., PARÉ, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DICKE, M., and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M., and VET, L. E. M. 1999. Plant–carnivore interactions: Evolutionary and ecological consequences for plant, herbivore, and carnivore, pp. 483–520, *in* H. Olff, V. K. Brown, and R. H. Drent (eds.). Herbivores: Between Plants and Predators. University Press, Cambridge.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990. Plant strategies of manipulating predator-prey interactions through allelochemicals: Prospects for application in pest control. *J. Chem. Ecol.* 16:3091–3118.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in Lima bean plants. *J. Chem. Ecol.* 25:1907–1922.

ELZEN, G. W., WILLIAMS, H. J., BELL, A. A., STIPANOVIC, R. D., and VINSON, S. B. 1985. Quantification of volatile terpenes of glanded and glandless *Gossypium hirsutum* L. cultivars and lines by gas chromatography. *J. Agric. Food Chem.* 33:1079–1082.

FARMER, E. E., and RYAN, C. A. 1990. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci. U.S.A.* 87:7713–7716.

FELTON, G. W., SUMMERS, C. B., and MULLER, A. J. 1994. Oxidative responses in soybean foliage to herbivory by bean leaf beetle and three-cornered alfalfa hooper. *J. Chem. Ecol.* 20:639–650.

GERSHENZON, J., MAFFEI, M., and CROTEAU, R. 1989. Biochemical and histochemical localization of monoterpene biosynthesis in the glandular trichomes of spearmint (*Mentha spicata*). *Plant Physiol.* 89:1351–1357.

GOLS, R., POSTHUMUS, M. A., and DICKE, M. 1999. Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis*. *Entomol. Exp. Appl.* 93:77–86.

HATANAKA, A., KAJIWARA, T., and SEKIYA, J. 1987. Biosynthetic pathway for C_6-aldehydes formation from linolenic acid in green leaves. *Chem. Physical Lipids* 44:341–361.

HEATH, R. R., and MANUKIAN, A. 1994. An automated system for use in collecting volatile chemicals released from plants. *J. Chem. Ecol.* 20:593–607.

HENNEBERRY, T. J., and KISHABA, A. N. 1966. Cabbage loopers, pp. 461–478, *in* C. N. Smith (ed.). Insect Colonization and Mass Production, Academic Press, New York.

KARBAN, R., and MYERS, J. H. 1989. Induced plant responses to herbivory. *Annu. Rev. Ecol. Syst.* 20:331–348.

KOCH, T., KRUMM, T., JUNG, V., ENGELBERTH, J., and BOLAND, W. 1999. Differential induction of plant volatiles biosynthesis in the Lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiol.* 121:153–162.

KRUMM, T., BANDEMER, K., and BOLAND, W. 1995. Induction of volatile biosynthesis in the Lima bean (*Phaseolus lunatus*) by leucine- and isoleucine conjugates of 1-oxo- and 1-hydroxyindan-4-carboxylic acid: evidence for amino acid conjugates of jasmonic acid as intermediates in the octadecanoid signalling pathway. *FEBS Lett.* 377:523–529.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R. TURLINGS, T. C. J., and TUMLINSON, J. H. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. *Proc. Natl. Acad. Sci. U.S.A.* 91:11836–11840.

MCAUSLANE, H. J., and ALBORN, H. T. 1998. Systemic induction of allelochemicals in glanded and glandless isogenic cotton by *Spodoptera exigua* feeding. *J. Chem. Ecol.* 24:399–416.

MCCALL, P. J., TURLINGS, T. C. J., LEWIS, W. J., and TUMLINSON, J. H. 1993. Role of plant volatiles in host location by the specialist parasitoid *Microplitis croceipes* Cresson (Braconidae: Hymenoptera). *J. Insect Behav.* 6:625–639.

MCCALL, P. J., TURLINGS, T. C. J., LOUGHRIN, J., PROVEAUX, A. T., and TUMLINSON, J. H. 1994. Herbivore-induced volatile emissions from cotton (*Gossypium hirsutum* L.) seedlings. *J. Chem. Ecol.* 20:3039–3050.

NIST (National Institute of Standards and Technology). 1995. Mass Spectral Library on CD-rom, version 1.0. NIST, Gaithersburg, Maryland.

PARÉ, P. W., and TUMLINSON, J. H. 1996. Plant volatile signals in response to herbivore feeding. *Fla. Entomol.* 79:93–103.

PARÉ, P. W., and TUMLINSON, J. H. 1997a. Induced synthesis of plant volatiles. *Nature* 385:30–31.

PARÉ, P. W., and TUMLINSON, J. H. 1997b. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114:1161–1167.

PARÉ, P. W., and TUMLINSON, J. H. 1998. Cotton volatiles synthesized and released distal to the site of insect damage. *Phytochemistry* 47:521–526.

PARÉ, P. W., and TUMLINSON, J. H. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121:325–331.

RÖSE, U. S., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1996. Volatile semiochemicals released from undamaged cotton leaves. *Plant Physiol.* 111:487–495.

STASWICK, P. E., and LEHMAN, C. C. 1999. Jasmonic acid-signaled responses in plants, pp. 117–136, *in* A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses against Pathogens and Herbivores, APS Press, Minnesota.

STENHAGEN, E., ABRAHAMSSON, S., and MCLAFFERTY, F. W. 1974. Registry of Mass Spectral Data, Vol. 2. John Wiley & Sons, New York, p. 1026.

STOUT, M. J., WORKMAN, K. V., and DUFFEY, S. S. 1996. Identity, spatial distribution, and variation of induced chemical defenses in tomato plants. *Entomol. Exp. Appl.* 79:255–271.

THALER, J. S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399:686–688.

THALER, J. S., STOUT, M. J., KARBAN, R., and DUFFEY, S. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol.* 22:1767–1781.

TURLINGS, T. C. J., and TUMLINSON, J. H. 1992. Systemic release of chemical signals by herbivore-induced corn. *Proc. Natl. Acad. Sci. U.S.A.* 89:8399–8402.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., RÖSE, U.S.R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acd. Sci. U.S.A.* 92:4169–4174.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L., and WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta* 207:146–152.

WASTERNACK, C., MIERSCH, O., KRAMELL, R., HAUSE, B., WARD, J., BEALE, M., BOLAND, W., PARTHIER, B., and FEUSSNER, I. 1998. Jasmonic acid: Biosynthesis, signal transduction, gene expression. *Fett/Lipid* 100:139–146.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

RESPONSE OF SOME SCOLYTIDS AND THEIR PREDATORS TO ETHANOL AND 4-ALLYLANISOLE IN PINE FORESTS OF CENTRAL OREGON

GLADWIN JOSEPH,[1,2] RICK G. KELSEY,[3,]* ROBERT W. PECK,[3] and CHRIS G. NIWA[3]

[1]*Department of Forest Science*
Oregon State University
Corvallis, Oregon 97331

[3]*Pacific Northwest Research Station*
USDA Forest Service
3200 Jefferson Way, Corvallis, Oregon 97331

(Received July 10, 2000; accepted December 12, 2000)

Abstract—Lindgren multiple funnel traps were set up in pine forests of central Oregon to determine the response of scolytid bark beetles to ethanol and 4-allylanisole (4AA). Traps were baited with two release rates of ethanol (4.5 or 41.4 mg/hr) and three release rates of 4AA (0, 0.6, or 4.3 mg/hr) in a 2×3 factorial design. All traps also released a 1:1 mixture of α- and β-pinene at 11.4 mg/hr. Of 13,396 scolytids caught, *Dendroctonus valens* made up 60%, *Hylurgops* spp. 18.5%, *Ips* spp. 16%, *Hylastes* spp. 1.8%, *Ganthotrichus retusus* 0.9%, and bark beetle predators another 2.8%. Increasing the release rate of ethanol in the absence of 4AA increased the number of most scolytid species caught by 1.5–3.7 times, confirming its role as an attractant. *Ips latidens*, *Temnochila chlorodia*, and clerid predators were exceptions and did not show a response to higher ethanol release rates. Release of 4AA at the lowest rate inhibited attraction of most scolytids, with a significant reduction in *G. retusus*, *Hylastes macer*, and *Hylurgops porosus* when compared to traps without 4AA. A high release rate of 4AA further inhibited responses for most beetles compared to low 4AA. Seven species were significantly deterred by high 4AA, including the latter three, and *Hylastes longicollis*, *Hylastes nigrinus*, *Hylurgops reticulatus*, and *Ips latidens*. Exceptions include *Hylurgops subcostulatus*, which was significantly attracted to both low and high 4AA, and *I. pini*, which was attracted to low and high 4AA in combination with low ethanol, but unaffected by either release of 4AA with high ethanol. *Dendroctonus valens* was significantly attracted to low 4AA and

*To whom correspondence should be addressed. e-mail: rkelsey@fs.fed.us or kelseyr@fsl.orst.edu
[2]Current address: Ashoka Trust for Research in Ecology and the Environment (ATREE), 659, 5th A Main Rd, Hebbal, Bangalore 560024, India.

0098-0331/01/0400-0697$19.50/0

unaffected by high 4AA. Predators appeared to be less inhibited by 4AA than most bark beetles. Although 4AA can deter the attraction of some secondary bark beetles to ethanol in combination with α- and β-pinene, this inhibition could be weakened for certain species by increasing ethanol release rates. 4-Allylanisole may have some utility for managing the behavior of secondary bark beetles sensitive to this compound.

Key Words—Methyl chavicol, bark beetles, primary attraction, host selection, host volatiles.

INTRODUCTION

The compound 4-allylanisole [4AA; 1-methoxy-4-(2-propenyl)benzene] is present in oleoresin of various pine species (Drew and Pylant, 1966; Werner, 1972; Pierce et al., 1987; Hayes et al., 1994) and is a known deterrent to primary bark beetles (Hayes et al., 1994; Hayes and Strom, 1994; Werner, 1995). It has been tested extensively in the lab and field as a potential antiaggregation semiochemical. *Dendroctonus frontalis* Zimmerman (Hayes et al., 1994), *D. rufipennis* (Kirby) (Werner, 1995), *D. ponderosae* Hopkins, *D. brevicomis* LeConte (Hayes and Strom, 1994; Hobson, 1996), and *Ips pini* (Say) (Hayes and Strom, 1994) were all significantly inhibited by 4AA in the presence of their specific aggregation pheromones.

Oleoresin in healthy trees usually contains higher concentrations of 4AA than stressed trees. For instance, quantities of 4AA in lodgepole pine (*Pinus contorta* Dougl. ex. Loud. var. *latifolia* Engelm.) infected with Comandra blister rust (*Cronartium comandrae* Pk.) or armillaria [*Armillaria mellea* (Vahl.: Fr.) Kummer] were 43.6–63% less than in healthy trees (Nebeker et al., 1995). Similarly, concentrations of 4AA in ponderosa pine (*Pinus ponderosa* Dougl. ex. Laws.) damaged by smog were 71% less than in undamaged trees (Cobb et al., 1972). Lower quantities of 4AA in smog stressed pine could be partially responsible for their greater vulnerability to bark beetle attacks than healthy trees (Stark et al., 1968).

Ethanol also occurs naturally in trees and is an important host-derived semiochemical. It is a product of fermentative respiration in plant tissues and is usually associated with hypoxic or anoxic conditions (Davies, 1980; Bennett and Freeling, 1987; Harry and Kimmerer, 1991). In contrast to 4AA, ethanol accumulates in severely stressed, dying, or recently dead trees. High ethanol concentrations have been reported in tissues of flooded (Crawford and Baines, 1977; Crawford and Finegan, 1989; Joseph and Kelsey, 1997), mechanically injured (Sjödin et al., 1989), or diseased trees (Gara et al., 1993; Kelsey and Joseph, 1998; Kelsey et al., 1998), severely water stressed branches (Kelsey and Joseph, 2001), stumps (von Sydow and Birgersson, 1997; Kelsey and Joseph, 1999a), and logs (Kelsey, 1994a,b; Kelsey and Joseph, 1997, 1999b). Ethanol, released alone or in

combination with host terpenes, is a well-known primary attractant for various species of weevils and scolytid secondary bark beetles (Moeck, 1970; Klimetzek et al., 1986; Nordlander et al., 1986; Liu and McLean, 1989; Phillips et al., 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Sjödin et al., 1989; Byers, 1992; Kelsey, 1994a,b). If ethanol accumulates and 4AA declines in severely stressed pine trees, they are likely to be more susceptible to attack by secondary bark beetles than healthy trees.

While secondary bark beetles and weevils are normally less aggressive than primary bark beetles, they still cause a variety of problems in forest management that warrants mitigation. For example, the value of commercial logs and lumber can be reduced by pinhole-sized galleries and stain fungi introduced to the sapwood by ambrosia beetles (McLean, 1985). Numerous other species colonize woody debris or stumps and can damage or kill regeneration seedlings or saplings (Nordlander, 1987; Ciesla, 1988; Wilson et al., 1996; Salom, 1997). In some instances larger trees under stress can be killed (Furniss and Carolin, 1977). When secondary bark beetles or weevils are attracted to trees infected with certain root disease organisms, they can vector the pathogen to adjacent healthy trees or nearby healthy stands (Harrington et al., 1985; Witcosky et al., 1986a,b; Bedard et al., 1990; Nevill and Alexander, 1992a,b; Klepzig et al., 1991, 1995).

Although 4AA inhibits the response of various primary bark beetles to their pheromones, its effect on responses of secondary bark beetles to primary host attractants, such as ethanol, has not been thoroughly tested. In one study, *Dendroctonus valens* was unaffected by 4AA when *S*-(−)-β-pinene was used as the attractant (Hobson, 1996). The objective of our experiment was to determine whether 4AA deters attraction of scolytids to ethanol released simultaneously with a mixture of α- and β-pinene and whether the deterrency of 4AA is affected by ethanol release rates. A mixture of α- and β-pinene was used at a constant release rate because they occur in ponderosa pine tissues (Drew and Pylant, 1966) when ethanol is synthesized. Furthermore, the combined release of ethanol with α - and β-pinene will attract a broader range of scolytids and in larger numbers than ethanol alone (Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Phillips et al., 1988; Lindelöw et al., 1993).

METHODS AND MATERIALS

Chemicals and Release Rates. Release rates of 4AA (98%, Aldrich), ethanol (100% USP grade, Georgia Pacific), and a 1:1 (v/v) mixture of (±)-α-pinene (98%, Aldrich) and (1*S*)-(−)-β-pinene (99%, Aldrich) in the laboratory and field were determined gravimetrically. All compounds were released from glass scintillation vials (20 ml) inside a perforated plastic container with lid (50 ml). There were 12 holes (0.8 cm diam.) on its side and two on the bottom, allowing free exchange

of chemicals with surrounding air. This setup prevented rain from diluting the compounds and altering their release rates.

Laboratory release rates were determined in an incubator at 30°C, and on a bench at 21°C. To simulate field conditions on the bench an electric fan was used to generate a wind speed of 1.1 m/sec. Two release rates were tested for 4AA and ethanol and one release rate for the pinene mixture, each averaged from five replicates. A high release rate for 4AA was achieved by placing three string wicks into the liquid and draping them over the open vial rim. A low release rate for 4AA was achieved with an open vial without strings. Ethanol was released from vials with lids having a 0.7- or 0.2-cm-diam. hole for high and low rates. The α- and β-pinene mixture was released from a separate open vial. Field release rates were determined by weighing vials before and after use in traps. These vials were capped for transport to and from the field.

Our field design consisted of a 2×3 factorial combination with two release rates of ethanol (low or high) and three release rates of 4AA (0, low or high) for a total of six treatments. Each treatment had the same α- and β-pinene release rate.

Field Tests. Lindgren 16-unit multiple funnel traps (Phero Tech, Delta, British Columbia, Canada) were replicated at 10 sites in Central Oregon, east of La Pine at 1494 to 1525 m elevation. Five sites separated by distances of 1.0–7.2 km were located in an area near 43°43′N; 120°52′W. These sites were thinned in 1996 to obtain a species composition of about 80% ponderosa and 20% lodgepole pine with diameters of approximately 20–30 cm at breast height (dbh). About 40 km southwest, in the vicinity of 43°35′N; 121°21′W, were another five sites separated by distances of 0.4–2.2 km. These sites were thinned in 1995 to obtain a species compositon of 70–100% ponderosa and 0–30% lodgepole pine, with 10–18 cm dbh. Disturbance from thinning helped ensure adequate beetle populations were available to successfully conduct the experiment. Sites had various levels of burned or unburned slash remaining.

Temperature data were collected from one site in each of the two areas with sensors attached to a datapod (Omnidata, Logan, Utah). One sensor was placed inside a weather shelter to measure ambient temperatures, while another sensor was placed inside the plastic bait container to determine temperature changes near the chemicals. These containers were not sheltered and could have received direct sun periodically during the day depending on crown positions of adjacent trees, etc.

At each site, six funnel traps were placed a minimum of 50 m apart along a transect. Traps were hung 0.5–1.0 m above ground from rope tied between two closely spaced trees and tethered with twine to keep them from swaying with wind. Traps were baited randomly by securing a plastic container to the middle funnel. Baits were replaced with fresh chemicals every two weeks and rerandomized among traps (by moving baits only) within a site to minimize the influence of

trap location on beetle catch numbers. Collection cups contained a piece of plastic releasing 2,2-dichlorovinyl-dimethylphosphate (Peststrip, Loveland Industries, Greeley, Colorado) to kill trapped insects. Traps were initially baited on May 8, 1997, and rebaited with fresh chemicals on May 19 and 30, and June 12. Insects were collected on the latter three dates and finally on June 26. Beetles were sorted into major scolytid species or their predators and counted.

Statistical Analysis. Data were analyzed using SAS software (SAS Institute, 1989, 1996). Release rates were summarized with their mean, minimum, and maximum values. Beetle numbers for each treatment and site were summed over the four collection dates and the analyses conducted on these values. Data were analyzed as a randomized complete block design with 4AA and ethanol as two factors. Beetle numbers were square-root transformed to meet homogeneity of variance and normality assumptions. Means were back-transformed for presentation. Significant differences between means were separated using Fisher's protected LSD at $\alpha = 0.10$.

To help compare behavior among species, ratios of beetles attracted to ethanol (high–low ethanol when 4AA was absent), or 4AA with ethanol (low–zero 4AA, and high–zero 4AA) were calculated. 4AA ratios were calculated with beetle averages across low and high ethanol treatments within each level of 4AA. Values above 1 indicate beetle attraction, while values below 1 show the degree of deterrence to each chemical.

RESULTS

Release Rates. There was substantial variation between laboratory and field release rates (Table 1). All chemicals, except the high ethanol treatment, had lower field release rates than in the laboratory at 21°C with air movement. Ambient field

TABLE 1. RELEASE RATES OF HOST VOLATILES IN INCUBATOR, LABORATORY, AND FIELD

Volatile	Release rates (mean, mg/hr)[a]		
	Incubator (30°C)	Laboratory (21°C)	Field (min 3.3, max 19.7°C)
α- + β-Pinene mixture	6.1 (5.7–6.3)	25.2 (14.6–34.1)	11.4 (6.4–16.3)
High ethanol	34.2 (33.7–35.0)	30.8 (28.1–37.2)	41.4 (30.1–57.2)
Low ethanol	11.7 (11.1–12.8)	6.7 (5.7–8.0)	4.5 (3.7–5.0)
High 4-allylanisole	5.2 (3.3–6.6)	10.4 (7.3–14.9)	4.3 (3.1–5.9)
Low 4-allylanisole	0.5 (0.3–0.7)	0.8 (0.1–0.8)	0.6 (0.5–0.9)

[a]The incubator (30°C) had no air movement, but in the laboratory (21°C) a fan generated a wind speed of 1.1 m/sec. Wind speed in the field was not measured. Numbers in parentheses are the range of release rates.

TABLE 2. SCOLYTIDS AND PREDATORS COLLECTED FROM 60 FUNNEL TRAPS (4 COLLECTION DATES) BAITED WITH α- AND β-PINENE PLUS DIFFERENT RELEASE RATES OF ETHANOL AND 4-ALLYLANISOLE, MAY 8–JUNE 26, 1997, IN PINE FORESTS OF CENTRAL OREGON

Beetle species	Number trapped	% of total
Bark beetles (Scolytidae)		
Dendroctonus valens	7,962	59.4
Ips latidens	1,989	14.8
Hylurgops porosus	1,349	10.1
Hylurgops subcostulatus	972	7.3
Ips pini	214	1.6
Hylurgops reticulatus	150	1.1
Hylastes macer	130	0.9
Gnathotrichus retusus	122	0.9
Hylastes nigrinus	74	0.5
Hylastes longicollis	53	0.4
Predator beetles		
Cleridae	240	1.8
Temnochila chlorodia (Trogositidae)	141	1.0
Total number of beetles	13,396	100.0

temperatures in weather shelters ranged from 3.3 to 19.7°C (average min–max). Inside plastic bait containers, average minimum and maximum temperatures were 2.1°C cooler at night, or 2.6°C warmer during the day than ambient temperatures, probably because these containers were not sheltered.

Field Test. In ponderosa pine forests of central Oregon, 10 species of secondary scolytid beetles and some of their predators were attracted to ethanol released in combination with a mixture of α- and β-pinene. *Dendroctonus valens* LeConte was the most abundant scolytid species trapped, comprising 59.4% of the total beetles caught (Table 2). Other abundant speices included *Ips latidens* LeConte at 14.8%, *Hylurgops porosus* (LeConte) 10.1%, and *Hylurgops subcostulatus* (Mannerheim) 7.3%. No primary bark beetles such as *Dendroctonus brevicomis* or *D. ponderosae* were caught in any traps. Aerial survey maps for 1996 and 1997 indicated endemic levels of activity for *D. brevicomis* and *D. ponderosae* in the forest surrounding our sites. Bark beetle predators such as clerids and *Temnochila chlorodia* (Mannerheim) were a small fraction of total beetles caught.

Dendroctonus valens responded about four times more frequently to traps with high ethanol compared to low ethanol regardless of 4AA levels (Figure 1). Low 4AA treatment caught significantly more *D. valens* than either the high ($P < 0.002$) or zero 4AA ($P = 0.029$). There was no difference in beetle numbers between high and zero 4AA ($P = 0.270$).

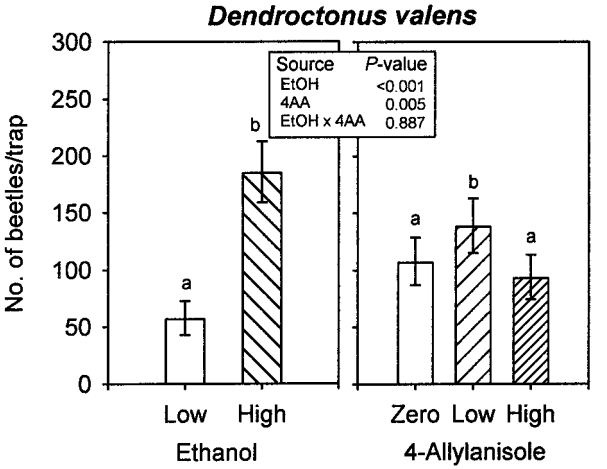


FIG. 1. Response of *D. valens* to traps baited with α- and β-pinene, ethanol, and 4-allylanisole. Vertical bars are means $\pm$ SE, $N = 10$.

Ips latidens was unaffected by ethanol treatments ($P = 0.288$), but was deterred at high 4AA compared to zero ($P = 0.004$) and low 4AA ($P = 0.037$, Figure 2). Beetle catches were not different between low and zero 4AA ($P = 0.385$). In contrast, *I. pini* showed a significant interaction between ethanol and 4AA (Figure 2). When 4AA was absent, *I. pini* numbers were greater at high ethanol than low ethanol ($P = 0.035$). The opposite was observed with low 4AA present ($P = 0.061$), and there was no difference in trap catches between ethanol release rates at high 4AA ($P = 0.974$). Low ethanol with either low or high 4AA attracted more *I. pini* than low ethanol alone ($P = 0.017$ and 0.054, respectively). Attraction of *I. pini* to high ethanol was unaffected by the presence of 4AA. There was a decrease in numbers in combination with low 4AA, but this was not significantly lower than trap catches for high ethanol with zero or high 4AA ($P = 0.113$, and 0.154, respectively).

All three *Hylurgops* species responded more strongly to high ethanol than low ethanol, regardless of amounts of 4AA (P values in Figure 3), but they each responded differently to 4AA. High and low 4AA inhibited *Hylurgops porosus* compared to zero 4AA ($P < 0.001$ and $P = 0.092$, respectively), whereas both release rates attracted *H. subcostulatus* ($P = 0.008$ and 0.052, respectively). *Hylurgops reticulatus* Wood was inhibited by high 4AA ($P < 0.014$), but not low 4AA ($P = 0.858$). Trap catches with high 4AA were smaller than those with low 4AA for *H. porosus* ($P < 0.030$) and *H. reticulatus* ($P = 0.009$), but not *H. subcostulatus* ($P = 0.432$).

Hylastes longicollis Swaine showed a significant 4AA and ethanol interaction (Figure 4). High ethanol caught more beetles than low ethanol at both zero

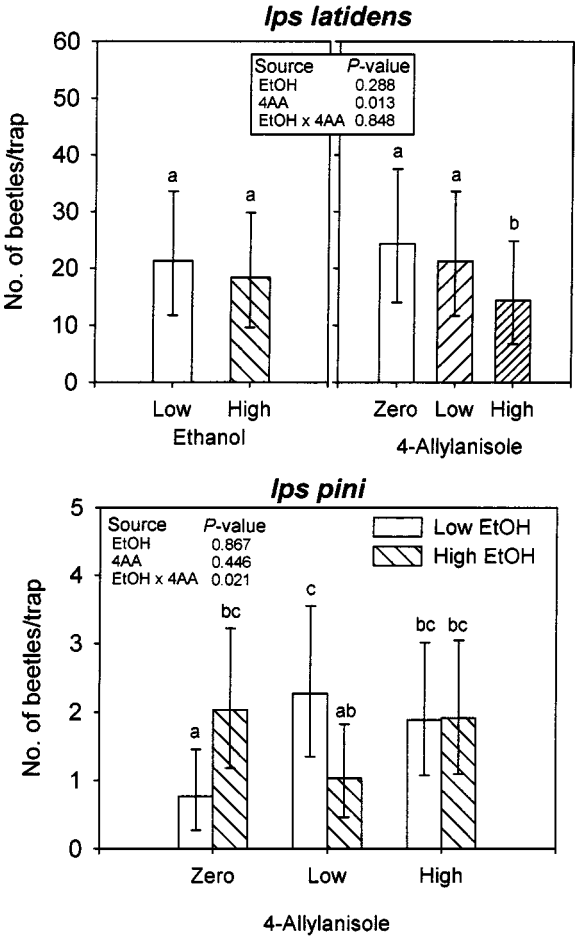


FIG. 2. Response of *Ips* spp. to traps baited with α- and β-pinene, ethanol, and 4-allylanisole. Vertical bars are means $\pm$ SE, $N = 10$.

($P < 0.001$) and low 4AA ($P = 0.025$), but not at high 4AA ($P = 1.000$). Low ethanol attracted the same number of *H. longicollis* regardless of 4AA treatments ($P \geq 0.727$). However, when the ethanol release rate was high, the high 4AA treatment reduced beetle numbers compared to zero ($P = 0.001$) and low 4AA ($P = 0.011$). *Hylastes macer* LeConte and *H. nigrinus* (Mannerheim) responded more strongly to high ethanol than low ethanol release rates (*P* values in Figure 4). Increasing the 4AA release rate from zero to low, and low to high, reduced *H. macer* almost linearly (all $P \leq 0.062$), whereas high, but not low 4AA reduced trap catches for *H. nigrinus* ($P = 0.074$ and 0.187, respectively).

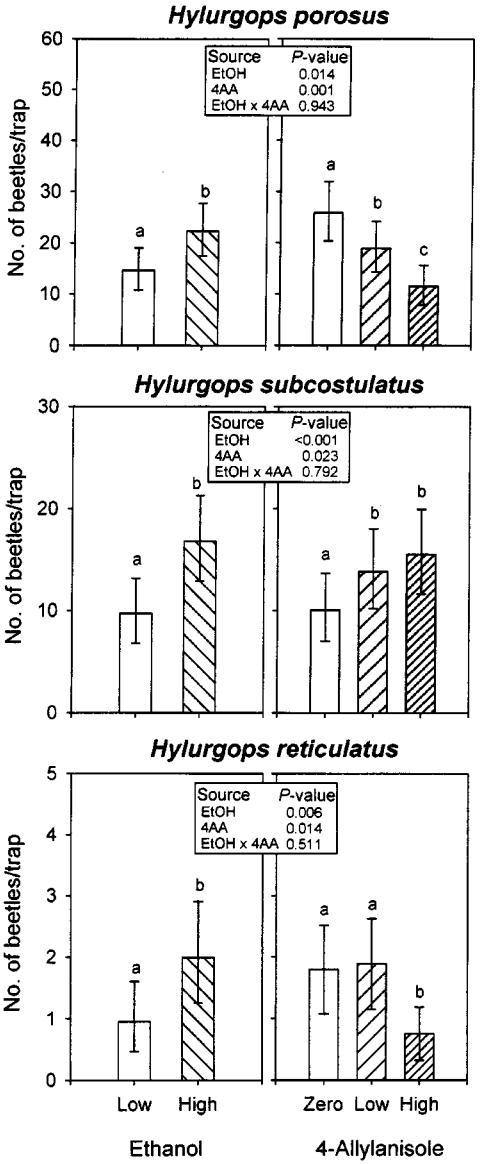


FIG. 3. Response of *Hylurgops* spp. to traps baited with α- and β-pinene, ethanol, and 4-allylanisole. Vertical bars are means $\pm$ SE, $N = 10$.

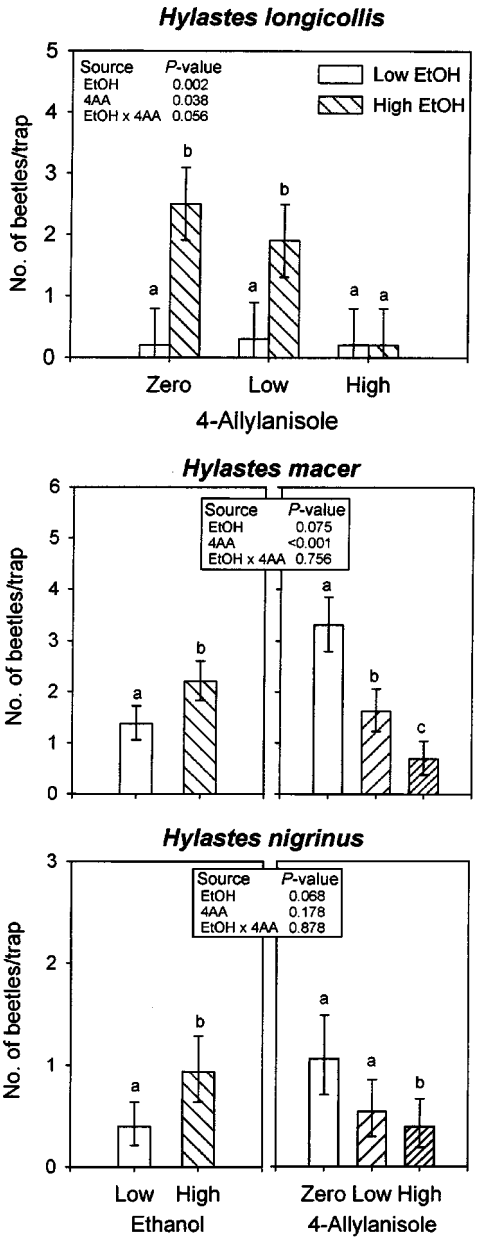


FIG. 4. Response of *Hylastes* spp. to traps baited with α- and β-pinene, ethanol, and 4-allylanisole. Vertical bars are means $\pm$ SE, $N = 10$.

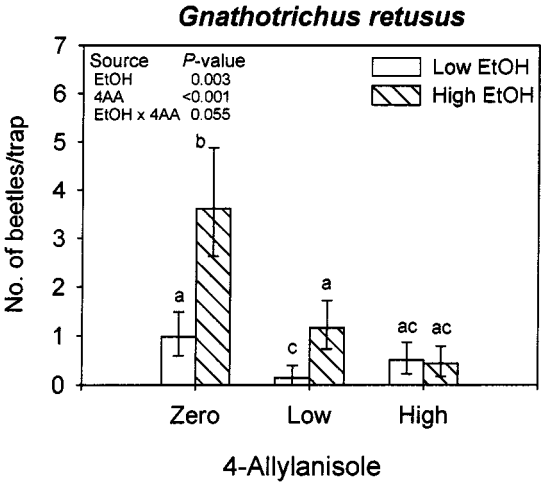


FIG. 5. Response of *G. retusus* to traps baited with α- and β-pinene, ethanol, and 4-allylanisole. Vertical bars are means $\pm$ SE, $N = 10$.

Gnathotrichus retusus (LeConte) numbers showed a significant interaction between 4AA and ethanol (Figure 5) similar to *Hylastes longicollis*. When 4AA was absent or low, the high ethanol release rate caught more beetles than low ethanol ($P = 0.003$ and $P = 0.014$), but when 4AA was high there was no difference in beetle numbers between ethanol treatments ($P = 0.858$). When ethanol was low, trap catches were inhibited by low 4AA, but not high 4AA, compared with zero 4AA ($P = 0.031$ and 0.287, respectively). When ethanol was high, trap catches of *G. retusus* were reduced by both low and high 4AA compared with zero 4AA ($P = 0.008$ and <0.001, respectively).

Clerids and *Temnochila chlorodia* were the only predators caught. Most clerids were either *Enoclerus* spp. or *Thanasimus* spp. However, neither clerids nor *Temnochila chlorodia* responded significantly to ethanol or 4AA treatments (*P* values are in Figure 6).

Relative responses of all species to ethanol and 4AA are shown in Figure 7. Most beetles responded 1.5–3.7 times more frequently to traps with high ethanol release rates (41.4 mg/hr) than those with low release rates (4.5 mg/hr) when 4AA was absent, except *Hylastes longicollis*, which was 12.5 times higher. Responses of *Ips latidens*, *T. chlorodia*, and clerids were not affected by ethanol release rates. Low release of 4AA reduced trap catches of four species by 25% or more, with significant inhibition for three of them compared to zero 4AA (*Gnathotrichus retusus*, *Hylastes macer*, and *Hylurgops porosus*). In contrast, *Hylurgops subcostulatus*, *I. pini*, and *D. valens* were attracted to low 4AA. Increasing the release rate of 4AA enhanced repellency, with significant inhibition for seven species. At high

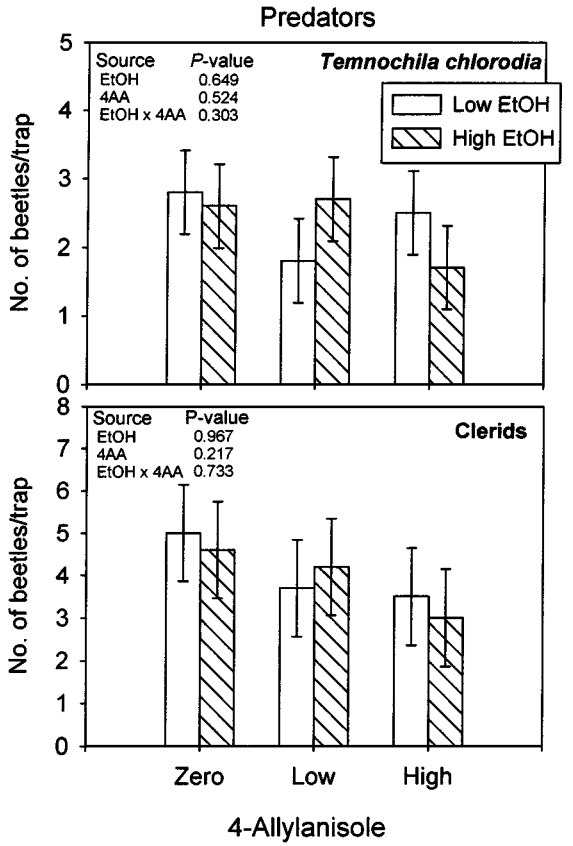


FIG. 6. Response of *T. chlorodia* and clerid predators to traps baited with α- and β-pinene, ethanol, and 4-allylanisole. Vertical bars are means $\pm$ SE, $N = 10$.

4AA, *Hylastes longicollis*, *Hylastes macer*, and *G. retusus* were most inhibited, while *Hylurgops subcostulatus* and *I. pini* (with low ethanol only) were attracted. Increasing the release rate of 4AA from 0.6 to 4.3 mg/hr had its greatest impact on *Hylastes longicollis* and *Hylurgops reticulatus* and least affect on *G. retusus*, *Hylastes nigrinus*, *Hylurgops subcostulatus*, and *I. pini*.

DISCUSSION

Ethanol, released in combination with α- and β-pinene, is an important primary attractant for many secondary bark beetles in ponderosa pine forests of central Oregon. Attraction of *Dendroctonus valens* to a similar combination was previously demonstrated in Wisconsin forests where traps baited with a 1:1

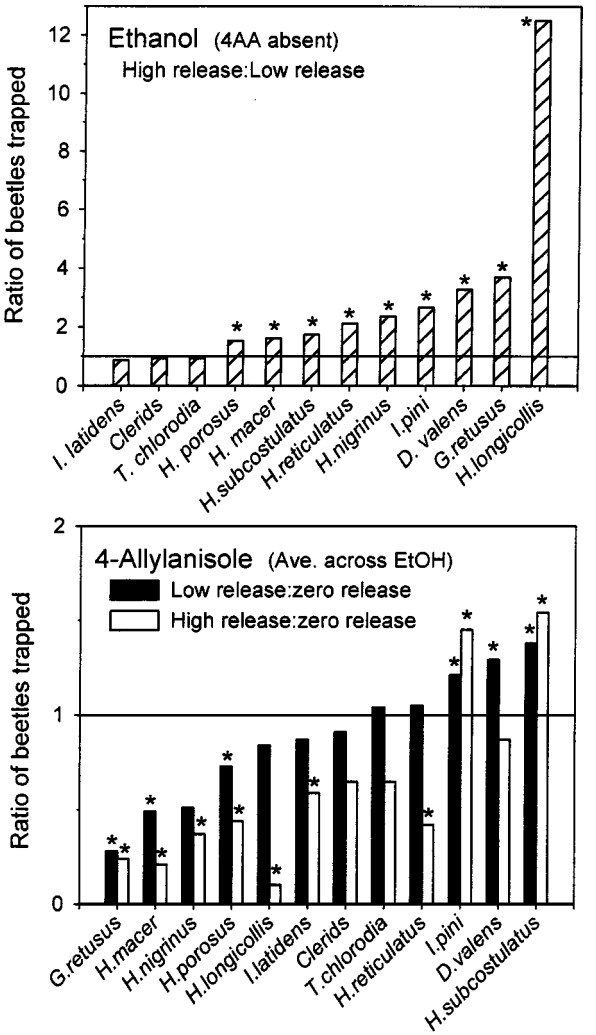


FIG. 7. Ratios of beetles trapped by different release rates of ethanol or 4AA. For species with a significant interaction between 4AA and ethanol, we used the main effect means for 0, low, and high 4AA. Values greater than 1 (horizontal line) indicate attraction and values less than 1 indicate deterrence. Asterisks indicate species where the number of beetles captured by treatments used in the numerator and denominator of a ratio were significantly different by ANOVA. *Ips pini* was attracted to 4AA only in combination with low ethanol release rates (see Figure 2 for details).

ethanol–terpentine (α- and β-pinene plus other terpenes) mixture captured 60 times more beetles than traps with terpentine alone (Klepzig et al., 1991). All *Hylastes* and *Hylurgops* at our sites preferred traps with the highest ethanol release rate, much like related species in Europe. For example, ethanol alone attracts *Hylurgops palliatus* (Gyll.) and the beetle numbers rise as the release rate increases (Klimetzek et al., 1986; Schroeder, 1988; Schroeder and Lindelöw, 1989; Byers, 1992). α-Pinene alone does not attract this species, but the combination of α-pinene and ethanol results in a synergistic response, with greater trap catches than the total from both compounds individually (Schroeder, 1988; Schroeder and Lindelöw, 1989; Byers, 1992). Although *Hylastes longicollis* in this experiment showed the largest proportional increase in trap catches between the two release rates of ethanol, this could have resulted, in part, from the low number of individuals trapped. Their typical response to ethanol is probably more similar to the other species. Numbers of *G. retusus* captured were low also, but their affinity for a high ethanol release rate reflects their preference for logs with high ethanol concentrations over logs with low concentrations (Kelsey, 1994a,b; Kelsey and Joseph, 1999b). α-Pinene most likely does not enhance, and may inhibit, the attraction of *Gnathotrichus* spp. to ethanol (Kelsey and Joseph, 1997).

Ips latidens was not attracted to ethanol, which is similar to *I. grandicollis* (Eichhoff) (Chénier and Philogène, 1989) and *I. typographus* (L.) (Klimetzek et al., 1986). For the latter species, combining ethanol with its pheromone reduced the beetle numbers compared to traps baited with pheromone alone, although the differences were not significant. In contrast, *I. pini* in the current experiment was attracted to ethanol. Why it responded differently than the *Ips* species listed above is unclear. Increasing the ethanol release rate also failed to enhance trap catches of clerids and *Temnochila chlorodia* in central Oregon. Other clerid predators, such as *Thanasimus formicarius* (L.) in Sweden (Schroeder, 1988) or *T. dubius* (Fabricius) in Canada (Chénier and Philogène, 1989), are attracted to α-pinene but not ethanol or their combination.

Effectiveness of 4AA in central Oregon as a repellent for secondary bark beetles attracted to host kairomones is dependent on the beetle species, and release rates of 4AA and ethanol. In general, 4AA was effective at inhibiting some beetles, such as *G. retusus* and the three species of *Hylastes.* In contrast, one or both release rates of 4AA attracted other species, including *Hylurgops subcostulatus*, *I. pini*, and *D. valens.* Hobson (1996) reports 4AA failed to inhibit attraction of *D. valens* to β-pinene. His release rate of 4AA was nearly 19 times (80 mg/hr) greater than our highest level. The response of *D. valens* to traps baited with ethanol and 4AA is consistent with their preference for colonizing the base of diseased ponderosa pine boles (Moeck et al., 1981), which can have lower amounts of 4AA (Nebeker et al., 1995) and higher ethanol concentrations than healthy trees (Kelsey et al., 1998).

Attraction of bark beetles to 4AA has been reported previously for *I. grandicollis* (Eichh.), particularly the males (Werner, 1972). In the current study, *I. pini*

was also attracted to 4AA, but only when ethanol release rates were low, whereas *I. latidens* showed no signs of attraction to 4AA. In studies where traps were baited with pheromone attractants, 4AA has inhibited the response of *I. pini.* Hobson (1996) reduced trap catches of *I. pini* by 29% with a release rate of 80 mg/hr 4AA, but it was not significant. In contrast, Hayes and Strom (1994) reported a 43% reduction in numbers of *I. pini* caught by traps in Wisconsin releasing 4AA at 6.6 mg/hr, or 1.5 times greater than our highest treatment. Attraction to 4AA also was previously reported for the bark beetle predator *T. chlorodia* when tested in Oregon, but not when tested in California (Hayes and Strom, 1994). Our trap catches of *T. chlorodia* and clerid predators were reduced by a high release rate of 4AA, but not significantly. Thus, it appears some bark beetle predators are not adversely impacted by 4AA.

It is becoming apparent that ethanol accumulates in woody tissues more often than anyone previously suspected. It can be synthesized by many tree species (Kimmerer and MacDonald, 1987; Kimmerer and Stringer, 1988; Kelsey, 1996) and by all of their tissues (Kimmerer and Stringer, 1988; Kelsey et al., 1998) under appropriate conditions. Synthesis of ethanol in small quantities is probably an important physiological process for normal tree growth (Harry and Kimmerer, 1991; Joseph and Kelsey, unpublished data). However, ethanol can accumulate to abnormally high concentrations when trees are subjected to various types of severe stress or when tissues are dying, as in logs and stumps (Kimmerer and Kozlowski, 1982; Sjödin et al., 1989; Kelsey, 1994a,b; von Sydow and Birgersson, 1997; Kelsey and Joseph, 1999a,b, 2001). Quantities of ethanol synthesized in wood residues are highly variable and strongly influenced by environmental parameters such as precipitation, and temperature (Kelsey and Joseph, 1999a,b). This variability can provide opportunities to manipulate ethanol production and mitigate the behavior of some secondary bark beetles. One alternative is to minimize slash and woody debris, but stumps are usually left intact, and current forest practices are moving toward leaving more coarse woody debris in the forest to allow important ecological processes to function normally and sustainably (Schowalter and Filip, 1993). Thus, other alternatives may be needed to help manage secondary bark beetles.

4AA is an allomone with potential utility for management because it inhibits responses for a wide range of scolytid beetles to primary and secondary attractants (Hayes and Strom, 1994; Werner, 1995). It might have the added advantage of not adversely interfering with the attraction of bark beetle predators, as observed here and by others (Hayes and Strom, 1994). 4AA also has shown some promise in suppressing or reducing growth of small infestations of *D. frontalis* (Hayes and Clarke, 1998). Consequently, it seems worthwhile to further examine the use of 4AA for protecting high-value logs, individual trees, and possibly small stands or plantations from unwanted attack and colonization by secondary bark beetles that are sensitive to this compound.

Acknowledgments—We thank the staff of Bend/Ft Rock Ranger District, especially Matt Deppmeier, for assistance in locating suitable sites for this experiment. We thank Dr. J. L. Hayes and Dr. R. A. Werner for reviewing the manuscript, and Dr. Lisa Ganio and Manuela Huso for statistical advice. The use of trade, firm, or corporation names is for information and convenience of the reader. Such use does not constitute an official endorsement or approval by the US Department of Agriculture of any product or service to the exclusion of others that may be suitable.

REFERENCES

BEDARD, W. D., FERRELL, G. T., WHITMORE, M. C., and ROBERTSON, A. S. 1990. Trapping evaluation of beetle vectors of black stain root disease in Douglas fir. *Can. Entomol.* 122:459–468.

BENNETT, D. C., and FREELING, M. 1987. Flooding and the anaerobic stress response, pp. 79–84, *in* D.W. Newman and K.G. Wilson (eds.). Models in Plant Physiology and Biochemsitry, Vol. III. CRC Press, Boca Raton, Florida.

BYERS, J. A. 1992. Attraction of bark beetles, *Tomicus piniperda, Hylurgops palliatus* and *Trypodendron domesticum* and other insects to short-chain alcohols and monoterpenes. *J. Chem. Ecol.* 18:2385–2402.

CIESLA, W. M. 1988. Pine bark beetles: A new pest management challenge for Chilean foresters. *J. For.* 86:27–31.

CHÉNIER, J. V. R., and PHILOGÈNE, B. J. R. 1989. Field responses of certain forest Coleoptera to conifer monoterpenes and ethanol. *J. Chem. Ecol.* 15:1729–1745.

COBB, F. W., Jr., ZAVARIN, E., and Bergot, J. 1972. Effect of air pollution on the volatile oil from leaves of *Pinus ponderosa. Phytochemistry* 11:1815–1818.

CRAWFORD, R. M. M., and BAINES, M. A. 1977. Tolerance of anoxia and the metabolism of ethanol in tree roots. *New Phytol.* 79:519–526.

CRAWFORD, R. M. M., and FINEGAN, D. M. 1989. Removal of ethanol from lodgepole pine roots. *Tree Physiol.* 5:53–61.

DAVIES, D. D. 1980. Anaerobic metabolism and the production of organic acids, pp. 581–611, *in* D. D. Davies (ed.). The Biochemistry of Plants, Vol. 2, Metabolism and Respiration. Academic Press, New York.

DREW, J., and PYLANT, G. D., Jr. 1966. Turpentine from the pulpwoods of the United States and Canada. *Tappi* 49:430–438.

FURNISS, R. L., and CAROLIN, V. M. 1977. Western forest insects. USDA For. Serv. Misc. Pub. 1339. US Government Printing Office, Washington, DC.

GARA, R. I., LITTKE, W. R., and RHODES, D. F. 1993. Emission of ethanol and monoterpenes by fungal infected lodgepole pine trees. *Phytochemistry* 34:987–990.

HARRINGTON, T. C., COBB, F. W., Jr., and LOWNSBERY, J. W. 1985. Activity of *Hylastes nigrinus*, a vector of *Verticicladiella wageneri*, in thinned stands of Douglas-fir. *Can. J. For. Res.* 15:519–523.

HARRY, D. E., and KIMMERER, T. W. 1991. Molecular genetics and physiology of alcohol dehydrogenase in woody plants. *For. Ecol. Manage.* 43:251–272.

HAYES, J. L., and CLARKE, S. 1998. Development and evaluation of host-compound use in disruption of southern pine beetle infestation growth: Proceedings, 49th Annual Western Forest Insect Work Conference, USDA, For. Serv., Rocky Mtn. Res. Sta., Ogden, Utah, p. 55.

HAYES, J. L., and STROM, B. L. 1994. 4-Allylanisole as an inhibitor of bark beetle (Coleoptera: Scolytidae) aggregation. *J. Econ. Entomol.* 87:1586–1594.

HAYES, J. L., STROM, B. L., ROTOM, L. M., and INGRAM, L. L. Jr. 1994. Repellent properties of the host compound 4-allylanisole to the southern pine beetle. *J. Chem. Ecol.* 20:1595–1615.

HOBSON, K. R. 1996. Interruption of bark beetle aggregation by a vigor-dependent *Pinus* host compound, pp. 228–233, *in* W. J. Mattson, P. Niemela, and M. Rousi (eds.). Dynamics of Forest Herbivory: Quest for Pattern and Principle. USDA For. Serv. Gen. Tech. Rep. NC-183.

JOSEPH, G., and KELSEY, R. G. 1997. Ethanol synthesis and water relations of flooded *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) seedlings under controlled conditions. *Int. J. Plant Sci.* 158:844–850.

KELSEY, R. G. 1994a. Ethanol synthesis in Douglas-fir logs felled in November, January, and March and its relationship to ambrosia beetle attack. *Can. J. For. Res.* 24:2096–2104.

KELSEY, R. G. 1994b. Ethanol and ambrosia beetles in Douglas fir logs with and without branches. *J. Chem. Ecol.* 20:3307–3319.

KELSEY, R. G. 1996. Anaerobic induced ethanol synthesis in the stems of greenhouse-grown conifer seedlings. *Trees* 10:183–188.

KELSEY, R. G., and JOSEPH, G. 1997. Ambrosia beetle host selection among logs of Douglas fir, western hemlock, and western red cedar with different ethanol and α-pinene concentrations. *J. Chem. Ecol.* 23:1035–1051.

KELSEY, R. G., and JOSEPH., G. 1998. Ethanol in Douglas-fir with black-stain root disease (*Leptographium wageneri). Can. J. For. Res.* 28:1207–1212.

KELSEY, R. G., and JOSEPH., G. 1999a. Ethanol and water in *Pseudotsuga menziesii* and *Pinus ponderosa* stumps. *J. Chem. Ecol.* 25:2779–2792.

KELSEY, R. G., and JOSEPH., G. 1999b. Ethanol and ambrosia beetles in Douglas fir logs exposed or protected from rain. *J. Chem. Ecol.* 25:2793–2809.

KELSEY, R. G., and JOSEPH., G. 2001. Attraction of *Scolytus unispinosus* bark beetles to ethanol in water-stressed Douglas-fir branches. *For. Ecol. Manage.* In press.

KELSEY, R. G., JOSEPH, G., and THIES, W. G. 1998. Sapwood and crown symptoms in ponderosa pine infected with black-stain and annosum root disease. *For. Ecol. Manage.* 111:181–191.

KIMMERER, T. W., and KOZLOWSKI, T. T. 1982. Ethylene, ethane, acetaldehyde, and ethanol production by plants under stress. *Plant Physiol.* 69:840–847.

KIMMERER, T. W., and MACDONALD, R. C. 1987. Acetaldehyde and ethanol biosynthesis in leaves of plants. *Plant Physiol.* 84:1204–1209.

KIMMERER, T. W., and STRINGER, M. A. 1988. Alcohol dehydrogenase and ethanol in the stems of trees. *Plant Physiol.* 87:693–697.

KLEPZIG, K. D., RAFFA, K. F, and SMALLEY, E. B. 1991. Association of an insect-fungal complex with red pine decline in Wisconsin. *For. Sci.* 37:1119–1139.

KLEPZIG, K. D., SMALLEY, E. B, and RAFFA, K. F. 1995. *Dendroctonus valens* and *Hylastes porculus* (Coleoptera: Scolytidae): Vectors of pathogenic fungi (Ophiostomatales) associated with red pine decline disease. *Great Lakes Entomol.* 28:81–87.

KLIMETZEK, D., KÖHLER, J., VITÉ, J. P., and KOHNLE, U., 1986. Dosage response to ethanol mediates host selection by "secondary" bark beetles. *Naturwissenschaften* 73:270–272.

LINDELÖW, Å., EIDMANN, H. H., and NORDENHEM, H. 1993. Response on the ground of bark beetle and weevil species colonizing conifer stumps and roots to terpenes and ethanol. *J. Chem. Ecol.* 19:1393–1403.

LIU, Y.-B., and MCLEAN, J. A. 1989. Field evaluation of responses of *Gnathotrichus sulcatus* and *G. retusus* (Coleoptera: Scolytidae) to semiochemicals. *J. Econ. Entomol.* 82:1687–1690.

MCLEAN, J. A. 1985. Ambrosia beetles: A million dollar degrade problem of sawlogs in coastal British Columbia. *For. Chron.* 61:295–298.

MOECK, H. A. 1970. Ethanol as the primary attractant for the ambrosia beetle *Trypodendron lineatum* (Coleoptera: Scolytidae). *Can. Entomol.* 102:985–995.

MOECK, H. A., WOOD, D. L., and LINDAHL, K. Q., Jr. 1981. Host selection behavior of bark beetles (Coleoptera: Scolytidae) attacking *Pinus ponderosa*, with special emphasis on the western pine beetle, *Dendroctonus brevicomis. J. Chem.Ecol.* 7:49–83.

NEVILL, R. J., and ALEXANDER, S. A. 1992a. Transmission of *Leptographium procerum* to eastern white pine by *Hylobius pales* and *Pissodes nemorensis* (Coleoptera: Curculionidae). *Plant Dis.* 76:307–310.

NEVILL, R. J., and ALEXANDER, S. A. 1992b. Root- and stem-colonizing insects recovered from eastern white pines with procerum root disease. *Can. J. For. Res.* 22:1712–1716.

NEBEKER, T. E., SCHMITZ, R. F., Tisdale, R. A., and HOBSON, K. R. 1995. Chemical and nutritional status of dwarf mistletoe, Armillaria root rot, and Comandra blister rust infected trees which may influence tree susceptibility to bark beetle attack. *Can. J. Bot.* 73:360–369.

NORDLANDER, G. 1987. A method for trapping *Hylobius abietis* (L.) with a standardized bait and its potential for forecasting seedling damage. *Scand. J. For. Res.* 2:199–213.

NORDLANDER, G., EIDMANN, H. H., JACOBSSON, U., NORDENHEM, H., and SJÖDIN, K. 1986. Orientation of the pine weevil *Hylobius abietis* to underground sources of host volatiles. *Entomol. Exp. Appl.* 41:91–100.

PHILLIPS, T. W. WILKENING, A. J., ATKINSON, T. H., NATION, J. L, WILKINSON, R. C., and FOLTZ, J. L. 1988. Synergism of turpentine and ethanol as attractants for certain pine-infesting beetles (Coleoptera). *Environ. Entomol.* 17:456–462.

PIERCE, H. D., Jr., CONN, J. E., OEHLSCHLAGER, A. C., and BORDEN, J. H. 1987. Monoterpene metabolism in female mountain pine beetles, *Dendroctonus ponderosa E*, Hopkins, attacking ponderosa pine. *J. Chem. Ecol.* 13:1455–1480.

SALOM, S. M. 1997. Status and management of pales weevil in the eastern United States. *Tree Planters' Notes* 48:4–11.

SAS INSTITUTE INC. 1989. SAS/STAT User's Guide, Ver. 6, 4th ed., Vols. 1 & 2. SAS Institute Inc., Cary, North Carolina.

SAS INSTITUTE INC. 1996. SAS/STAT Software: Changes and Enhancements Through Release 6.11. SAS Institute Inc., Cary, North Carolina.

SCHOWALTER, T. D., and FILIP, G. M. 1993. Bark beetle–pathogen–conifer interactions: An overview, pp. 3–19, *in* T. D. Schowalter and G. M. Filip (eds.). Beetle–Pathogen Interactions in Conifer Forests. Academic Press, San Diego, California.

SCHROEDER, L. M. 1988. Attraction of the bark beetle *Tomicus piniperda* and some other bark- and wood-living beetles to the host volatiles α-pinene and ethanol. *Entomol. Exp. Appl.* 46:203–210.

SCHROEDER, L. M., and LINDELÖW, Å. 1989. Attraction of scolytids and associated beetles by different absolute amounts and proportions of α-pinene and ethanol. *J. Chem. Ecol.* 15:807–817.

SJÖDIN, K., SCHROEDER, L. M., EIDMANN, H. H., NORIN, T., and WOLD, S. 1989. Attack rates of scolytids and composition of volatile wood constituents in healthy and mechanically weakened pine trees. *Scand. J. For. Res.* 4:379–391.

STARK, R. W., MILLER, P. R., COBB, F. W., Jr., WOOD, D. L., and PARMETER, J. R., Jr. 1968. Incidence of bark beetle infestation in injured trees. *Hilgardia* 59:121–126.

VON SYDOW, F., and BIRGERSSON, G. 1997. Conifer stump condition and pine weevil (*Hylobius abietis*) reproduction. *Can. J. For. Res.* 27:1254–1262.

WERNER, R. A. 1972. Aggregation behavior of the beetle, *Ips grandicollis*, in response to host-produced attractants. *J. Insect. Physiol.* 18:423–437.

WERNER, R. A. 1995. Toxicity and repellency of 4-allylanisole and monoterpenes from white spruce and tamarack to the spruce beetle and eastern larch beetle (Coleoptera: Scolytidae). *Environ. Entomol.* 24:372–379.

WILSON, W. L., DAY, K. R., and HART, E. A. 1996. Predicting the extent of damage to conifer seedlings by the pine weevil (*Hylobius abietis* L.): A preliminary risk model by multiple logistic regression. *New For.* 12:203–222.

WITCOSKY, J. J., SCHOWALTER, T. D., and HANSEN, E. M. 1986a. *Hylastes nigrinus* (Coleoptera: Scolytidae), *Pissodes fasciatus*, and *Steremnius carinatus* (Coleoptera: Curculionidae) as vectors of black-stain root disease of Douglas-fir. *Environ. Entomol.* 15:1090–1095.

WITCOSKY, J. J., SCHOWALTER, T. D., and HANSEN, E. M. 1986b. The influence of time of precommercial thinning on the colonization of Douglas-fir by three species of root-colonizing insects. *Can. J. For. Res.* 16:745–749.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

ANTITERMITIC ACTIVITY OF ESSENTIAL OILS AND COMPONENTS FROM TAIWANIA (*Taiwania cryptomerioides*)

SHANG-TZEN CHANG,[1,]* SEN-SUNG CHENG,[1] and SHENG-YANG WANG[2]

[1]*Department of Forestry*
National Taiwan University, No. 1, Section 4
Roosevelt Road, Taipei 106, Taiwan

[2]*Institute of BioAgricultural Sciences*
Academia Sinica, No. 128, Section 2
Academy Road, Taipei 115, Taiwan

(Received August 28, 2000; accepted December 12, 2000)

Abstract—Antitermitic activity of Taiwania (*Taiwania cryptomerioides* Hayata) against *Coptotermes formosanus* Shiraki was demonstrated in laboratory tests. Blocks of sapwood and heartwood from *T. cryptomerioides* exhibited antitermitic activity. Bioassays revealed that heartwood essential oil exhibited the highest antitermitic activity, followed by sapwood essential oil and then the *n*-C_6H_{14} soluble fraction when tested at 10 mg/g. The order of termite mortality of three compounds purified from *n*-C_6H_{14} soluble extracts of heartwood was cedrol > α-cadinol > ferruginol. The termite resistance of *T. cryptomerioides* wood can be attributed to the termiticidal activity of cedrol and α-cadinol.

Key Words—*Taiwania cryptomerioides* Hayata, *Coptotermes formosanus* Shiraki, antitermitic activity, essential oils, cedrol, α-cadinol, ferruginol.

INTRODUCTION

Biodegradation is recognized as one of the most significant problems for wood utilization. Among several factors leading to biodegradation, termites are one of the most damaging to wooden structures worldwide. There are several properties that determine wood's natural resistance to termite attack. For example, hardness of wood affects the termite's ability to fragment the wood with its mandibles (Bultman et al., 1979). It is also well known that extractives have a significant effect on the durability of wood (Chang et al., 1999b). Certain extractives from wood

*To whom correspondence should be addressed. e-mail peter@ms.cc.ntu.edu.tw

0098-0331/01/0400-0717$19.50/0

tissues can provide protection against harmful insects. For nondurable woods, it may be necessary to use inorganic compounds or synthetic pesticides to preserve woods and prolong their application life. To avoid environmental pollution and health problems caused by the use of traditional wood preservatives or synthetic pesticides, there is a trend to search for naturally occurring toxicants in plants. Accordingly, many researchers are focusing on the separation and identification of extractives from some durable woods and examining bioactivities.

Taiwania (*Taiwania cryptomerioides* Hayata) (*Taxodiaceae*) is an endemic tree that grows at elevations of 1800–2600 m in Taiwan's central mountains. The heartwood of *T. cryptomerioides* is yellowish red with distinguished purplish pink streaks. *T. cryptomerioides* timbers are well known in Taiwan for their decay resistance and excellent durability. Recently, we have illustrated the relationships between extractives and wood properties, including the photodiscoloration, antifungal, and antibacterial activity in *T. cryptomerioides* heartwood (Chang et al., 1998, 1999a,b, 2000a,b). To our knowledge, there are no prior studies of the antitermitic activity of chemical constituents from *T. cryptomerioides. Coptotermes formosanus* Shiraki is the termite mainly responsible for wood destruction in many countries, including Taiwan, Japan, and parts of the United States. Thus, many researchers have investigated antitermitic compounds of wood species using *C. formosanus* as a test termite (Yaga and Kinjo, 1986; Kinjo et al., 1988; Yaga et al., 1991; Ohtani et al., 1997; Sogabe et al., 2000a,b). In this study, we examined the antitermitic activity of the essential oils and dominant constituents isolated from *T. cryptomerioides* against *C. formosanus*.

METHODS AND MATERIALS

Wood Samples. Test blocks, 2.0 × 2.0 × 2.0 cm, were cut from the sapwood and heartwood of a 27-year-old *T. cryptomerioides* tree collected from the Experimental Forest of National Taiwan University. The blocks were oven-dried (60 ± 2°C, 24 hr) and weighed before treatment. Heartwood and sapwood blocks were extracted in a Soxhlet apparatus with alcohol–toluene (1 : 2) for 24 hr.

Termites. The test termites were from a *C. formosanus* colony from S.-J. Lin's laboratory at the Taiwan Forest Research Institute. The colony has been reared on wood pieces in the dark at 26.5°C and 85% relative humidity for more than two years.

General Procedures. Merck (Germany) Kieselgel $60F_{254}$ sheets were used for analytical thin layer chromatography (TLC). High performance liquid chromatography (HPLC) was performed with a Jasco model PU980 pump equipped with a Jasco RI-930 RI detector and Hibar Lichrosorb Si-60 column (25 × 1 cm ID). FTIR spectra were recorded on a Bio-Rad model FTS-40 spectrophotometer, and the MS was obtained using a Finnigan MAT-95s Mass spectrometer. The ^{13}C and ^{1}H NMR spectra were recorded on a Bruker Avance-500 MHz FT-NMR.

Extraction and Isolation. T. cryptomerioides heartwood chips were prepared from the freshly cut tree. The essential oils from sapwood and heartwood of this tree were obtained by water distillation. The air-dried heartwood chips (5.7 kg) were exhaustively extracted with methanol (MeOH). The extracts were condensed to 286.4 g by rotary evaporation, followed by extraction with *n*-hexane (*n*-C_6H_{14}), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) successively. After removing solvents from the combined extracts, the *n*-C_6H_{14}, $CHCl_3$, EtOAc, and MeOH soluble fractions and MeOH insoluble fraction were obtained. The *n*-hexane soluble fraction (5 g) of methanol extracts was fractionated initially by gradient elution with EtOAc/*n*-C_6H_{14} on a silica gel column (800 g). Fractions were tracked by TLC, and compounds with similar R_f values were pooled to give 41 subfractions (H1–H41). α-Cadinol (**1**) (27.6 min retention time) was isolated and purified from H16 to H22 by semipreparative HPLC [Si-60 column, EtOAc–*n*-C_6H_{14} (30 : 70) mobile phase, 1.0 ml/min flow rate]. Ferruginol (**2**) (16.2 min retention time) was collected from H2–H8 with the same HPLC system. The mobile phase was changed to EtOAc–*n*-C_6H_{14} (10 : 90). Cedrol (**3**) (20.0 min retention time) was isolated from H10 with the same HPLC system. The mobile phase was changed to EtOAc–*n*-C_6H_{14} (20 : 80), 1.0 ml/min flow rate. Structures of compounds isolated from *T. cryptomerioides* were identified using FTIR, MS, and NMR spectrometry. Their spectral data are consistent with those reported in the literature (Chang et al., 1998, 2000a,b).

*α-Cadinol (**1**)*. Colorless needle crystal; mp 74–75°C; EI-MS for $C_{15}H_{26}O$ found 222; IR ν_{max}: 3372, 3046, 1660 cm^{-1}; ^1H NMR (in $CDCl_3$): δ (ppm) 0.74 (d, $J = 7.0$, H-12), 0.89 (d, $J = 7.0$, H-13), 1.08 (s, H-14), 1.64 (s, H-15), 2.13 (m, H-11), 5.47 (s, H-4); ^{13}C NMR: δ (ppm) 15.13 (C-12), 20.77 (C-14), 21.51 (C-13), 22.00 (C-1), 22.68 (C-7), 23.81 (C-15), 26.00 (C-11), 30.95 (C-2), 39.89 (C-5), 42.22 (C-8), 46.73 (C-6), 50.04 (C-10), 72.47 (C-9), 122.35 (C-4), 134.94 (C-3).

*Ferruginol (**2**)*. Yellow oil; EI-MS for $C_{20}H_{30}O$ found 286; IR ν_{max}: 3370, 1612, 1501, 1440 cm^{-1}; ^1H NMR (in $CDCl_3$): δ (ppm) 0.89 (3H, s, H-18), 0.93 (3H, s, H-19), 1.15 (3H, s, H-20),1.22 (3H, d, $J = 7.0$ Hz, H-16), 1.29 (3H, d, $J = 7.0$ Hz, H-17), 2.77 (1H, ddd, $J = 17.0$, 10.5, 7.0 Hz, H-7a), 2.81(1H, ddd, $J = 2.0$, 6.5, 17.0 Hz, H-7b), 3.11 (sept, $J = 7.0$ Hz, H-15), 6.62 (1H, s, H-11), 6.81 (1H, s, H-14); ^{13}C NMR: δ (ppm) 19.12 (C-6), 19.18 (C-2), 21.46 (C-19), 22.50 (C-16), 22.67 (C-17), 24.60 (C-20), 26.47 (C-15), 29.60 (C-7), 33.17 (C-18), 33.23 (C-4), 37.27 (C-10), 38.65 (C-1), 41.57 (C-3), 50.23 (C-5), 110.86 (C-11), 126.47 (C-14), 126.27 (C-8), 131.69 (C-13), 148.16 (C-9), 150.95 (C-12).

*Cedrol (**3**)*. Colorless needle crystal; mp 87°C; EI-MS for $C_{15}H_{26}O$ found 222; IR ν_{max}: 3342, 1373 cm^{-1}; ^1H NMR (in $CDCl_3$): δ (ppm) 0.81 (d, $J = 7.2$, H-15), 0.97 (s, H-13), 1.23 (s, H-14), 1.29 (s, H-12); ^{13}C NMR (in $CDCl_3$): δ (ppm) 15.54 (C-15), 25.34 (C-13), 27.61 (C-14), 28.89 (C-12), 30.16 (C-11), 31.58 (C-9), 35.33 (C-10), 36.99 (C-3), 41.45 (C-7), 41.96 (C-4), 43.38 (C-2), 54.08 (C-1), 56.51 (C-5), 61.03 (C-6), 75.10 (C-8).

Termiticidal Tests with Wood Blocks. Sapwood and heartwood (extracted and unextracted) blocks were tested using the no-choice test methods described by the American Wood-Preservers' Association (AWPA, 1997) against *C. formosanus. Pinus taiwanensis* sapwood is well recognized in Taiwan for its poor durability (Chang and Wang, 1995). It was selected as the control in this assay. Oven-dried wood blocks as well as 45 workers and 5 soldiers of *C. formosanus* were placed into the test container (75 mm diameter $\times$ 65 mm high). Three replicates of each treatment were tested in a growth chamber maintained at 26.5°C and 80% relative humidity for four weeks. Termite mortality (percent) and weight loss (percent) of the blocks were recorded periodically up to four weeks, using the following equations:

$$\text{Termite mortality (\%)} = \frac{\text{Number of dead termites}}{\text{Total number of test termites}} \times 100$$

$$\text{Weight loss (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

where W_1 is the oven-dried weight before termiticidal test (grams) and W_2 is the oven-dried weight after termiticidal test (grams).

We used Duncan's multiple-range test to examine the difference of the results of termiticidal test. Results with $P < 0.05$ were considered statistically significant.

Termiticidal Test with Essential Oils and Compounds. The termiticidal test followed the method of Kang et al. (1990). Each compound (**1, 2** and **3** as shown in Figure 1) and the essential oils (sapwood and heartwood) were dissolved in acetone, to make various concentrations of solutions, which were then applied to filter paper (8.6 cm diameter, Whatman No. 3) and air-dried at room temperature. Control filter paper was treated with solvent only. Fifty active termites (45 workers and 5 soldiers) were put onto the filter paper impregnated with the test materials in the Petri dish (9 cm diam. $\times$ 1.5 cm high). The test dishes were placed in a growth chamber maintained at 26.5°C and 80% realtive humidity for 14 days. A few drops of water were periodically put onto the bottom edge of the dishes. Three replicates were made for each sample, and termite mortality was determined daily. Paired Student's *t* tests were used to evaluate the difference of the results of termiticidal test. Results with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Antitermitic Activity of Woods. Unextracted sapwood of *Pinus taiwanensis* (PSN), used as a control, showed no antitermitic activity (Table 1). In contrast, both the sapwood and heartwood of *T. cryptomerioides* displayed antitermitic activity. Termite mortalities for THN (unextracted *T. cryptomerioides* heartwood), THE (alcohol–toluene-extracted *T. cryptomerioides* heartwood), TSN (unextracted

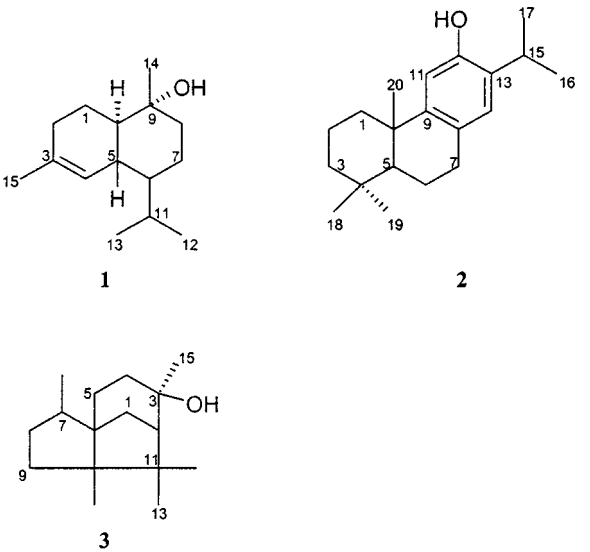


FIG. 1. Structures of α-cadinol (**1**), ferruginol (**2**), and cerdol (**3**) isolated from *T. cryptomerioides* heartwood.

T. cryptomerioides sapwood) and TSE (alcohol–toluene-extracted *T. cryptomerioides* sapwood) after four weeks were 57%, 50%, 48% and 43%, respectively. Duncan's multiple range test showed significant differences in termite mortality between extracted and unextracted wood ($P < 0.05$). Weight losses of heartwood and sapwood blocks extracted by alcohol–toluene (8.2% and 7.8%) were larger than those of unextracted blocks (1.1% and 1.3%) after four weeks. These differences were significant at $P < 0.05$. We conclude that the presence of

TABLE 1. TERMITE MORTALITY AND WEIGHT LOSS OF WOOD BLOCKS ATTACKED BY *Coptotermes formosanus*[*]

Samples[**]	Termite mortality (%)	Weight loss (%)
TSE	43 ± 1.2c	7.8 ± 0.2b
TSN	48 ± 2.0b	1.3 ± 0.1c
THE	50 ± 1.6b	8.2 ± 0.1b
THN	57 ± 2.3a	1.1 ± 0.1c
PSN	9 ± 2.1d	29.1 ± 0.7a

[*]Means ($N = 3$) using 50 termites per replicate. Numbers followed by the different letter (a, b, c, d) are significantly different at the level of $P < 0.05$ according to Duncan's multiple range test.

[**]TSE: alcohol–toluene-extracted *T. cryptomerioides* sapwood; TSN: unextracted *T. cryptomerioides* sapwood; THE: alcohol–toluene-extracted *T. cryptomerioides* heartwood; THN: unextracted *T. cryptomerioides* heartwood; PSN: unextracted pine sapwood.

T. cryptomerioides wood extractives decreases the weight loss of wood in the presence of termites.

Antitermitic Activity of Essential Oils and Fractions. In our previous studies, the essential oils and *n*-C_6H_{14}-soluble fraction of MeOH extracts of *T. cryptomerioides* were demonstrated to have antifungal and antibacterial activity (Chang et al., 1998, 1999b, 2000a,b). Therefore, the antitermitic activities of essential oils (heartwood and sapwood) and the *n*-C_6H_{14}-soluble fraction of MeOH extracts from *T. cryptomerioides* were determined in this study.

After 14 days, the order of termite mortality from essentials oils and the *n*-C_6H_{14}-soluble fraction at 10 mg/g was: heartwood essential oil (56%) > sapwood essential oil (32%) > *n*-C_6H_{14}-soluble fraction (22%) (Figure 2). All samples demonstrated significant termiticidal activity compared to controls (termite mortality = 4%). According to our previous data, the cadinanes (T-cadinol, T-muurolol, α-cadinol) were the main constituents in heartwood essential oils (66.7%), followed by sapwood essential oils (37.1%), and then *n*-C_6H_{14} extracts (27.4%) (Chang et al., 2000a). These results suggest that antitermitic activity of *T. cryptomerioides* may be related to the cadinane constituents of essential oils or the *n*-C_6H_{14} soluble fraction.

Although the antitermitic activity of the *n*-C_6H_{14}-soluble fraction at 10 mg/g was lower than heartwood and sapwood essential oils, termite mortality increased to 72% when the dosage increased to 50 mg/g.

Antitermitic Activity of Isolated Compounds. The *n*-C_6H_{14} soluble fraction from *T. cryptomerioides* had excellent antitermitic activity at 50 mg/g. HPLC was used to isolate and purify α-cadinol, ferruginol and cedrol for further bioassay. Figure 3 shows the antitermitic activity of the three dominant compounds. The order of antitermitic activity against *C. formosanus* was cedrol, followed by α-cadinol, and then ferruginol. Termite mortalities (at 5 mg/g after 14 days) were

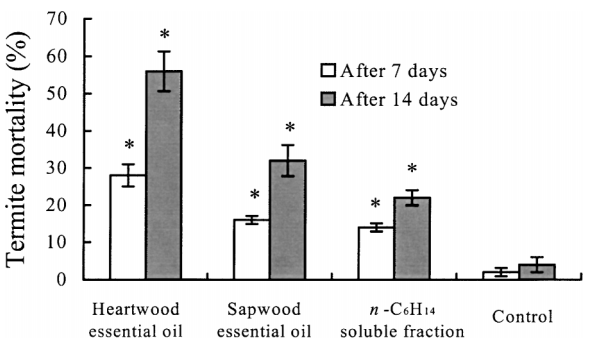


FIG. 2. Antitermitic activity of essential oils and the *n*-C_6H_{14} soluble fraction from *T. cryptomerioides* (bars = SD). $^*P < 0.05$; statistically different from the data of control.

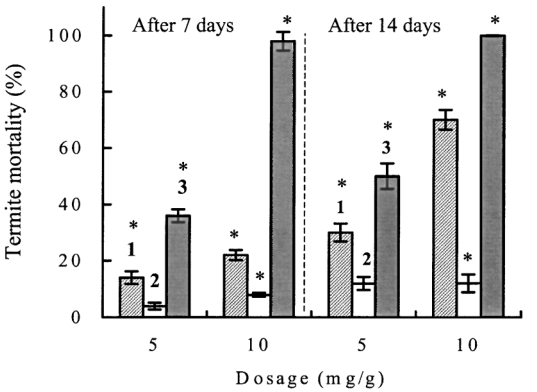


FIG. 3. Antitermitic activity of α-cadinol (**1**), ferruginol (**2**), and cedrol (**3**), (bars = SD). $^{*}P < 0.05$; statistically different from the data of control.

50%, 30%, and 12%, respectively. The antitermitic activities of 10 mg/g α-cadinol and cedrol increased after 14 days, whereas no dosage-dependent-effects occurred with ferruginol. The decreasing order of termite mortality at 10 mg/g dosage for 14 days was cedrol (100%) > α-cadinol (70%) > ferruginol (12%). Overall, cedrol possessed the strongest antitermitic activity.

During the test periods all the termites were killed within eight days at a dosage of 10 mg/g. This suggests that the resistance of *T. cryptomerioides* wood to termite attack may be attributed to the action of both cedrol and α-cadinol. Yaga and Kinjo (1986) previously showed that cedrol from *Sciadopitys verticillata* S. et Z was termiticidal. They also found that α-cadinol was the main termiticidal constituent of *Chamaecyparis obtusa* Endl (Kinjo et al., 1988).

Acknowledgments—This study was supported in part by grant NSC-89-2313-B-002-121 from the National Science Council of the Republic of China. The authors thank Mr. H.-Y. Lin (The Experimental Forest of National Taiwan University) for supplying *T. cryptomerioides* wood and Mr. S.-J. Lin (Taiwan Forestry Research Institute) for providing us with termites.

REFERENCES

AWPA. 1997. Standard method for laboratory evaluation to determine resistance to subterranean termites. Standard E1-97. American Wood-Preservers' Association. pp. 279–282.

BULTMAN, J. D., RAYMOND, H. B., and AMPONG, F. F. K. 1979. Natural resistance of some tropical African woods to *Coptotermes formosanus* Shiraki. *For. Prod. J.* 29:46–51.

CHANG, S.-T., and WANG, S.-Y. 1995. Introduction of the termiticidal wood extractives. *For. Prod. Ind.* 14:149–159 (in Chinese).

CHANG, S.-T., WU, C.-L., WANG, S.-Y., SU, Y.-C., and KUO, Y.-H. 1998. Studies on the antifungal compounds in the heartwood extractives of Taiwania (*Taiwania cryptomerioides* Hayata) (I) Isolation

and identification of antifungal compounds in hexane soluble fraction. *For. Prod. Ind*. 17:287–304. (in Chinese with English abstract).

CHANG, S.-T., WANG, S.-Y., SU, Y.-C., and KUO, Y.-H. 1999a. Chemical constituents and mechanisms of discoloration of Taiwania (*Taiwania cryptomerioides* Hayata) heartwood—(I) The structure reconfirmation and conversion mechanism of Taiwanin A. *Holzforschung* 53:142–146.

CHANG, S.-T., WANG, S.-Y., WU, C.-L., SU, Y.-C., and KUO, Y.-H. 1999b. Antifungal compounds in the ethyl acetate soluble fraction of the extractives of Taiwania (*Taiwania cryptomerioides* Hayata) heartwood. *Holzforschung* 53:487–490.

CHANG, S.-T., WANG, S.-Y., WU, C.-L., CHEN, P.-F., and KUO, Y.-H. 2000a. Comparisons of the antifungal activities of cadinane skeletal sesquiterpenoids from Taiwania (*Taiwania cryptomerioides* Hayata) heartwood. Holzforschung 54:241–245.

CHANG, S.-T., CHEN, P.-F., and CHANG, S.-C. 2000b. Antibacterial activity of essential oils and extractives from Taiwania (*Taiwania cryptomerioides* Hayata). *Q. J. Chin. For*. 33:119–125. (in Chinese with English abstract).

KANG, H. Y., MATSUSHIMA, N., SAMESHIMA, K., and TAKAMURA, N. 1990. Termite resistance tests of hardwoods of Kochi growth I. The strong termiticidal activity of kagonoki (*Litsea coreana* Léveillé). *Mokuzai Gakkaishi* 36:78–84.

KINJO, K., DOUFUKU, Y., and YAGA, S. 1988. Termiticidal substances from the wood of *Chamaecyparis obtusa. Mokuzai Gakkaishi* 34:451–455.

OHTANI, Y., HAZAMA, M., and SAMESHIMA, K. 1997. Crucial chemical factors of the termiticidal activity of Hinoki wood (*Chamaecyparis obtusa*) III. Contribution of α-terpinyl acetate to the termiticidal activity of hinoki wood. *Mokuzai Gakkaishi* 43:1022–1029.

SOGABE, A., KINJO, K., ABE, F., YAMAUCHI, T., and YAGA, S. 2000a. Termiticidal substances from the wood and bark of *Wikstroemia retusa* A. Gray. *Mokuzai Gakkaishi* 46:47–53.

SOGABE, A., KINJO, K., ABE, F., YAMAUCHI, T., and YAGA, S. 2000b. Termiticidal substances from the heartwood of *Cryptomeria japonica* D. Don. *Mokuzai Gakkaishi* 46:124–131.

YAGA, S., and KINJO, K. 1986. Termiticidal substances from *Sciaopitys verticillata* S. et Z. *Mokuzai Gakkaishi* 32:720–723.

YAGA, S., KINJO, K., FERNZNDEZ, E. C., ZERRUDO, J. V., and CASTILLO, S. A. 1991. The termite resistance of Okinawan timbers X. Termiticidal substances from *Bruguiera gymnorrhiza. Mokuzai Gakkaishi* 37:358–362.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

TANNINS IN *Puccinellia arctica*: POSSIBLE DETERRENTS TO HERBIVORY BY CANADA GEESE

TRENT J. VOLZ and THOMAS P. CLAUSEN*

Department of Chemistry and Biochemistry
University of Alaska Fairbanks
Box 6160, Fairbanks, Alaska 99775-6160

(Received June 12, 2000; accepted December 12, 2000)

Abstract—Urban populations of Canada geese, *Branta canadensis*, pose a nuisance problem throughout most of the eastern United States and in other parts of the United States and Canada. *Puccinellia arctica* is a species of arctic grass that is unpalatable to Canada geese on the North Slope of Alaska and may prove to be an effective long-term and nonlethal means of controlling the growing populations of urban Canada geese. A comparative study of the secondary metabolites of both *P. arctica* and *Puccinellia langeana* and *Poa pratensis*, two palatable grass species that Canada geese generally consume, revealed no significant differences. However, ellagitannin levels were higher in *P. arctica* than in the palatable grass species and may be contributing to its unpalatability to Canada geese. These results support the potential to use *P. arctica* in public areas to control geese intrusions.

Key Words—*Branta canadensis*, carbon nutrient balance, chemical defense, herbivory, *Poa pratensis*, *Puccinellia arctica*, *Puccinellia langeana*, tannins.

INTRODUCTION

Urban populations of Canada geese, *Branta canadensis*, pose a nuisance problem throughout most of the eastern United States and in other parts of the United States and Canada (Conover, 1985). Grazing on lawns has become one of the principal food sources for geese in urban areas (Conover and Kania, 1991). This has created a problem when the geese damage turf by foraging on grass in backyards, golf courses, parks, landscaped areas, and playing fields (Cummings et al., 1991).

The feces the geese leave behind, up to three pounds per goose per day (Sinnott, personal communication), diminishes the aesthetic and recreational value

*To whom correspondence should be addressed.

0098-0331/01/0400-0725$19.50/0

of the grazed areas (Cummings et al., 1991). The accumulated feces can decrease water quality and may have deleterious effects upon public health (Conover and Chasko, 1985). In fact, several public parks and beaches have been closed due to health concerns stemming from goose feces (Conover, 1985).

Puccinellia arctica is a species of arctic grass that is unpalatable to Canada geese on the North Slope of Alaska (McKendrick, 1997; Scorup, personal communication). It is being tested as a goose deterrent in Anchorage, Alaska, in an urban environment. By planting the grass in urban areas where Canada geese forage, *P. arctica* may prove to be an effective long-term and nonlethal means of controlling the growing populations of urban Canada geese.

As part of the testing of *P. arctica*, its secondary metabolite profile was compared to the metabolite profiles of two palatable grass species that Canada geese do eat to determine if *P. arctica* may be chemically defended. Gas chromatography–mass spectroscopy (GC-MS) and thin-layer chromatography (TLC) showed no significant differences between *P. arctica* and the palatable grass species (Volz, 2000). Alkaloids, which may defend tall fescue (*Festuca arundinacea*) from herbivory by Canada geese (Conover and Messmer, 1996), were screened for and not detected (Volz, 2000).

This report details condensed tannin, gallotannin, and ellagitannin assays that were performed on *P. arctica* as well as on *Poa pratensis* and *Puccinellia langeana*, two palatable grass species that Canada geese favor (Scorup, personal communication). These assays were done to investigate whether tannins, which can in some cases deter bird herbivory in plants such as grain sorghum (Watterson and Butler, 1983), are contributing to the unpalatability of *P. arctica* to Canada geese.

Plant samples were also analyzed to investigate factors that may influence tannin levels in *P. arctica.* Since *P. arctica* may eventually be used in an urban setting, some plants were subjected to routine mowing to study the changes in tannin levels. Seedling *P. arctica* samples were analyzed to examine whether plant maturity affects tannin levels. Samples of *P. arctica* growing in the nutrient limited wet sedge tundra of Alaska's North Slope (Shaver and Chapin, 1995) were tested to study the affects of low soil nutrient levels on tannin levels.

METHODS AND MATERIALS

Chemicals. Ellagic acid and gallic acid were purchased from Aldrich. Quebracho tannin was obtained from Pilar River Plate Corporation. All chemicals and solvents were reagent grade, and water was deionized.

Plant Samples. All *P. arctica* samples were collected between May and August 1999. The nutrient-limited wet sedge tundra samples were collected on August 5

near Prudhoe Bay, Alaska. The remaining *P. arctica* samples were collected near Anchorage, Alaska (May 27, July 12, and August 19). The mown *P. arctica* samples were cut to a height of 9 cm an average of once a week in the 1999-growing season and collected on July 12.

The palatable *P. langeana* samples were collected from Prudhoe Bay, Alaska on August 5, 1999. The palatable *P. pratensis* samples were collected near Anchorage, Alaska between May and July 1999. After collection, plant samples were placed in coolers and chilled with freezer packs for transport to the laboratory. Once in the laboratory, plant material was stored in a freezer at -20°C prior to analysis.

Tannin Assays. To quantify condensed tannin levels, 0.20 g of lyophilized plant material was first extracted with 3 ml of 50% (v/v) MeOH in water (Harborne, 1984) for 1 hr at room temperature. The extraction was repeated twice, and the aqueous MeOH extracts were combined. A 1-ml portion of the aqueous MeOH extract was then analyzed using the method of Porter et al. (1986). Condensed tannin levels were reported in terms of the percentage of condensed tannin equivalents (quebracho tannin) by dry weight. Interspecific differences in condensed tannin levels should be interpreted with caution, since tannins from different plant species yield varying responses with this assay (Wisdom et al., 1987).

To extract, hydrolyze, and analyze gallotannins, a 0.20-g portion of lyophilized plant material was extracted with 3 ml of 70% (v/v) acetone in water for 30 min at room temperature. The filtered extract was placed in an ampule with 5 ml of 1 M H_2SO_4, and the sealed ampule was then heated in an oven for 26 hr at 100°C. After cooling, the ampule was opened and the hydrolysate was diluted to 50 ml with water. A 1-ml volume of the diluted hydrolysate was then analyzed by the method of Inoue and Hagerman (1988). This provided a measure of total gallic acid, both free and esterified, in the plant sample.

A second 0.20-g portion of the lyophilized plant material was extracted, diluted to 50 ml with water, and then assayed without hydrolysis to measure the amount of free gallic acid in the plant sample. The amount of free gallic acid was then subtracted from the amount of total gallic acid to determine the amount of esterified gallic acid in the plant sample. Gallotannin levels were reported in terms of the percentage of esterified gallic acid equivalents by dry weight.

To extract, hydrolyze, and analyze ellagitannins, a 0.10-g portion of lyophilized plant material was placed in an ampule with 8 ml of 1 M H_2SO_4. The sealed ampule was then heated in an oven for 24 hr at 100°C, and the concentration of ellagic acid was measured using the method of Wilson and Hagerman (1990). Ellagitannin levels were reported in terms of the percentage of ellagic acid equivalents by dry weight.

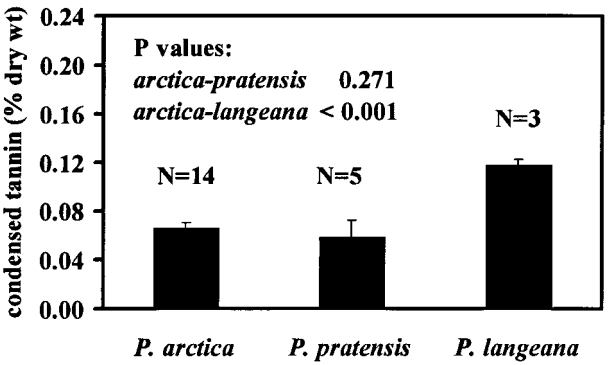


FIG. 1. Mean condensed tannin concentration in *P. arctica* and palatable plants. Vertical lines represent +1 SD, and *N* is the number of samples. One-tailed *P* values from unpaired *t* tests are reported.

RESULTS AND DISCUSSION

There were no seasonal effects or trends on the tannin measures of *P. arctica* and *P. pratensis* (data not shown). Thus, samples from different collection times were pooled in making the following comparisons between species and treatments.

Figure 1 displays the results of the condensed tannin assay done on *P. arctica* and the palatable plants. Condensed tannin levels were low in all of the plant samples. There was no statistically significant difference in condensed tannin levels between *P. arctica* and the palatable *P. pratensis* ($P_{1\text{-tail}} = 0.271$). The condensed tannin levels were 79% higher ($P_{1\text{-tail}} < 0.001$) in the palatable *P. langeana* than in *P. arctica.* Therefore, unless the condensed tannins in *P. arctica* have either a high level of biologically activity (Clausen et al., 1990; Ayres et al., 1997) or are insensitive to the proanthocyanidin assay (Wisdom et al., 1987), they are not primarily responsible for its unpalatability to Canada geese.

Figure 2 shows the results of the condensed tannin assay done on *P. arctica* control and test plants. The condensed tannin levels in the seedlings were 75% higher than in the control grass ($P_{1\text{-tail}} = 0.004$). This may be expected, since a plant's developmental stage may influence the amount of tannins it contains (Hagerman and Butler, 1991).

The *P. arctica* plants grown in nutrient limited soils had condensed tannin levels that were 70% higher than the control plants ($P_{1\text{-tail}} = 0.002$), as would be predicted by the carbon-nutrient balance (CNB) hypothesis (Coley et al., 1985, Bryant et al., 1983). The condensed tannin levels in the mown plants were 95% higher than in the control plants ($P_{1\text{-tail}} = 0.007$).

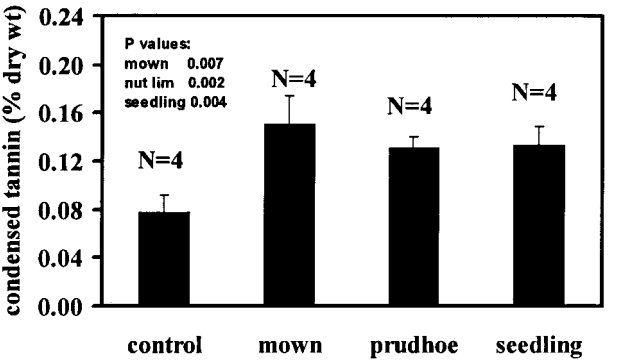


FIG. 2. Mean condensed tannin concentration in *P. arctica* control and test plants. The mown and control samples were collected from a common plot at the same time. Vertical lines represent +1 SD, nut lim = nutrient-limited samples, and *N* is the number of samples. One-tailed *P* values from paired *t* tests are reported.

The response of condensed tannins to mowing also may be explained using the CNB hypothesis. In the unmown control grass, the lower leaves of the grass are blocked from the sun by grass that is growing above them. Thus, a sizable percentage of the unmown control grass leaves have lowered levels of photosynthesis and therefore fix less carbon. In the mown grass, the leaves are exposed to more sunlight and can fix more carbon. The amount of carbon fixed per mass of leaf is thus higher in the mown grass, thus resulting in more tannin being produced per mass of leaf and in higher assayed condensed tannin levels. (Interestingly, this same argument may be used to explain the previously noted elevated condensed tannin levels in the *P. arctica* seedlings).

Figure 3 displays the results of the gallotannin assay done on *P. arctica* and the palatable plants. Gallotannin levels were low in all of the plant samples. There was no statistically significant difference in gallotannin levels between *P. arctica* and the palatable *P. langeana* ($P_{1\text{-tail}} = 0.072$). The gallotannin levels were 118% higher ($P_{1\text{-tail}} < 0.001$) in the palatable *P. pratensis* than in *P. arctica.* As was seen with condensed tannins, unless the gallotannins in *P. arctica* have a high level of biologically activity or are insensitive to the assay, they are not primarily responsible for its unpalatability to Canada geese (Clausen et al., 1990; Ayres et al., 1997).

Figure 4 displays the results of the ellagitannin assay done on *P. arctica* and the palatable plants. *P. arctica* plants contained 105% more ellagitannin than the palatable *P. pratensis* plants ($P_{1\text{-tail}} < 0.001$) and in the *P. arctica* plants contained 90% more ellagitannin than the palatable *P. langeana* plants ($P_{1\text{-tail}} < 0.001$). These elevated ellagitannin levels in *P. arctica* may be contributing to its unpalatability to Canada geese.

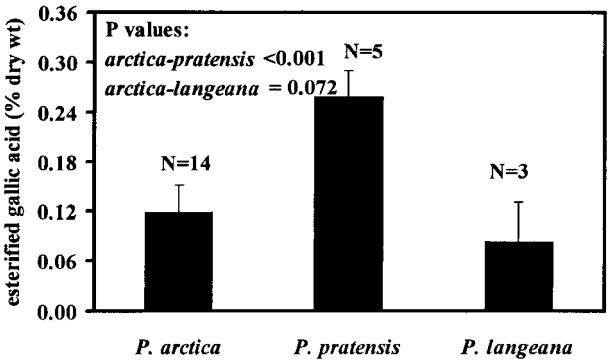


FIG. 3. Mean gallotannin concentration in *P. arctica* and palatable plants. Vertical lines represent +1 SD, and *N* is the number of samples. One-tailed *P* values from unpaired *t* tests are reported.

Figure 5 shows the results of the gallotannin and ellagitannin assays done on *P. arctica* control and test plants. There was no statistically significant difference between the gallotannin and ellagitannin levels of the mown and nutrient-limited test plants and the control plants ($P_{1\text{-tail}} = 0.242$ and 0.198 for gallotannins, $P_{1\text{-tail}} = 0.173$ and 0.305 for ellagitannins). The seedling *P. arctica* plants contained 33% more ellagitannin than the control plants ($P_{1\text{-tail}} = 0.030$) and 174% less gallotannin than the control plants ($P_{1\text{-tail}} = 0.045$). While small, the effects of the test plots on gallotannins are qualitatively different from those of condensed and ellagitannins. This suggests that *P. arctica* has significant control over how it allocates resources to its secondary metabolism while under stress.

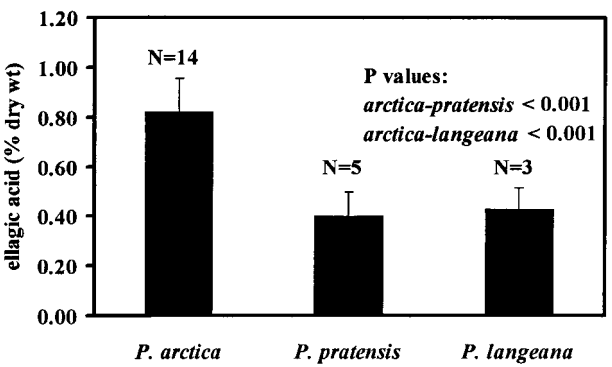


FIG. 4. Mean ellagitannin concentration in *P. arctica* and palatable plants. Vertical lines represent +1 SD, and *N* is the number of samples. One-tailed *P* values from unpaired *t* tests are reported.

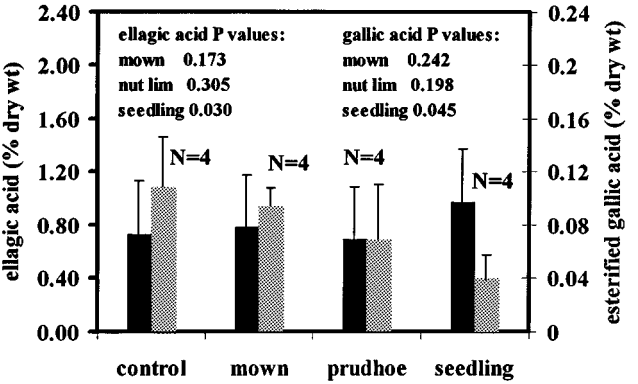


FIG. 5. Mean ellagitannin (dark bars) and gallotannin (light bars) concentration in *P. arctica* control and test plants. Vertical lines represent +1 SD, nut lim = nutrient limited samples, and *N* is the number of samples. One-tailed *P* values from paired *t* tests are reported.

CONCLUSIONS

GC-MS and TLC showed no significant differences between the secondary metabolites of *P. arctica* and grass species that Canada geese usually find palatable (Volz, 2000). Alkaloids were screened for and not detected (Volz, 2000). However, ellagitannin levels were higher in *P. arctica* than in the palatable plant samples. The elevated ellagitannin levels in *P. arctica* may be contributing to its unpalatability to Canada geese. It should be noted that the presence of moderate levels of tannin does not necessarily protect plants from herbivory (Clausen et al., 1992). For confirmation, the tannins would have to be isolated and used in feeding trials. These results suggest that *P. arctica* is, barring the existence of some trace and highly toxic substance, appropriate to plant in areas such as playing fields, lawns, and playgrounds for use as a deterrent to Canada geese.

REFERENCES

AYRES, M. P., CAUSEN, T. P., MACLEAN, S. F., REDMAN, A. M., and REICHARDT, P. B. 1997. Diversity of structure and antiherbivore activity in condensed tannins. *Ecology* 78:1696–1712.

BRYANT, J. P., CHAPIN, F. S., III, and KLEIN, D.R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CLAUSEN, T. P., PROVENZA, F. D., BURRITT, E. A., REICHARDT, P. B., and BRYANT, J. P. 1990. Ecological implications of condensed tannin structure: A case study. *J. Chem. Ecol.* 16:2381–2392.

CLAUSEN, T. P., REICHARDT, P. B., BRYANT, J. P., and PROVENZA, F. D. 1992. Condensed tannins in plant defense: a perspective on classical theories, pp. 639–651, *in* R. W. Hemingway and P. E. Laks (eds.). Plant Polyphenols: Synthesis, Properties, Significance. Plenum Press, New York.

COLEY, P. D., BRYANT, J. P., and CHAPIN, F. S., III. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

CONOVER, M. R. 1985. Alleviating nuisance Canada goose problems through methiocarb-induced aversive conditioning. *J. Wildl. Manage.* 49:631–636.

CONOVER, M. R., and CHASKO, G. G. 1985. Nuisance Canada goose problems in the eastern United States. *Wildl. Soc. Bull.* 13:228–233.

CONOVER, M. R., and KANIA, G. S. 1991. Characteristics of feeding sites used by urban-suburban flocks of Canada geese in Connecticut. *Wildl. Soc. Bull.* 19:36–38.

CONOVER, M. R., and MESSMER, T. A. 1996. Feeding preferences and changes in mass of Canada geese grazing endophyte-infected tall fescue. *Condor* 98:859–862.

CUMMINGS, J. L., MASON, J. R., OTIS, D. L., and HEISTERBERG, J. F. 1991. Evaluation of dimethyl and methyl anthranilate as a Canada goose repellent on grass. *Wildl. Soc. Bull.* 19:184–190.

HAGERMAN, A. E., and BUTLER, L. G. 1991. Tannins and lignins, pp. 355–387, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites, 2nd ed. Academic Press, New York.

HARBORNE, J. B. 1984. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 2nd ed. Chapman and Hall, London.

INOUE, K. H., and HAGERMAN, A. E. 1988. Determination of gallotannin with rhodanine. *Anal. Biochem.* 169:363–369.

MCKENDRICK, J. D. 1997. Long-term tundra recovery in Northern Alaska, pp. 503–518, *in* R. M. M. Crawford (ed.). Disturbance and Recovery in Arctic Lands. Kluwer Academic, Dordrecht, Netherlands.

PORTER, L. J., HIRSTICH, L. N., and CHAN, B. G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223–230.

SHAVER, G. R., and CHAPIN, F. S. 1995. Long-term responses to factorial, NPK fertilizer treatment by Alaskan wet and moist tundra sedge species. *Ecography* 18:259–275.

VOLZ, T. J. 2000. Phytochemical comparison of *Puccinellia arctica* to *Poa pratensis, Puccinellia langeana*, and *Puccinellia phryganodes* for evidence of chemical defense. MS thesis. University of Alaska Fairbanks.

WATTERSON, J. J., and BUTLER, L. G. 1983. Occurrence of an unusual leucoanthocyandin and absence of proanthocyanidins in sorghum leaves. *J. Agric. Food Chem.* 31:41–45.

WILSON, T. C., and HAGERMAN, A. E. 1990. Quantitative determination of ellagic acid. *J. Agric. Food Chem.* 38:1678–1683.

WISDOM, C. S., GONZALEZ-COLOMA, A., and RUNDEL, P. W. 1987. Ecological tannin assays: Evaluation of proanthocyanidins, protein binding assays and protein precipitating potential. *Oecologia* 72:395–401.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

RELEASE RATES FOR PINE SAWFLY PHEROMONES FROM TWO TYPES OF DISPENSERS AND PHENOLOGY OF *Neodiprion sertifer*

BJÖRN G. JOHANSSON,[1,6] OLLE ANDERBRANT,[1,*] JIRI SIMANDL,[2,7] NIKOLAOS D. AVTZIS,[3] CHRISTINA SALVADORI,[4] ERIK HEDENSTRÖM,[5] HELÉN EDLUND,[5] and HANS-ERIK HÖGBERG[5]

[1]*Department of Ecology*
Lund University, Ecology Building
SE-223 62 Lund, Sweden

[2]*Institute of Entomology*
Czech Academy of Sciences
Branisovska 31, Ceské Budejovice
Czech Republic

[3]*T.E.I. Kavalas*
Department of Forestry in Drama
Proastio, 66100 Drama, Greece

[4]*Forestry Unit–Research Centre of ISMA*
38010 San Michele all'Adige
Trento, Italy

[5]*Department of Chemistry and Process Technology*
Mid Sweden University
SE-851 70 Sundsvall, Sweden

(Received March 10, 2000; accepted December 12, 2000)

Abstract—Comparisons of release rates, duration in the field, and catch efficiency of polyethylene and cotton roll dispensers for the sex pheromones of sawflies (Hymenoptera: Diprionidae) were conducted. The release rates of the *Neodiprion sertifer* (Geoffr.) and *Diprion pini* (L.) sex pheromones, the acetates of pentadecanol and (2*S*,3*S*,7*S*)-3,7-dimethyl (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol from polyethylene dispensers were measured at different temperatures in the laboratory. The release rates for the substances depended on both the temperature and initial load in the vials. The catch from cotton rolls baited with 100 μg of the acetate or propionate of 3,7-dimethyl-2-pentadecanol was

*To whom correspondence should be addressed. e-mail: olle.anderbrant@ekol.lu.se
[6]Present address: Department of Animal Ecology, Evolutionary Biology Centre, Norbyv. 18d, SE-752 36 Uppsala, Sweden.
[7]Present address: Netolická 8/1143, 37005 Ceské Budéjovice, Czech Republic.

0098-0331/01/0400-0733$19.50/0

compared to the catch from regularly renewed cotton rolls baited with 10 μg of the same acetate. The catch was higher for the 100-μg cotton rolls for, at most, 45 days, and there was no significant difference in catch between the acetate and the propionate. The catch in traps baited with polyethylene or cotton roll dispensers loaded with the acetate of 3,7-dimethyl-2-pentadecanol was compared and showed that cotton roll traps mirrored the decreasing release of the substance rather than the actual flight activity. The length of the flight period of *N. sertifer* in Sweden, the Czech Republic, Italy, and Greece did not exceed 100 days in any of the countries. By adjusting the initial pheromone load of the polyethylene vials to the expected temperatures, it should be possible to get a constant and sufficiently high release rate during the entire flight period.

Key Words—Hymenoptera, Diprionidae, *Diprion pini*, *Neodiprion sertifer*, 3,7-dimethyl-2-pentadecanol, diprionol, 3,7-dimethyl-2-tridecanol.

INTRODUCTION

In addition to the chemical composition, the amount of substance released is of great importance in pheromone based systems for control or monitoring of insect species. Different kinds of dispensers have been tried with respect to constancy and predictability in release rate. An ideal dispenser should have a constant release rate during the whole flight period of the target insect (Byers, 1988).

Although the knowledge of pheromones in sawflies is generally sparse (Anderbrant, 1999), the pheromone systems of *Neodiprion sertifer* (Geoffr.) (Kikukawa et al., 1983; Anderbrant et al., 1992a) and *Diprion pini* L. (Bergström et al., 1995; Anderbrant et al., 1995a) (Diprionidae) are comparatively well understood. The sex pheromone of *N. sertifer*, the most thoroughly investigated species of sawflies, is the acetate or propionate of 3,7-dimethyl-2-pentadecanol (diprionol), a substance it shares with most other diprionids investigated so far (Anderbrant, 1999). The sex pheromone of *D. pini* differs with respect to chain length, viz. 13 instead of 15 carbons, and in stereochemistry.

In an earlier study (Anderbrant et al., 1992b), the release rates of the acetate of 3,7-dimethyl-2-pentadecanol from glass capillaries and dental cotton rolls were measured, and the cotton rolls were found to be the more practical dispenser for this substance. Dental cotton rolls have been used in most field studies on diprionids (Anderbrant et al., 1992b), although rubber septa have been tried and rejected, due to too low release rate, for *D. pini* (Bergström et al., 1995).

Since *N. sertifer* is one of the major defoliators of pine trees, *Pinus spp.*, in parts of its wide distribution area, it would be valuable to have monitoring systems for more efficient control of the species (Anderbrant, 1998). Such monitoring systems, based on sex pheromone trapping with cotton rolls as dispensers, have recently been attempted by Lyytikäinen-Saarenmaa et al. (1999), who pointed out a need to develop and document the release rate of more long-lasting dispensers,

e.g. polyethylene vials. In the present study, such measurements for the *N. sertifer* and *D. pini* pheromones were performed. The attractivity and duration of diprionol acetate and propionate as baits for *N. sertifer* were compared. Parallel with these trials, the phenology of the flight period of *N. sertifer* was studied in different parts of Europe. From these studies we conclude that the release rate of 3,7-dimethyl-2-pentadecanol esters from polyethylene dispensers is reasonably constant for the length of the *N. sertifer* flight period.

METHODS AND MATERIALS

Chemicals

The acetate of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol, synthesized at the Mid Sweden University according to Högberg et al. (1990), was used in all field tests, with the addition of the propionate in 1994. Both compounds contained 1% of the 2*S*,3*S*,7*R* isomer and less than 0.08% of the other isomers (Högberg et al., 1990; Anderbrant et al., 1992a). Release measurements were made with the acetates of (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol and (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol (Bergström et al., 1995), also synthesized at the Mid Sweden University.

Pheromone Release Rates

Polyethylene Vials. Closed polyethylene vials (No. 730, Kartell; 32 mm long $\times$ 8 mm OD $\times$ 6 mm ID) were loaded with neat pheromones and placed in environmental cabinets with temperatures of 12°C, 18–21°C, or 27–30°C. The amount of pheromone in each vial varied from 1.18 to 4.18 mg (mean = 2.35, SD = 0.86). An empty vial was used as a control for each of the filled vials. The vials were weighed at regular intervals with an accuracy of measurement of 10 μg (Mettler AE 240 scale). The weight change of the control vial was subtracted from the weight of its paired pheromone containing vial. The release rate was determined by fitting a linear regression model to the linear part of the curves formed by the corrected weights of pheromone containing vials. To get data on release rates in the field, one pheromone vial with control was weighed immediately before and after its use in the field in Italy 1996.

Cotton Rolls. In order to get an estimate of the pheromone release from the cotton rolls used in the field tests below, a regression model presented by Anderbrant et al. (1992b), $y = a \exp[b \ln(x + 1)]$, where y is the remaining amount and x is the time in days, was used. In all computations the b value (-0.685) calculated by Anderbrant et al. (1992b) for cotton rolls loaded with 60 μg 3,7-dimethyl-2-pentadecyl acetate and released for 33.5 days in the field was used.

Field Tests

In all field tests we used the Lund-I trap (Anderbrant et al., 1989), in which insects are trapped on a horizontally oriented cardboard bottom (21 × 21 cm) covered with insect glue (Stikem Special, Seabright Enterprises) on the upper side. Dental cotton rolls (Celluron No. 2, Paul Hartman SA) and polyethylene vials (No. 730, Kartell) were used as dispensers.

Duration of Cotton Rolls. The experiment was made in a pure, mature Scots pine (*Pinus sylvestris* L.) stand near Ceské Budéjovice, southern Bohemia, Czech Republic, from July 15 to October 30, 1994. Two replicates were made, each consisting of four sets of traps, placed at different sites with an interval of one month. Each set consisted of four traps, placed at the corners of a square with 30-m sides, and baited with cotton rolls containing 100 μg of the acetate of 3,7-dimethyl-2-pentadecanol, 100 μg of the propionate of 3,7-dimethyl-2-pentadecanol, 10 μg of the acetate of 3,7-dimethyl-2-pentadecanol, respectively. One trap in each set was an unbaited control. The 10-μg acetate baits were changed every 10 days, thus giving a more constant release of the pheromone than the other traps that were only examined at the same time interval.

Comparison Between Cotton Rolls and Polyethylene Vials. In 1996, experiments were made in Italy (September 10–November 27) and Greece (September 11–December 4). The Greek trial was made in a 20-year-old *Pinus brutia* Ten. forest at the Forest Research Institute of Thessaloniki in northern Greece. Four traps were set in the corners of a 40-m square at each site, two baited with polyethylene vials containing about 2 mg of the acetate of 3,7-dimethyl-2-pentadecanol each, and two baited with cotton rolls containing about 100 μg of the same substance. The Italian trial was carried out in an ~15-year-old Scots pine forest on Monte Baldo, Trentino, in the Italian Central Alps. The four traps, baited as in the Greek trial, were placed with an intertrap distance of 100–150 m. The cotton rolls were changed after 41 (Italy) or 28 days (Greece), and the sticky bottoms were changed and examined with an interval of about one week.

Phenology of Neodiprion sertifer. In the Czech Republic, catch data from the two 10-μg traps that were used during the whole test period were used to obtain phenological data, whereas the traps baited with polyethylene vials served the same purpose in Italy and Greece. In 1989, a number of traps for studies on the pheromone biology of *N. sertifer* were set up near Valdemarsvik in the province of Östergötland, southeastern Sweden (e.g., Anderbrant et al., 1992a). Catch data from three of these traps, placed in an 8 to 10-year-old Scots pine forest with an intertrap distance of about 30 m, were used in this study. The traps were baited August 2 with dental cotton rolls containing 100 μg of the acetate of 3,7-dimethyl-2-pentadecanol each. Baits and sticky bottoms were changed at intervals varying from one to two weeks until October 4. The catches from each collection were

TABLE 1. RELEASE RATES OF PINE SAWFLY PHEROMONES FROM POLYETHYLENE VIALS (MEAN ± SD)

Temperature	Release rate (μg/day)	Days (N)	r^2 (range)	Vials (N)
Acetate of 3,7-dimethyl-2-pentadecanol				
12°C	5.4 ± 1.1	100	0.737–0.774	2
18–21°C	10.0 ± 1.6	105 ± 10	0.971–0.989	4
27–30°C	15.6 ± 1.6	45 ± 4	0.977–0.989	2
Acetate of 3,7-dimethyl-2-tridecanol				
12°C	20.6 ± 0.1	100	0.957–0.978	2
18–21°C	25.8 ± 6.4	65	0.968–0.988	2
27–30°C	38.4 ± 1.3	42	0.971–0.975	2

divided by the number of days since the last examination to get a value of the mean catch per day for each site.

RESULTS

Pheromone Release Rates

Polyethylene Vials. The model gave a fairly good approximation of release rates, as can be seen by the coefficients of determination in Table 1. The release rate was constant for the acetate of 3,7-dimethyl-2-pentadecanol during the first 100 days at the two lower temperatures and decreased after this time period. However, at 27–30°C, the linear part of the curve encompassed only 45 days (Figure 1A). The data from one of the vials used during the field studies in Italy gave, when fitted to the linear model, a daily release rate of 9.4 μg, which seems to be in accordance with the laboratory data (Figure 1A). The release rate of the acetate of 3,7-dimethyl-2-tridecanol was higher and the length of the linear part of the curves consequently shorter (Figure 1B).

Cotton Rolls. The computed curve of release from cotton rolls loaded with 100 μg of the acetate of 3,7-dimethyl-2-pentadecanol, described as amount released substance per day, is inserted in Figure 3A and 3C below. The model was also used for comparison of the release from the 100 μg and 10 μg baits in the Czech field test.

Field Tests

Duration of Cotton Rolls. The unbaited traps did not catch any sawflies during the trial, which was finished October 30 due to night frost. There was no significant

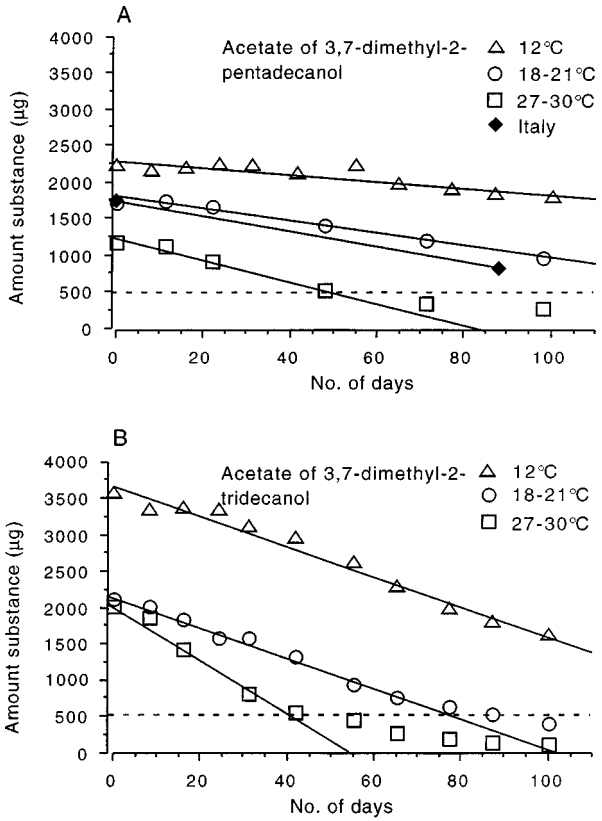


FIG. 1. Residual quantity of the acetates of 3,7-dimethyl-2-pentadecanol (A) and 3,7-dimethyl-2-tridecanol (B) in seven polyethylene vials at different temperatures in the laboratory and in the field (Italy). Dotted line represents proposed minimum amount of substance for linear release.

difference in trap catch between the traps baited with the acetate and propionate of 3,7-dimethyl-2-pentadecanol in any of the sets, according to a Wilcoxon signed-rank test (Wilcoxon, 1945). On average the capture of sawflies in a 100-μg trap was 0.96 per day (SD = 0.92, $N = 16$), while the capture in a 10-μg trap was 0.94 (SD = 0.38, $N = 8$). The relative catch from the traps baited with 100 μg of the acetate or propionate of 3,7-dimethyl-2-pentadecanol, respectively, was computed by dividing it with the catch from the 10-μg traps for the same time period (Figure 2). Thus, a relative catch >1 indicates that the 100-μg bait, without bait renewal, was more efficient than the regularly changed 10-μg bait. The catch in the 100-μg traps was at least as high as the catch from the 10-μg traps and stayed higher for at most 45 days from the onset of the flight period in mid-August. In the

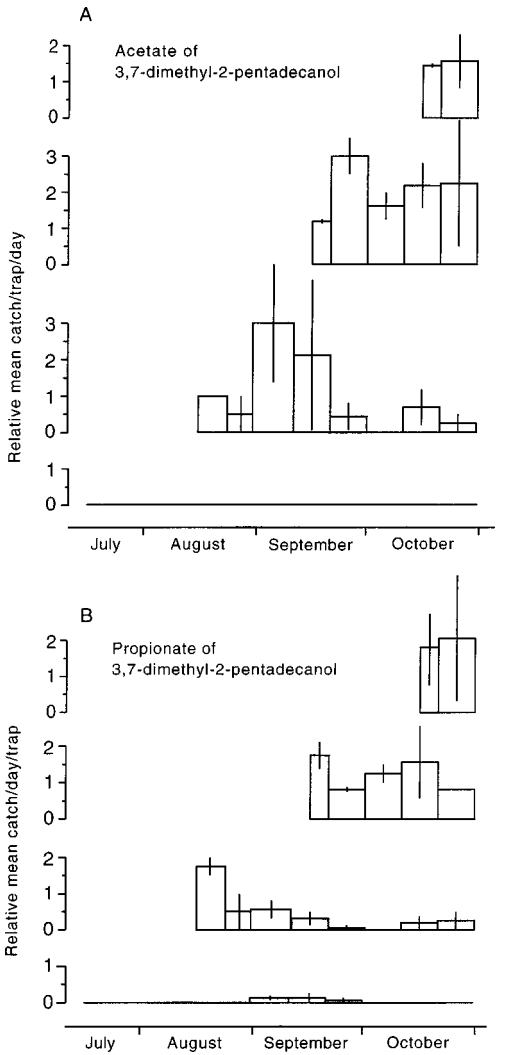


FIG. 2. Catches of male *Neodiprion sertifer* (mean ± SE) per trap per day in traps baited with 100-μg of the acetate (A) or propionate (B) of 3,7-dimethyl-2-pentadecanol divided by the catch in traps baited with 10-μg of the acetate. Each line of histograms represents a set of two 100-μg traps and two 10-μg traps, in which the 10-μg traps were renewed every tenth day. The four sets of each ester were set up with an interval of one month.

first sets of 100-μg traps, the lure did not remain efficient long enough to attract males during the peak flight.

Comparison Between Cotton Rolls and Polyethylene Vials. Two peaks of trap catch were observed for the traps with cotton roll dispensers in both Italy and Greece. These coincided with the change of dispensers but had no correspondence in the catch from polyethylene vials (Figure 3).

Phenology of Neodiprion sertifer. Both the onset and end of the flight period shifted towards later in the season with decreasing latitude (Figure 4). The length of the flight period, if counted as the number of days with a mean catch per trap and day of one or more, seems to be about the same (57.0 $\pm$ 11.4 days, mean $\pm$ SD) at all sites.

DISCUSSION

Dental cotton roll dispensers have been used with satisfactory results in many studies on *N. sertifer* (e.g., Anderbrant et al., 1992a; Jönsson and Anderbrant, 1993; Wedding et al., 1995; Simandl and Anderbrant, 1995). Since the objectives of these studies mainly were to evaluate different factors influencing trap catch (such as weather, trap position, and different amounts and compositions of lures), the long-term constancy of release rates was not a major issue. The limitations of cotton roll dispensers have been brought to light in studies of mating disruption and monitoring. Anderbrant et al. (1995b) found a decreased disruption effect of cotton roll dispensers after five weeks, and Lyytikäinen-Saarenmaa et al. (1999) propose a more reliable monitoring system if dispensers with a longer and more constant attracting power are used.

The dispensers in the studies cited above, mainly loaded with 100 μg of the acetate of 3,7-dimethyl-2-pentadecanol, were changed at intervals varying from a week to a month. Our results from the Czech field test suggest that the latter time period is about as long as the cotton roll dispensers can be expected to be efficient. The release from the 10 μg baits, which were changed at a 10-day interval, will follow the same regression model as the unchanged 100-μg baits since the proportional release rate from cotton rolls at these loads is independent of the initial load (Anderbrant et al., 1992b). Thus, as the release from the 100-μg baits decreases, the release from the 10-μg baits will be proportionally higher. Using the model of Anderbrant et al. (1992b), it will take about 30 days before the release from the 10-μg baits becomes higher than the release from the 100-μg baits for a whole 10-day period, although it will be temporarily higher already at the first change of baits. After a month, the daily release from the 100-μg baits should be about 0.2 μg and the remaining substance reduced to about 10% of the initial load. These shifting patterns of relative release could probably account for the variations in relative catch shown in Figure 2.

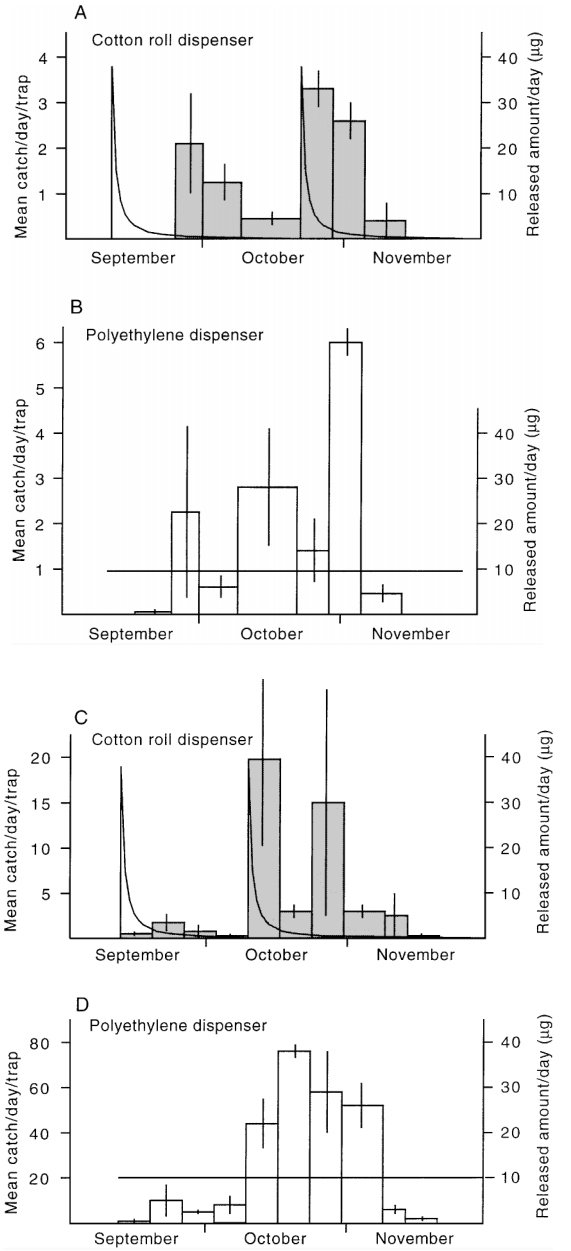


FIG. 3. Catches of male *Neodiprion sertifer* (mean ± SE) per trap per day (filled bars) in traps with cotton roll dispensers ($N = 2$) baited with 100 μg of the acetate of 3,7-dimethyl-2-pentadecanol in Italy (A) and Greece (C) and calculated release of the substance (curves). Corresponding catches (empty bars) in traps with polyethylene vial dispensers ($N = 2$) in Italy (B) and Greece (D) and calculated release of the substance (horizontal line). Note different scales at *y* axes.

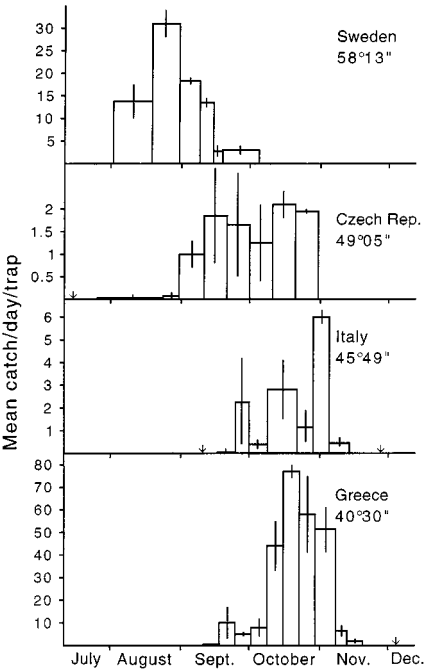


FIG. 4. Catches of male *Neodiprion sertifer* (mean ± SE) in pheromone traps in Sweden ($N = 3$), the Czech Republic ($N = 2$), Italy ($N = 2$) and Greece ($N = 2$). Arrows indicates start and end of trapping when not evident from bars. Note different scales at *y* axes.

This approximate duration of the cotton roll dispensers is further confirmed by the trap catch in Italy and Greece, although the period of efficient trapping seems to be longer later in the season in Greece. Lower temperatures probably have a leveling effect on the release curve. When compared to the trap catch from polyethylene vials, the pattern shown by the cotton rolls seems more to reflect the decreasing release of the pheromone than the actual flight activity (Figure 3).

The indifference of *N. sertifer* males to whether the acetate or propionate of 3,7-dimethyl-2-pentadecanol is used as an attractive component (Figure 2) has been shown previously in Sweden (Anderbrant et al., 1992b) and North America (e.g., Kikukawa et al., 1983) and is discussed by Anderbrant (1999).

From the laboratory studies, it seems reasonable to assume that the length of the period with a constant release rate depends on both temperature and the initial load of the polyethylene vials. The latter conclusion is based on the observation that the release rates for both substances investigated decrease when the residual amount of substance in the vials is about 500 μg (Figure 1). This could be due to the decrease in surface area wetted by the substance (Brown et al., 1992). If there is at least 500 μg of substance left in the vial at the end of a chosen time period,

the release rate should be constant for a given temperature in the interval 12–30°C. The initial load could be computed with the linear regression model $y = a - bx$, where y is the remaining amount of substance, a the initial load, b the release rate, and x the number of days. Thus, if we chose 100 days as the time period that encompasses the length of flight for *N. sertifer* and take the release rates from Table 1, the initial load of the acetate of 3,7-dimethyl-2-pentadecanol at a temperature of 18–21°C should be $y + bx$ or about 1.5 mg and at 27–30°C about 2 mg.

The relative constancy in the length of the flight period is in contrast to the preceding summer diapause which varies from 1–2 months in Scandinavia to 5–6 months in the Mediterranean (Pschorn-Walcher, 1982). According to Kolomiets et al. (1979), the length of the flight period varies from about one and a half months to three, but from the results of the present study this variation seems not to be correlated with latitude. However, the onset and end of the flight is clearly influenced by latitude and, as Kolomiets et al. (1979) suggested, two approximate zones can be recognized: the first encompassing eastern and northwestern Europe with a flight period from the beginning of August to October and the second western and southwestern Europe where the species fly from September to the middle of November.

D. pini has a longer flight period that can start at the end of April and continue, through several flight waves, to the end of August (Pschorn-Walcher, 1982). In a recent monitoring study of *D. pini*, Herz et al. (2000) tried both polyethylene vials and cotton rolls. The polyethylene vials caught very few animals in comparison with the cotton rolls in this study, in which the cotton rolls were loaded with new pheromone solution weekly and the polyethylene vials just once. This might be due to the fast release of the acetate of 3,7-dimethyl-2-tridecanol and the long exposure period of the polyethylene vials. If we apply the formula for computing initial loads presented above and choose 180 days as the length of the flight period for *D. pini*, the polyethylene vials should be loaded with about 5 mg of 3,7-dimethyl-2-tridecyl acetate 18–21°C, and about 7.4 mg at 27–30°C. The longer flight period of *D. pini*, and the higher volatility of the pheromone may call for an even slower release than from the present polyethylene dispensers.

For applications such as mating disruption and monitoring, the polyethylene vial is a sufficiently reliable and constant dispenser, with respect to release rate and the length of the *N. sertifer* flight period. However, cotton roll dispensers are useful for short-term studies and, furthermore, have the advantage that the proportional release rate is independent of the load (Anderbrant et al., 1992b). Thus, within a given temperature interval, the release rate from cotton rolls will follow the same regression curve no matter which initial amount of pheromones that are loaded, in contrast to the case in polyethylene vials.

Acknowledgments—We thank John Byers for constructive comments on earlier versions of the manuscript. The C. F. Lundström Foundation, Carl Trygger Foundation, and the Swedish Council for Forestry and Agricultural Research (SJFR) provided financial support.

REFERENCES

ANDERBRANT, O. 1998. Monitoring pine sawflies with pheromone traps, pp. 75–79, *in* W. Grodzki, M. Knizek and B. Forster (eds.). Methodology of Forest Insect and Disease Survey in Central Europe. Proceedings from the IUFRO WP 7.03.10 workshop. IBL, IUFRO, Warsaw.

ANDERBRANT, O. 1999. Sawflies and seed wasps, pp. 199–226, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Wallingford.

ANDERBRANT, O., LÖFQVIST, J., JÖNSSON, J., and MARLING, E. 1989. Effects of pheromone trap type, position and colour on the catch of the pine sawfly *Neodiprion sertifer* (Geoff.) (Hym., Diprionidae). *J. Appl. Entomol.* 107:365–369.

ANDERBRANT, O., LÖFQVIST, J., HÖGBERG, H.-E., HEDENSTRÖM, E., WASSGREN, A.-B., BERGSTRÖM, G., BENGTSSON, M., and MAGNUSSON, G. 1992a. Field response of the pine sawfly *Neodiprion sertifer* to the pheromone (2S,3S,7S)-diprionyl acetate and its stereoisomers. *Entomol. Exp. Appl.* 62:169–181.

ANDERBRANT, O., BENGTSSON, M., LÖFQVIST, J., and BAECKSTRÖM, P. 1992b. Field response of the pine sawfly *Neodiprion sertifer* to controlled release of diprionyl acetate, diprionyl propionate and *trans*-perillenal. *J. Chem. Ecol.* 18:1707–1725.

ANDERBRANT, O., HANSSON, B. S., HALLBERG, E., GERI, C., VARAMA, M., HEDENSTRÖM, E., HÖGBERG, H.-E., FÄGERHAG, J., EDLUND, H., WASSGREN, A.-B., BERGSTRÖM, G., and LÖFQVIST, J. 1995a. Electrophysiological and morphological characteristics of pheromone receptors in male pine sawflies, *Diprion pini* (Hymenoptera: Diprionidae), and behavioural response to some compounds. *J. Insect Physiol.* 41:395–401.

ANDERBRANT, O., LÖFQVIST, J., HÖGBERG, H.-E., and HEDENSTRÖM, E. 1995b. Development of mating disruption for control of pine sawfly populations. *Entomol. Exp. Appl.* 74:83–90.

BERGSTRÖM, G., WASSGREN A.-B., ANDERBRANT, O., FÄGERHAG, J., EDLUND, H., HEDENSTRÖM, E., HÖGBERG, H.-E., GERI, C., VARAMA, M., HANSSON, B. S., and LÖFQVIST, J. 1995. Sex pheromone of the pine sawfly *Diprion pini* (Hymenoptera: Diprionidae): Chemical identification, synthesis and biological activity. *Experientia* 51:370–380.

BROWN, D. F., KNIGHT, A. L., HOWELL, J. F., SELL, C. R., KRYSAN, J. L., and WEISS, M. 1992. Emission characteristics of a polyethylene pheromone dispenser for mating disruption of codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 85:910–917.

BYERS, J. A. 1988. Novel diffusion-dilution method for release of semiochemicals: testing pheromone component ratios on Western pine beetle. *J. Chem. Ecol.* 14:199–212.

HERZ, A., HEITLAND, W., ANDERBRANT, O., EDLUND, H., and HEDENSTRÖM, E. 2000. First use of pheromones to detect phenology patterns and density relationships of pine sawflies in German forests. *Agric. For. Entomol.* 2:123–129.

HÖGBERG, H.-E., HEDENSTRÖM, E., WASSGREN, A.-B., HJALMARSSON, M., BERGSTRÖM, G., LÖFQVIST, J., and NORIN, T. 1990. Synthesis and gas chromatographic separation of the eight stereoisomers of diprionol and their acetates, components of the sex pheromone of pine sawflies. *Tetrahedron* 46:3007–3018.

JÖNSSON, P., and ANDERBRANT, O. 1993. Weather factors influencing catch of *Neodiprion sertifer* (Hymenoptera: Diprionidae) in pheromone traps. *Environ. Entomol.* 22:445–452.

KIKUKAWA, T., MATSUMURA, F., OLAIFA, J., KRAEMER, M., COPPEL, H. C., and TAI, A. 1983. Field evaluation of chiral isomers of the sex pheromone of the European pine sawfly, *Neodiprion sertifer*. *J. Chem. Ecol.* 9:673–693.

KOLOMIETS, N. G., STADNITSKII, G. V., and VORONTZOV, A. I. 1979. The European pine sawfly. Amerind Publishing Co., New Dehli.

LYYTIKÄINEN-SAARENMAA, P., ANDERBRANT, O., LÖFQVIST, J., HEDENSTRÖM, E., and HÖGBERG, H.-E. 1999. Monitoring European pine sawfly population densities with pheromone traps in young pine plantations. *For. Ecol. Manage.* 124:113–121.

PSCHORN-WALCHER, W. 1982. Unterordnung Symphyta, Pflanzenwespen, pp. 4–234, *in* W. Schwenke (ed.). Die Forstschädlinge Europas. IV: Hautflügler und Zweiflügler. Paul Parey Verlag, Berlin.

SIMANDL, J., and ANDERBRANT, O. 1995. Spatial distribution of flying *Neodiprion sertifer* (Hymenoptera: Diprionidae) males in a mature *Pinus sylvestris* stand as determined by pheromone trap catch. *Scand. J. For. Res.* 10:51–55.

WEDDING, R., ANDERBRANT, O., and JÖNSSON, P. 1995. Influence of wind conditions and intertrap spacing on pheromone trap catches of male European pine sawfly, *Neodiprion sertifer. Entomol. Exp. Appl.* 77:223–232.

WILCOXON, F., 1945. Individual comparisons by ranking methods. *Biometrics* 1:80–83.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

ALLELOPATHY, DIMBOA PRODUCTION AND GENETIC VARIABILITY IN ACCESSIONS OF *Triticum Speltoides*

MOTIUL QUADER,[1,3] GRANT DAGGARD,[1] RUSSELL BARROW,[1,4] STEVEN WALKER,[2] and MARK W. SUTHERLAND[1,*]

[1]*Centre for Rural and Environmental Biotechnology and Department of Biological and Physical Sciences The University of Southern Queensland Toowoomba, Queensland 4350*

[2]*Leslie Research Centre, Toowoomba, Queensland 4350, Australia*

(Received August 21, 2001; accepted December 15, 2000)

Abstract—A screening was conducted to study the allelopathic potential of Australian-held accessions of *Triticum speltoides*. Of 26 accessions, four were found to inhibit root growth in the indicator species, lettuce (*Lactuca sativa*). The methanol leaf extracts of these accessions significantly reduced the root length of wild oat (*Avena* spp.). In all but one case, alellopathic accessions contained higher amounts of DIMBOA than did nonallelopathic accessions. Since some variation in allelopathic response was detected within lines, random amplified polymorphic DNA (RAPD) markers were used to estimate genetic diversity between and within the allelopathic accessions of *Triticum speltoides* L. The average genetic similarity between all possible pairs of selected accessions was found to be 55% and ranged from 44% to 88%. Comparison of DNA extracted from different single seedlings within the same accession revealed significant intraaccession genetic diversity (4–24%), although this was much less than that observed between accessions tested. This intraaccession diversity has significant implications for the selection of *T. speltoides* accessions in breeding or screening programs.

Key Words—Allelopathy, wheat, RAPD, genetic diversity, weed, wild oats, lettuce.

*To whom correspondence should be addressed. e-mail: marksuth@usq.edu.au

[3]Present address: Department of Applied and Molecular Ecology, The University of Adelaide, SA5064, Australia.

[4]Present address: Department of Chemistry, The Australian National University, Canberra, ACT 0200 Australia.

INTRODUCTION

Weed management is a significant problem in agriculture and accounts for some 30% of the variable cost of wheat production in Australia (Medd, 1997). Despite the overwhelming economic benefits, the heavy reliance on chemical herbicides is considered a potential threat to public health and to the environment (Jiwan and Gates, 1994). Furthermore, the appearance of herbicide-resistant weeds is an increasing problem (Boutsalis and Powles, 1995). These factors have prompted research into alternative means of weed control. Allelopathy, the release of biomolecules into the environment by one plant, which subsequently influences the growth and development of neighboring plants, has been proposed as a possible alternative weed management strategy (Einhellig, 1995).

Allelopathic potential has been identified in most major cereal crops (Lovett and Hoult, 1995). One of the mechanisms through which allelopathic potential is recognized is by the plant exuding active metabolites from roots into the environment. Such activity can be observed in soils during or following the presence of the crop (Lovett and Hoult, 1995). In barley, the exudates of living plants and living roots were found to be more inhibitory than aqueous leachate of dead roots (Rice, 1979). Liu and Lovett (1993) found lower germination and reduction in radicle length of white mustard (*Sinapis alba* L.), when it was grown alongside germinating barley seeds. Hoffman et al. (1996) found that germinating sorghum reduced the radicle length of several weed species.

While the biomolecules responsible for allelopathy in many species are unknown, in maize the major allelochemical is 2,4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) (Niemeyer and Perez, 1995), a product that has also been identified in *Triticum speltoides*, where it is present in significantly higher quantities than in commercial wheats, *Triticum aestivum* (Niemeyer, 1988).

Although insect and disease resistance genes have been incorporated into wheat cultivars through introgression from *Triticum speltoides*, there has been no attempt to screen wheat lines crossed to *T. speltoides* for allelopathy. Significantly, one recent study has indicated that *T. speltoides* does segregate for allelopathic effects. In an *in vitro* screening experiment with 29 accessions, Hashem and Adkins (1998) found that one accession of *T. speltoides* reduced radicle length of wild oat by 50%, while another two accessions reduces radical length of Indian hedge mustard (*Sisymbrium orientale* L.).

Based on these possibilities, the intentions of this study were to screen Australian collections of *T. speltoides* for allelopathic potential and to determine DIMBOA levels and genetic diversity, assessed by random amplified polymorphic DNA (RAPD) analysis, in a selection of these accessions. The assessment of genetic diversity became necessary when a significant level of intraaccession variability in allelopathic expression became evident.

METHODS AND MATERIALS

Plant Materials. Twenty-six accessions of *T. speltoides* (Ts) were screened. The heads containing seeds of *T. speltoides* and seeds of barley (cv. Tallon), wheat (cv. Hartog), and wild oat (*Avena ludoviciana*) were obtained from collections at the Leslie Research Centre, Toowoomba, Queensland, and from the Australian Winter Cereals Collection, Tamworth, New South Wales. Lettuce seeds (*Lactuca sativa* L., cv. *Salad Mix*) were obtained from Arthur Yates & Co Ltd, Milperra, New South Wales. Sorghum seeds (PAC. 8306) were obtained from Pacific Seeds, Toowoomba.

Each individual seed of *T. speltoides* was manually dehusked. The wild oat seed husks were removed by agitating for a few seconds in a blender. All seeds were surface sterilized with 70% ethanol for three min then 4% (v/v) sodium hypochlorite solution for five min (lettuce) or 15 min (all other seeds). Seeds were then rinsed and dried under sterile conditions.

Screening for Allelopathic Potential. The procedure was adapted from the plant box (Fujii et al., 1995) and double filter paper (Latto and Wright, 1995) methods. Under sterile conditions, 0.5% water agar (15 ml) was poured into plastic Petri dishes, then covered by a 9-cm filter paper. A single seed of *T. speltoides* was placed in the middle of each Petri dish, which was then sealed with Parafilm. For control dishes, seeds were omitted. All treatments were placed in the dark for three days at $20 \pm 2^{\circ}$C to stimulate germination and root elongation. After three days, Petri dishes were transferred to a 16-hr light (200 μmol/m^2/sec^1)–8-hr dark cycle for 14 days at $20^{\circ}\text{C} \pm 5^{\circ}$C. After this time, the filter paper and seedlings were removed, nine lettuce seeds were placed within 2 cm of where the seedlings had been positioned, and the dish was resealed and incubated in the dark at $20 \pm 2^{\circ}$C for three days before being transferred to a 16 hr light–8-hr dark cycle for two days. The root lengths of individual lettuce seedlings were recorded.

Effects of Methanol Leaf Extracts of T. speltoides on Test Species. These experiments were based on the method of Jimenez-Osorino et al. (1996) to observe the effect of leaf extracts from selected accessions of *T. speltoides* on lettuce and weed species. The 0.2 g (per treatment) of immature green leaves of 90-day-old accessions were extracted by grinding in 98.8 % ice-cold methanol with a mortar and pestle. Extracts were collected into a 10 centrifuge tube. Final volume of the extracts was adjusted to 7 ml, and centrifuged at 3000 rpm for 10 min. Two milliliters of supernatant was added to a 5.5-cm filter paper (Whatman No. 1) placed on a sterile 9-cm Petri dish, and the methanol was allowed to evaporate. Each treatment was replicated three times. Washed sand (10 g) was placed in a clean Petri dish, and lettuce or weed seeds were sown into the sand. A filter paper containing dried leaf extract was placed on top of the sand, and analytical grade water (3 ml) was added onto the filter paper. Petri dishes were placed in the dark at $20 \pm 2^{\circ}$C. Filter papers were removed from the dishes after 24 hr for lettuce and

72 hr for other seeds. After three days (for lettuce) or five days (wild oat), the root lengths were recorded.

Estimation of DIMBOA Content in T. speltoides Accessions. Five allelopathic and five randomly selected nonallelopathic accessions (based on experiments described above) together with the wheat cultivar Hartog were studied in this experiment. DIMBOA extraction was performed by the method of Bohidar et al. (1986). The *T. speltoides* accessions and wheat plants were grown in MS (Murashige and Skoog) media under a 16L:8D hrs cycle at 26°C for 14 days. Seedlings were collected and frozen at −20°C. Plant tissue (0.2 to 1.5 g) was macerated with a mortar and pestle in deionized water (3 × 2 ml), filtered through cheesecloth, and left for 15 min at room temperature. The extract was adjusted to pH 3 with 1 M HCl and centrifuged at 10,000g for 10 min. The supernatant was extracted three times into equal volumes (6 ml) of diethyl ether, and the organic phases were evaporated to dryness. Ferric chloride reagent (3 ml) (50 g $FeCl_3 \cdot 6H_2O$, 500 ml 95% ethanol, and 5 ml 10 M HCl) was added, and the absorbency of extracts was measured at 590 nm. Each extraction was replicated three times. The concentration of DIMBOA in the tissue was determined by comparing the absorbance of extracts with a DIMBOA standard, which had previously been purified from 7-day-old seedlings of maize by the method of Tripton et al. (1967).

DNA Extraction and RAPD Analysis. All seeds were grown in Petri dishes containing 0.5% water agar under sterile conditions. Seeds were allowed to grow for 14 days (accessions) or 7 days (wheat) under dark conditions. Genomic DNA was extracted from duplicate samples from a single seedling by the method of Weining et al. (1994). Six 10mer primers (up to 70% GC) were selected from a previous study on the basis of polymorphisms revealed in hybrid wheat lines (Ma, 2000). The polymerase chain reaction (PCR) was performed in a 12.5-μl reaction mixture, as in Williams et al. (1990). The operon (OP) random primers used were OPC7, OPI18, OPI4, OPA4, OPW3, OPW8. A polyacrylamide gel electrophoresis (PAGE) method (Weising et al., 1995) was used to separate different sizes of RAPD-PCR products. The gels were silver stained for visualization of DNA bands by using a method developed by Bassam and Caetano-Anolles (1993). Only RAPD bands duplicated from independent DNA extractions were scored for the estimation of genetic diversity.

Statistical Analysis. Analyses of variance (ANOVA) were carried out by using the computer package SPSS 8.0 for Windows. For LSD, group comparison and pair comparisons were performed for means with equal replications and unequal replications, respectively (Sokal and Rohlf, 1981).

Genetic data analyses were performed by using the computer package NTSYS (Numerical Taxonomy System) PC version 1.8 (Rohlf, 1993). The data matrix was used to compute Jaccard's similarity coefficients for interval measurement within and between accessions by using the SIMQUAL program of NTSYS-PC (Link et al., 1995). These coefficients were used as operational taxonomic units

(OTUs) to construct dendrograms by the unweighted pair group method (UPGMA) (Sneath and Sokal, 1973) and the SAHN (sequential, agglomerative, hierarchical, and nested clustering) routine to produce a dendrogram for each individual primer. Finally, for comparisons between accessions, a consensus tree was constructed by combining all individual band sizes, generated by each primer, into one input file for SIMQUAL and subsequently for SAHN-clustering programs. Similarly, a tree was constructed from the band sizes of seedlings of accessions and the wheat cultivar Hartog, generated by primer Operon C07. The wheat cultivar Hartog was used as a homozygous control.

RESULTS

Screening for Allelopathic Potential. Of 26 accessions, four (Ts9, Ts10, Ts22 and Ts25) significantly reduced the mean root lengths of the test species lettuce (Table 1). Inhibition ranged from 34% (Ts9) to 63% (Ts25).

Effect of Methanol Leaf Extracts of T. speltoides on Test Species. Leaf extracts of the four inhibitory accessions in Table 1 and from Ts1 and Ts8 were tested for their ability to inhibit root growth in lettuce and wild oats. Accessions Ts8, Ts9, Ts10, Ts22, Ts25, and barley significantly reduced (20–67%) the root length of lettuce, while all accessions of barley and wheat reduced the root length of wild oats by between 36 and 87% compared to controls (Table 2). Notably, extracts from all *T. speltoides* accessions inhibited root lengths in both test species, when compared to the effects of wheat extracts.

Estimation of DIMBOA Content in T. speltoides Accessions. The amounts of DIMBOA in 14-day-old seedlings of 10 accessions, five of which had yielded methanol extracts that suppressed lettuce root growth (Table 2: Ts8, Ts9, Ts10, Ts22, Ts25) and five accessions which had not suppressed root growth (Table 1: Ts1, Ts7, Ts14, Ts15, and Ts20), were estimated (Table 2). Four of the five that gave suppressive extracts ranked higher than the nonallelopathic accessions with regard to their DIMBOA levels. Only Ts9 did not fit this pattern.

Genetic Diversity Between Accessions. A subset of five accessions (Ts1, Ts8, Ts9, Ts22, and Ts25), comprising four allelopathic and one nonallelopathic accession, were used to study variability among and within accessions. One randomly selected seedling per accession was used. Each of the six RAPD primers amplified an average of eight bands per accession of *T. speltoides*, ranging from 4 to 12 bands per accession. A total of 231 bands were scorable, of which 196 were polymorphic (Table 3). All 231 bands were used to calculate the genetic similarity (GS) among the five accessions. The average GS was found to be 55% for all possible pairs of accessions and ranged from 44% to 88% (Table 4). This revealed the presence of a significant variation among the accessions of *T. speltoides*. The highest similarity (88%) was observed between accessions Ts22 and Ts25, while lowest similarity (44%) was recorded between accessions Ts1 and Ts22.

TABLE 1. ROOT LENGTH INHIBITION OF LETTUCE BY *T. speltoides* ACCESSIONS

Accession/cultivar	Root lengths (mm, mean ± SE)[a]
Ts25	7.5 ± 1.0[b]
Ts22	9.6 ± 1.4[b]
Ts10	11.3 ± 2.3[b]
Ts9	13.5 ± 1.7[b]
Ts26	15.5 ± 1.8
Ts24	17.7 ± 0.6
Ts27	17.8 ± 1.8
Ts8	18.2 ± 0.3
Ts20	18.5 ± 0.9
Ts4	18.7 ± 6.9
Ts12	19.0 ± 1.0
Ts15	19.4 ± 1.0
Ts23	20.3 ± 0.8
Control	20.5 ± 1.7
Ts5	20.6 ± 1.1
Ts1	20.7 ± 1.4
Ts13	20.9 ± 0.7
Ts21	21.1 ± 0.6
Ts29	21.2 ± 1.3
Ts11	21.5 ± 0.4
Ts28	21.5 ± 0.5
Ts3	21.9 ± 2.1
Ts2	22.0 ± 4.4
Ts14	22.7 ± 1.0
Ts7	22.8 ± 0.9
Ts17	23.6 ± 1.3
Ts6	23.7 ± 0.8
Hartog	24.9 ± 4.3

[a] Total sample size = 13–21/treatment.
[b] Significant at 1% level, compared to control.

Genetic Homogeneity Within Accessions. Five randomly selected seedlings of promising allelopathic accessions, one nonallelopathic accession of *T. speltoides* (Ts1), and the wheat cultivar Hartog (*T. aestivum*) were assessed to observe the genetic homogeneity within the accessions. The DNA fingerprints generated from each of the five randomly selected individual seedlings of each accession and the wheat variety Hartog, by the operon C7 primer, were evaluated. High levels of polymorphism were detected among individuals of each accession (Figure 1). Twenty-four RAPD bands were used to estimate a GS within the seedlings of accessions. Accession Ts8 showed the highest level of polymorphism (75%) among individual plants, while Ts22 and Ts25 showed the lowest (10%). The highest numbers of genotypes were found in accessions Ts1, Ts8, and Ts10 while,

TABLE 2. EFFECT OF METHANOL LEAF EXTRACTS ON ROOT GROWTH OF LETTUCE AND WILD OAT AND RELATIONSHIP TO SEEDLING DIMBOA CONTENT

Accession/ cultivar	Root length (mm/plant) against methanol leaf extract[a]		DIMBOA content of Ts seedlings (mmol/kg fresh wt)[b]
	Lettuce	Wild oat	
Ts25	11.8 ± 0.2c	9.5 ± 0.5e	12.6 ± 0.1a
Ts10	6.5 ± 0.3de	7.0 ± 0.3fg	10.4 ± 0.7ab
Ts22	4.6 ± 0.3f	6.0 ± 0.3g	9.8 ± 0.4bc
Ts8	7.5 ± 0.3d	13.9 ± 0.4d	9.4 ± 0.7bcd
Ts20			8.2 ± 2.2bcd
Ts1	14.6 ± 0.55b	7.9 ± 0.96e	7.2 ± 0.5de
Ts7			6.1 ± 0.4def
Ts9	10.6 ± 0.6c	23.6 ± 0.6c	6.0 ± 0.4def
Ts15			5.1 ± 0.4efg
Wheat	20.0 ± 0.3a	29.9 ± 0.7b	3.9 ± 0.3fgh
Ts14			3.7 ± 0.2h
Barley	5.29 ± 0.29e	7.05 ± 0.30fg	
Control	15.4 ± 0.8b	47.5 ± 0.9a	

[a] Sample size 13-21/treatment.
[b] Mean of three replicate experiments/extractions. Measurements followed by different letters differ significantly at 5% level.

as expected, no variability was recorded among the five seedlings of wheat cultivar Hartog (Table 5).

Clustering. Accessions Ts22 and Ts25 were grouped into one main group, while Ts8, Ts9, and Ts10 were grouped into another (Figure 2). Accession Ts1 and cultivar Hartog were grouped into two individual main groups. The GS levels among accessions ranged from 61% to 95%. The GS level among seedlings of the accessions varied from 76% to 100%. Seedlings of accessions Ts1, Ts8, Ts9, Ts10, and wheat cv. Hartog separated into five distinct groups according to their accession, while seedlings of the accessions Ts22 and Ts25 were grouped closely together and did not segregate according to accession.

TABLE 3. TOTAL NUMBER OF BANDS AND NUMBER OF POLYMORPHIC BANDS GENERATED BY SIX PRIMERS IN FIVE ACCESSIONS OF *Triticum speltoides*

Primer	Sequence	Total bands	Polymorphic bands	Polymorphisims (%)
OP I04	CCGCCTAGTC	52	27	52
OP C07	GTCCCGACGA	39	39	100
OP W08	GACTGCCTCT	34	34	100
OP W03	GTCCGGAATG	36	31	86
OP A04	AATCGGGCTG	43	43	100
OP I18	TGCCCAGCCT	27	22	82
Total		231	196	

TABLE 4. GENETIC SIMILARITY MATRIX FOR RAPD FINGERPRINTS IN ACCESSIONS TS1, TS8, TS9, TS22 AND TS25 GENERATED BY PRIMERS OPA04, OPW08, OPC07, OPW03, OPI04, OPI18

	Ts1	Ts8	Ts9	Ts22	Ts25
Ts1	1.0000				
Ts8	0.53370	1.00000			
Ts9	0.54494	0.65168	1.00000		
Ts22	0.44382	0.48314	0.49438	1.00000	
Ts25	0.45505	0.49438	0.49438	0.87640	1.00000

TABLE 5. RAPD ANALYSIS OF INDIVIDUALS WITHIN *T. Speltoides* ACCESSIONS REVEALED BY PRIMER OPC7

Accession	Seedlings tested (*N*)	RAPD bands		Polymorphisms (%)	Genotypes observed (*N*)
		Total	Polymorphic		
Ts1	5	9	4	44	4
Ts8	5	8	6	75	4
Ts9	5	9	2	22	2
Ts10	5	15	10	67	4
Ts22	5	10	1	10	2
Ts25	5	10	1	10	2
Hartog	5	9	0	0	1

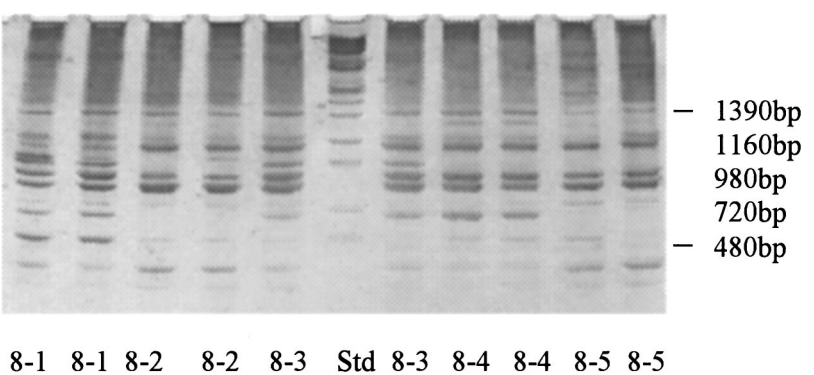


FIG. 1. RAPD pattern of five seedlings of *Triticum speltoides* (Accession No. Ts8) generated by primer OP7. Left to right: 8–1 to 8–5 are duplicate seedlings DNA, Std is standard DNA.

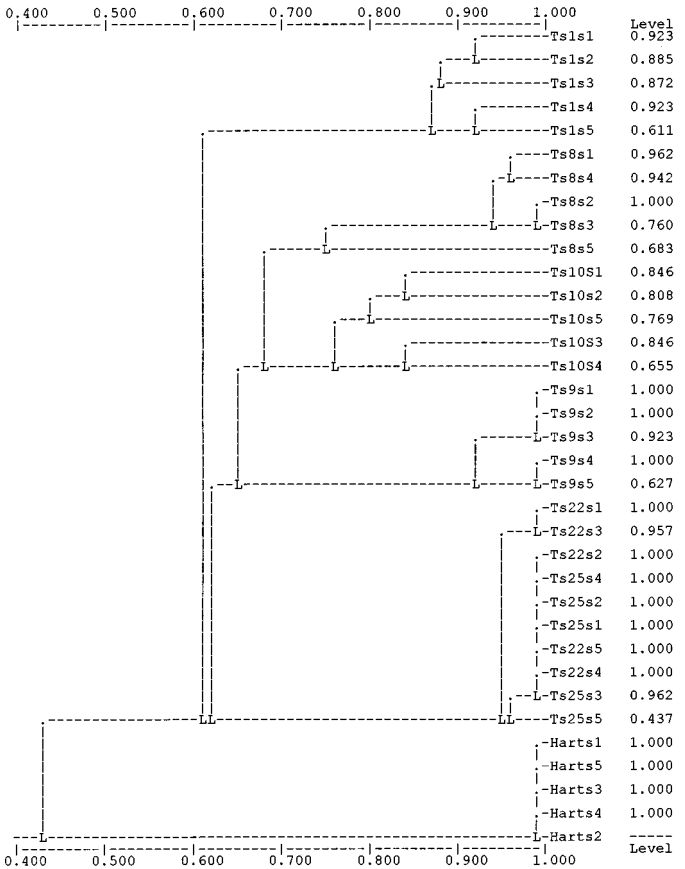


FIG. 2. Dendrogram illustrating general relationships among 35 seedlings of six accessions of *T. speltoides* and wheat (*Triticum estivum*) cultivar Hartog.

DISCUSSION

Screening for Allelopathy. Many of the problems encountered in separating allelopathy from resource competition can be attributed to a lack of convincing methodology (Fuerst and Putnam, 1983). One early survey of published bioassays revealed that the results may depend as much on the choice of methods as on the choice of species (Stowe, 1979). Clearly, the actual bioassay technique is a major determining factor in a screening program seeking to draw conclusions about the allelopathic potential of the species being assessed. The key criteria for developing convincing methods must be that the investigations are on intact plants and that experiments should reasonably simulate the real situation of plant interference.

Germinating seeds of cereal crops are able to take up 88% of their dry weight in water (Albabushev, 1977). For example, the average water uptake by germinating barley can be 83% of the initial dry weight on filter paper (Liu and Lovett, 1993). The amount of water absorbed by barley at barley densities of 0.5 seeds/cm^2 or more (Liu and Lovett, 1993) could certainly alter water availability for other species. Therefore, addition of water to compensate for water depletion by germinating seeds is essential in a bioassay when an attempt is made to reduce physical competition, especially in a limited test medium such as Petri dishes. On the other hand, addition of water can disturb the osmotic potential gradients between test species and allelochemical(s) released by suspected allelopathic plants. This study has addressed those problems by using 0.5% water agar media, which ensured equal water uptake and enabled conclusive separation of competition for water from allelopathic interference. In addition, accessions were grown for much longer periods of time in Petri dishes containing 0.5% water agar to create nutritional stress, which could influence the defence mechanism leading to expression of allelopathy by the accessions (if any). There is evidence that any kind of stress may induce expression of allelopathy in plant communities and enhance the production of allelochemicals (Putnam, 1985).

All promising accessions showed allelopathic potential in both bioassay systems, with the exception of Ts8. This was an encouraging finding because lack of reproducibility is a common phenomenon in allelopathy research (Latto and Wright, 1995). Veronneau et al. (1997) have suggested that reproducible results from two or more bioassay systems are required to indicate allelopathic potential in tested plant species. The allelopathic accessions identified in this study are consistent with the findings of previous work that used a similar method (Hashem and Adkins, 1998), where allelopathic effect was detected in accessions Ts8, Ts9, and Ts10 of *T. speltoides*. Similarly, in rice, allelopathic accessions have been identified that inhibited lettuce root length by 75% or more (Fujii, 1993).

Since numerous organic compounds are present within plants, the results obtained from solvent extractions or from maceration and grinding of plant materials have their limitations. These approaches may result in the liberation of chemicals that would not be released in the field by living tissue (Lovett, 1982). Nevertheless, such assays do indicate allelopathic potential where it occurs, both during the life of the plants and during subsequent decomposition of plant residues.

The significant growth-suppressing activity of the methanol leaf extract of the accessions tested suggests high levels of the allelochemical in these extracts. Leaf extracts of other allelopathic crops, such as rye and barley, can also reduce the growth of certain test and weed species. A methanol leaf extract of rye (0.33 g rye shoot/Petri dish) reduced lettuce seed germination up to 100% in filter paper placed on a 10-cm Petri dish (Creamer et al., 1996). Wardle et al. (1996) found that the aqueous extract of 10 grassland forage species severely inhibited the root elongation of a common test species *Carduus nutans* L.

The higher levels of DIMBOA in the putative allelopathic accessions suggest the possibility of a relationship between the allelopathic potential of the accession and the amount of DIMBOA in the accession. Considerable variation was noted in DIMBOA levels in *T. speltoides* accessions. Nevertheless, DIMBOA levels were, in all cases but one (Ts14), higher than Hartog. DIMBOA levels detected in Hartog were two to four times greater than levels measured in the range of wheat cultivars studied by Niemeyer (1988). The significance of these results is clouded by the variability we observed within and among different assays (e.g., for Ts8 and Ts9), especially those based on allelopathic effects due to single seedlings of the source species. To address this issue, it was essential to investigate the underlying genetic diversity of the accessions being examined.

Genetic Homogeneity Study. Bustos et al. (1998) have successfully used RAPD markers to investigate the genetic diversity within and among wild populations of species of the genus *Hordeum* (Poaceae). The high level of intraaccession genetic variability observed in our study might be due to the outbreeding reproductive system of *T. speltoides*. Morrison (1993) has observed different morphological forms, especially in inflorescence structure and disarticulation mode, within accessions of *T. speltoides*. Reproducibility is a critical factor in evaluating the usefulness of a DNA fingerprinting technique. It is a major issue in the use of RAPD-based techniques in wheat (Devos and Gale, 1992). However, by careful screening and replication, this study has demonstrated that RAPD fingerprints of *T. speltoides* and wheat are reproducible. Similarly, Ma (2000) has generated reproducible RAPD fingerprints in a doubled haploid population derived from the F_1 progeny of a cross between the wheat line WW21MMT70 and the cultivar Mendos.

The wheat cultivar Hartog, as expected, showed no polymorphism among the individuals tested. This result reflects the genetic homogeneity of commercial cultivars of inbred wheat (Chen et al., 1994). The genetic diversity within and among *T. speltoides* accessions observed in this study confirms *T. speltoides* as a potentially useful source of genetic variation for introgression into commercial wheats in which low levels of diversity are widely acknowledged by cereal breeders (Chen et al., 1994).

The significant genetic heterogeneity observed within the accessions tested opens the possibility of segregation within accessions for allelopathic characteristics, consistent with the variability in phenotype observed during experimentation. Clearly, there is a need for careful screening of individuals within these accessions before material can be selected for experiments attempting to cross allelopathic character into commercial wheat backgrounds.

Apart from the observed genetic diversity within the accessions of *T. speltoides* in the current study, the outbreeding character (Morrison, 1993) of *T. speltoides* has implications for germplasm maintenance. Germplasm curators must take extreme care to maintain individual accessions and recognize the possibility

of genetic drift over time within accessions. Additionally, in the case of screening or genetic studies, researchers may have to select at the single-plant level within accessions.

In the future, identification and purification of allelochemical(s) from a variety of growth media, and a study of the effect of the identified allelochemical(s) on weed species will be required to establish unequivocally the presence of allelopathy in *T. speltoides* (Fuerst and Putnam, 1983). Research is required to attempt the transfer of allelopathy gene(s) from selected *T. speltoides* material into cultivated wheat. This should be possible by conventional breeding, as *T. speltoides* is known to suppress the activity of the *Ph1* gene in hybrids with wheat, resulting in homologous pairing (Riley, 1996). If it is confirmed that DIMBOA is responsible for *T. speltoides* allelopathy, then a robust and cost-effective technique must be developed to identify DIMBOA gene(s). One of the possible techniques would be use of expressed sequenced tag (EST) technology, which involves partial cDNA sequencing to identify the gene of interest. This technique is becoming widely used in the identification of gene(s) of interest in plants.

REFERENCES

ALBABUSHEV, V. A. 1977. Water absorption by crop seeds during germination. *Field Crop Abstr*. 32:251.

BASSAM, B. J., and CAETANO-ANOLLES, G. 1993. Silver staining of DNA in polyacrylamide gels. *Appl. Biochem. Biotechnol.* 42:181–188.

BOHIDAR, K., WRATTEN, S. D., and NIEMEYER, H. M. 1986. Effects of hydroxamic acids on the resistance of wheat to the aphid *Sitobion avenae. Ann. Appl. Biol.* 109:193–198.

B0UTSALIS, P., and POWLES, S. B. 1995. Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. *Theor. Appl. Genet.* 35:149–155.

BUSTOS, A. D., CASANOVA, C., SOLER, C., and JOUVE, N. 1998. RAPD variation in wild populations of four species of the genus *Hordeum* (Poaceae). *Theor. Appl. Genet.* 96:101–111.

CHEN, P. D., TSUJIMOTO, H., and GILL, B. S. 1994. Transfer of *Ph1* genes promoting homologous pairing from *Triticum speltoides* to common wheat. *Theor. Appl. Genet.* 88:97–101.

CREAMER, N. G., BENNETT, M. A., STINNER, B. R., CARDINA, J., and REGNIER, E. E. 1996. Mechanisms of weed suppression in cover crop-based production systems. *Hortic. Sci.* 31:410–413.

DEVOS, K. M., and GALE, M. D. 1992. The use of random amplified polymorphic DNA markers in wheat. *Theor. Appl. Genet.* 84:567–572.

EINHELLIG, F. A. 1995. Mechanism of action of allelochemicals in allelopathy, pp. 96–116, *in* Inderjit, K. M. M. Dakshini and F. A. Einhellig (eds.). Allelopathy, Organisms, Processes, and Applications. American Chemical Society, Washington, DC.

FUERST, E. P., and PUTNAM, A. R. 1983. Separating the competitive and allelopathic components of interference. Theoretical principles. *J. Chem. Ecol.* l9:937–944.

FUJII, Y. 1993. The allelopathic effect of some rice varieties. Allelopathy in control of paddy weeds. ASPAC Food & Fertilizer Technology Centre, Technical Bulletin No. 134. Taipei, Taiwan. (ASPAC-Asia Pacific).

FUJII, Y., INOUE, H., ONO, S., SATO, K., KHAN, B. A., and WALLER, G. R. 1995. Screening of allelopathic cover crops and their application to abandoned fields. pp. 305–310, *in* Proceedings I (A), 15th Asian-Pacific Weed Science Society Conference, July 24–28, 1995. Tsukuba, Japan.

HASHEM, A., and ADKINS, S. W. 1998. Allelopathic effects of *Triticum speltoides* on two important weeds of wheat. *Plant Prot. Q*. 13:33–35.

HOFFMAN, M. L., WESTON, L. A., SNYDER, J. C., and REGNIER, E. 1996. Allelopathic influence of germinating seeds of cover crops on weed species. *Weed Sci.* 44:597–584.

JIMENEZ-OSORINO, F. M. V. Z., KUMAMOTO, J., and WASSER, C. 1996. Allelopathic activity of *Chenopodium ambrosioides* L. *J. Chem. Ecol.* 24:195–205.

JIWAN, J. S., and GATES, G. W. 1994. Water down under. Conference proceedings of the institute of engineers, Barton ACT, Australia, pp. 21–24.

LATTO, J., and WRIGHT, H. 1995. Allelopathy in seeds. *J. Biol. Educ*. 29:123–128.

LINK, W., DIXKENS, C., SINGH M., SCHWALL, A., and MELCHINGER, 1995. Genetic diversity in European and Mediterranean faba bean germplasms revealed by RAPD markers. *Theor. Appl. Genet.* 90:27–32.

LIU, D. L., and LOVETT, J. V. 1993. Biologically active secondary metabolites of barley. I: Developing techniques and assessing allelopathy in barley. *J. Chem. Ecol.* 19:2217–2230.

LOVETT, J. V. 1982. The effect of allelochemicals on crop growth and development, pp. 93–110, *in* J. S. McLaren (ed.). Chemical Manipulation of Growth and Development. Butterworths, London.

LOVETT, J. V., and HOULT, A. H. C. 1995. Allelopathy and self-defence in barley, pp. 170–183, *in* Inderjit, K. M. M. Dakshini and F. A. Einhellig (eds.). Allelopathy, Organisms, Processes, and Applications. American Chemical Society, Washington, DC.

MA, W. 2000. Molecular mapping of dough properties in wheat. PhD dissertation. University of Southern Queensland, Toowoomba.

MEDD, R. W. 1997. Biological change: weeds pp. 131–149, *in* A. L. Clarke and P. B. Wylie (eds.). Sustainable Crop Production in the Sub-tropics: An Australian Perspective. Queensland Department of Primary Industries, Brisbane.

MORRISON, L. A. 1993. *Triticum–Aegilops* systematics: Taking an integrative approach, pp. 59–66, *in* A. B. Damania and A Wiley-Sayca (ed.). Biodiversity and Wheat Improvement. ICARDA—International Centre for Agricultural Research in the Dry Areas. Aleppo, Syria.

NIEMEYER, H. M. 1988. Hydroxamic acids content of *Triticum* species. *Euphytica* 37:289–293.

NIEMEYER, H. M., and PEREZ, F. 1995. Potential of hydroxamic acids in the control of cereal pest, disease, and weeds, pp. 260–270, *in* Inderjit, K. M. M. Dakshini and F. A. Einhellig (eds.). Allelopathy, Organisms, Processes, and Applications. American Chemical Society, Washington, DC.

PUTNAM, A. R. 1985. Weed Physiology. Boca Raton, Florida.

RICE, E. L. 1979. Allelopathy—an update. *Bot. Rev.* 45:15–109.

RILEY, R. 1996. The genetic regulation of meiotic behaviour in wheat and its relatives. *Hereditas* 2(suppl.):395–408.

ROHLF, F. J. 1993. NTSYS-pc, Numarical Taxonomy and Multivariate Analysis System, Version 1.8. Applied Biostatistics, Inc., New York.

SNEATH, P. H. A., and SOKAL, R. R. 1973. Numerical Taxonomy. Freeman, San Francisco.

SOKAL, R. R., and ROHLF, F. J. 1981. Biometry. W. H. Freeman & Co, New York.

STOWE, L. G. 1979. Allelopathy and its influence on the distribution of plants in an Illinois old-field. *J. Ecol.* 67:1065–1085.

TRIPTON, C. L., KLUN, J. A., HUSTED, R. R., and PIERSON, M. D. 1967. Cyclic hydroxamic acids and related compounds from maize: Isolation and characterization. *Biochemistry* 6:2866–2870.

VERONNEAU, H., GREER, A. F., DAIGLE, S., and VINCENT, G. 1997. Use of mixtures of allelochemicals to compare bioassays using red maple, pin cherry, and American elm. *J. Chem. Ecol.* 23:1101–1117.

WARDLE, D. A., NICHOLSON, K. S., and RAHMAN, M. 1996. Use of a comparative approach to identify allelopathic potential and relationship between allelopathy bioassays and "competition" experiments for ten grassland and plant species. *J. Chem. Ecol.* 22:933–948.

WEINING, S., KO, L., and HENRY, R. J. 1994. Polymorphisms in the *α-amy1* gene of wild and cultivated barley revealed by the polymerase chin reaction. *Theor. Appl. Genet.* 89:509–513.

WEISING, K., NYBOM, H., WOLFF, K., and MEYER, W. 1995. DNA Fingerprinting in Plants and Fungi. CRC Press, Boca Raton, Florida.

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A., and TINGEY, S. V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531–6535.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

LIMITATIONS OF FOLIN ASSAYS OF FOLIAR PHENOLICS IN ECOLOGICAL STUDIES

HEIDI M. APPEL,* HEATHER L. GOVENOR,[1] MARK D'ASCENZO,[2] ERIN SISKA,[3] and JACK C. SCHULTZ

Pesticide Research Lab
The Pennsylvania State University
University Park, Pennsylvania 16802

(Received June 29, 2000; accepted December 12, 2000)

Abstract—We examined the response of the widely used Folin-Denis assay to purified tannins from 16 woody plant species and to three commercial polyphenol preparations often used as standards. The reagent's response to these chemical mixtures differed significantly among sources (tree species, commercial preparations) and sampling dates, even though the mixtures contained the same total dry weight of tannins. Response to commercial standards usually did not resemble response to actual plant tannin and produced estimates that differed from actual concentrations by as much as twofold. Species-based and seasonal differences in polyphenol composition are evidently responsible for these variable results. Reagents that depend on redox reactions, such as the Folin-Denis, do not produce reliable absolute or relative quantification of phenolics when different species or samples from different dates are compared, and use of commercial standards does not resolve this problem.

Key Words—Phenolics, tannins, polyphenols, Folin-Denis assay, plant allocation theory.

INTRODUCTION

Folin-type assays (FA; Folin-Denis, Folin-Ciocalteau, Prussian blue) are widely used to estimate amounts of total phenolics in vascular plant tissues. Plants produce a diverse array of phenolics, ranging in size from simple, substituted

*To whom correspondence should be addressed.
[1]Present address: Baker Environmental, Inc., 420 Rouser Road, Coraopolis, Pennsylvania 15108.
[2]Present address: Boyce Thompson Institute, Tower Road, Ithaca, New York 14853.
[3]Present address: Sonoma State University, Department of Biology, 1801 East Cotati Avenue, Rohnert Park, California 94928.

0098-0331/01/0400-0761$19.50/0

monomers to the complex polyphenols called tannins. Within each structural class there are hundreds to thousands of structurally distinct compounds. A single plant can produce a diverse mixture of phenolics, some with primary roles in metabolism, and some with sole or secondary functions in secondary metabolism. All vascular plant species produce a common set of monomeric phenolics, but there is substantial variation in which molecules predominate and in the conformation and substitution patterns of phenolics produced. For example, although virtually all vascular plants contain caffeic and chlorogenic acids, each plant taxon is usually characterized by one or a few particular types or groups of polyphenols (e.g., anthocyanins in *Arabidopsis*, phenolic glycosides in *Populus*). Similarly, among tannin-producing species, some produce only condensed tannins (e.g., grasses), some only hydrolyzable tannins (e.g., *Pelargonium*), and some produce both (e.g., oaks, maples, elms, birches, raspberries). These combinations often change as tissues age (e.g., Feeny, 1968; Scalbert and Haslam, 1987), and in response to a wide variety of biotic and abiotic stresses (Rossiter et al., 1988; Karban and Baldwin, 1997).

Probably because of their ubiquity among plant species and functions in defense, phenolics are among the most frequently studied plant secondary metabolites (Schultz, 1988). Ecologists have used the FA to estimate the quality of food for herbivores, the induction of polyphenols by herbivore feeding, and the investment by plants in defensive chemistry. Indeed, many influential ecological theories and generalizations have been based at least in part on the FA, including the plant apparency theory, optimal defense theory, resource availability hypothesis, carbon–nutrient balance hypothesis, and growth–differentiation balance hypothesis (e.g., Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1983; Coley et al., 1985; Herms and Mattson, 1992).

The chemistry underlying the FA and other redox-based assays limits their value in comparing different samples quantitatively. FAs employ one of several redox-based colorimetric reagents, e.g., Folin-Denis, Folin-Ciocalteau, Prussian blue (Folin and Denis, 1915; see Waterman and Mole, 1994). These reagents measure the ability of any mixture to reduce phosphomolybdic and phosphotungstic acids, which are blue when reduced (Swain and Hillis, 1959). At least as early as 1959, Swain and Hillis (1959) pointed out that Folin reagents may be inappropriate for comparisons of phenolic samples from different sources because the absorbance of the reagents is influenced by structural variation in phenolics within and among species, as well as by the presence of interfering metabolites and varying extraction and reaction conditions. Although phosphomolybdic and phosphotungstic acids are reduced by the aromatic hydroxyls characteristic of all phenolics, the degree to which they are depends on phenolic structure (Swain and Goldstein, 1964), and they may be reduced by many other plant constituents (e.g., alkaloids, proteins). If phenolics from different sources differ sufficiently in structure (especially the number or position of hydroxyls), or if plant extracts differ

in the presence of other reducing agents, Folin-reagent assays cannot accurately quantify differences in the amounts of phenolics present.

The FA is easy to perform and provides consistent estimates of the reducing power of phenolics within a sample, as evidenced by the high R^2 of standard curves relating absorbance to known concentrations of a standard and by the relatively low variance among aliquots from a sample. However, when samples from different sources (e.g., species, tissue, time, treatment) are compared, it is difficult to interpret FA values because different sources may comprise different mixes of phenolic structures as well as different amounts of the same structures. If those differences result in the same overall capacity to reduce the FA reagent, then the FA values will be the same. If the differences result in a different capacity to reduce the FA reagent, then the FA values will differ. Both the composition and amount of phenolics can differ among samples without a change in FA value.

Thus, although the FA is often used to measure the "amount" of phenolics in a mixture, it does not do so, but rather provides an estimate of the overall reducing capacity. The importance of this distinction between the amount of phenolics and their reducing capacity depends on the specific goals of the study and on what is known about the mode of action of the phenolics in the bioassay. If the goal is to measure the *amount* of phenolics, e.g., as a measure of plant investment in their production, then the FA is inappropriate. If the goal is to measure the redox-based *activity* of a mixture, then the FA can be very useful. FA results have been useful predictors of biological activity in several systems, either because they are correlated with some other key component of activity or because biological activity depends directly on the reducing capacity of the phenolic mixture.

The structural specificity of Folin reagents is also a major problem when selecting appropriate standards for these colorimetric assays. If the standard chosen fails to duplicate the phenolic extract's ability to reduce the Folin reagent—likely unless the standard is precisely the same as the extracted phenolics—then concentrations calculated from a standard curve do not reflect sample concentrations. Even relative concentrations derived this way may not be useful. We believe that dependence on Folin assays and the widespread use of convenient commercial standards by the ecological community has often produced misleading results in intraspecific or interspecific comparisons. Martin and Martin (1982), Hagerman and Butler (1989), and Wisdom et al. (1987) have suggested that purified phenolics from the plant and tissue being studied should be used as standards ("self" standards), but we have seen little adoption of this approach in recent publications.

Despite these chemical arguments, little experimental work has addressed the actual impact of qualitative structural variation on estimates of phenolic concentrations using Folin reagents (but see van Alstyne, 1995). To assess the limitations of Folin-type assays in intra- and interspecific comparisons and the value of using self standards, we compared results of FAs of known concentrations of purified tannins from 16 woody plant species and three commercial standards. We found

that the relationship between FA absorbance and the amount of self or commercial standard differed significantly with sample source, confirming our expectation of qualitative variation and demonstrating that a single standard cannot be used in comparisons of this sort. With few exceptions, using commercial standards significantly over- or underestimated actual polyphenols to a degree that would produce misleading ecological or evolutionary conclusions.

METHODS AND MATERIALS

Foliage Collection. We examined phenolic extracts from five oak species sampled on three dates in May, June, and July 1994. Three species were sampled from the subgenus Erythrobalanus (red oaks: red oak, *Quercus rubra* L.; black oak, *Quercus velutina* Lam.; and scarlet oak, *Quercus coccinea* Muench.), and two from the subgenus Lepidobalanus (white oaks: chestnut oak, *Quercus prinus* L.; and white oak, *Quercus alba* L.)

To examine interspecific differences in foliar tannins, foliage from 16 woody plant species was collected in July 1997: sugar maple, *Acer saccharum* Marsh.; red maple, *Acer rubrum* L.; striped maple, *Acer pensylvanicum* L.; yellow birch, *Betula lutea Michx. f.*; ironwood, *Ostrya virginiana* (Mill.) K. Koch; American ash, *Fraxinus americana* L.; black cherry, *Prunus serotina* Ehrh.; flowering dogwood, *Cornus florida* L.; witch hazel, *Hamamelis virginiana* L.; maple-leaf viburnum, *Viburnum acerfolium* L.; and blueberry, *Vaccinium angustifolium* Ait.

Leaves were collected haphazardly from 10 young trees (3–7 m in height) of each species in a mixed stand at Wind Ridge Farm, Union Township, Centre County, Pennsylvania. The leaf tissue was immediately frozen in liquid nitrogen in the field, transported to the lab on Dry Ice, freeze-dried, ground in an air-driven UDY Cyclone Mill (to reduce heating and oxidation) and the lyophilized powder stored at -20°C until use. For each species, leaves from the 10 trees were combined for tannin measures.

Tannin Extraction and Purification. Five grams of lyophilized leaf powder were washed in 100 ml of ether for 30 min to remove pigments and waxes, and then extracted three times in 125 ml 70% acetone at 45°C for 1 hr under sonication. Ascorbate (10 mM) was added to the acetone to prevent oxidation. Acetone was removed by evaporation under reduced pressure, and distilled water was added to the aqueous extracts to a constant volume of 125 ml. This procedure favors extraction of polymeric tannins (Hagerman and Klucher, 1986) and is not exhaustive; substantial amounts of polyphenols may remain covalently bound to cells walls or other cellular components (Appel and Schultz, unpublished data).

Tannins in the polyphenol extracts were separated from nontannin polyphenols by the method of Hagerman and Klucher (1986). A slurry of 50 g of Sephadex LH20 (Pharmacia, Piscataway, New Jersey) and approximately 1 liter of 95%

reagent-grade ethanol was equilibrated overnight and then mixed thoroughly with 125 ml of crude extract. Using a large Buchner funnel and vacuum filtration, nontannin, monomeric polyphenols were eluted from the slurry by washing it with 95% ethanol until the eluant contained no polyphenols. Polyphenols were detected by the ferric chloride assay (Waterman and Mole, 1994), in which a drop of the yellow ferric chloride reagent was mixed with a drop of eluant, immediately resulting in a blue color in the presence of polyphenols. Larger polymeric polyphenols (tannins) were subsequently eluted with 70% acetone until the eluant tested negative with ferric chloride, indicating an absence of polyphenols, irrespective of their composition. Acetone was removed from the filtrate by evaporation under reduced pressure, and the extract was freeze-dried and stored under nitrogen at -10°C. This is a crude purification. While nonphenolic materials are unlikely to be present (Hagerman and Klucher, 1986; Appel and Schultz, unpublished data), the product is merely a more representative sample of extractable polyphenols found in the actual plant than is a commercial standard from some other source. Yields of the off-white tannin powder averaged 5% of the dry weight of the leaf.

Three commercial sources of tannins commonly used by ecologists as standards in the Folin-Denis assay were also purified with LH20 to remove low-molecular-weight contaminants: two different sources of the hydrolyzable tannin mixture called "tannic acid" (Sigma T0125 prepared from Rhus sp., Anacardiaceae, and T8406 from *Caesalpinia spinosa*, Leguminosae tara tannin), and the condensed tannin quebracho (Leon Monnier, Inc., from *Schinopsis* spp., Anacardiaceae).

Polyphenol Assays. Purified tannins of each species were assayed for (1) *total phenolics* by the FA (Swain and Hillis, 1959; Swain and Goldstein, 1964), which measures the ability of phenolics to reduce a mixture of phosphomolybdic and phosphotungstic acids; (2) *condensed tannins* by the butanol HCl assay (Bate-Smith, 1977), which quantifies hydrolyzed proanthocyanidin residues; and (3) *hydrolyzable tannins* by the potassium iodate method modified for quantitative use (Schultz and Baldwin, 1982), which quantifies galloyl esters. Serial dilutions were made of each extract to provide final concentrations of dry weight leaf powder per milliliter of DD water of 2, 8, 14, and 20 mg/ml, and these were assayed in triplicate for each assay. The data are reported as averages, and standard deviations of triplicate measurements of absorbance of single extracts of each sample comprising foliage combined from 10 trees. To determine the impact of the use of commercial standards, we calculated the values obtained for a hypothetical absorbance of 0.200 using the regression equations for commercial standards and for individual species, and then expressed each pair of values as a proportion.

Statistics. Linear regression was used to construct standard curves relating absorbance of the FA reaction mixture to the known concentration (mg/ml) of tannin. The relationships among absorbance of the FA reaction, polyphenol concentration, species, sampling date, and their interactions were examined using

GLM (SAS 1996). Significant differences among species in their absorbance at each concentration were determined by Tukey's studentized range test, which corrects alpha for the number of pairwise comparisons. To determine how compositional variation (i.e., condensed versus hydrolyzable tannin contents) among the tree species might influence FA results, the relationships among absorbance of the FA reaction mixture, condensed tannin levels, hydrolyzable tannin levels, and their interactions were examined in a separate GLM analysis. To evaluate the relative impact of these polyphenol types on variation for each species, we conducted stepwise multiple regression (best model) of FA values with the variables condensed tannins, hydrolyzable tannins, and their sum (SAS Institute, 1996).

RESULTS

All Species. As expected, all relationships between FA absorbance and tannin concentration (all species) were strongly and significantly linear. The R^2 values ranged from 0.970 to 0.999 (all $P = 0.0001$), confirming that the FA can generate standard curves relating absorbance and polyphenol concentrations for a wide range of individual species. However, slopes ranged from 0.009 to 0.0328 (Figure 1) and intercepts from 0.005 to 0.030 among species, making the use of a single standard inappropriate for comparisons among them. GLM analysis revealed strong significant statistical interactions between polyphenol concentration

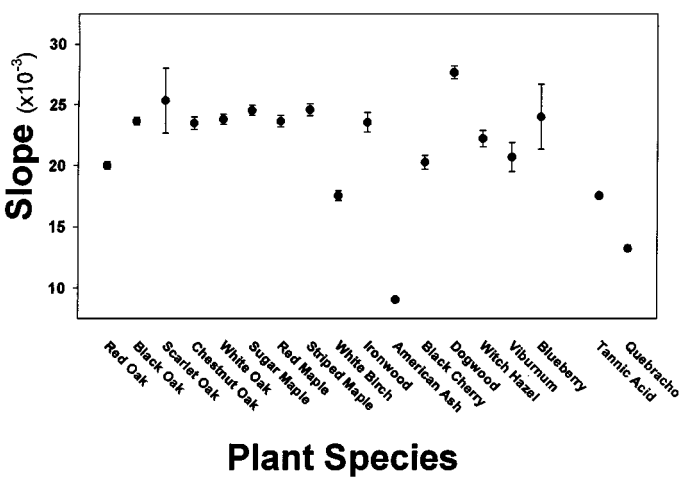


FIG. 1. Slopes and 95% confidence intervals for linear regressions of absorbance as a function of concentration (milligrams purified polyphenol per milliliter) in the Folin-Denis assay of total phenolics in foliage of 14 tree and two shrub species collected on July 14, 1998, and two commercial standards. Tannic acid used was Sigma T0125.

TABLE 1. GLM ANALYSIS OF EFFECTS OF SPECIES ON ESTIMATES OF FOLIN-DENIS REACTIVE PHENOLICS IN 14 FOREST TREES AND 2 SHRUBS AT 4 CONCENTRATIONS OF PURIFIED POLYPHENOLS FROM EACH SPECIES

Source	*df*	Type III SS	*F*	*P*
Concentration = 2 mg/ml				
Species	15	0.00930	249.01	0.0001
Concentration = 8 mg/ml				
Species	15	0.06145	278.01	0.0001
Concentration = 14 mg/ml				
Species	15	0.18192	73.70	0.0001
Concentration = 20 mg/ml				
Species	15	0.32462	1190.52	0.0001

and source (species) at every concentration (Table 1), and specific pairwise comparisons revealed significant sample absorbance differences among most species as a result (Tukey's studentized range test $P < 0.05$). The slopes of American ash and white birch were substantially lower and that of flowering dogwood much higher, than all the other species (Figure 1). No single standard could represent the phenolics in comparisons of all species. Single standards also are not representative in comparisons within genera. Among oaks, no single standard would be appropriate because the slope of red oak is much lower and that of scarlet oak much higher, than the black, chestnut, or white oaks (Figure 1). Note that the slopes do not segregate among subgenera. Slopes were more similar among the three species of maple than among the oaks, but they too produced statistically different absorbances at the $P < 0.05$ level in pairwise comparisons.

Interspecific differences in slope are likely due to different ratios of polyphenol types (Swain and Goldstein, 1964; Feeny, 1968). Both condensed and hydrolyzable tannins contributed significantly to FA results in these assays, with significant interactions between them (Table 2), indicating that the presence of each class of tannin influences the other's impact on FA absorbance. For some species, hydrolyzable tannins were the best predictor of FA values, for others condensed tannins or the sum of hydrolyzable and condensed tannins was the best predictor of FA values (Table 3).

TABLE 2. GLM ANALYSIS OF EFFECTS OF CONDENSED TANNIN LEVELS, HYDROLYZABLE TANNIN LEVELS, AND THEIR INTERACTIONS ON ESTIMATES OF FOLIN-DENIS REACTIVE PHENOLICS IN FOLIAGE OF 14 FOREST TREES AND 2 SHRUBS

Source	*df*	Type III SS	*F*	*P*
Condensed tannins	1	1.17141	164.76	0.0001
Hydrolyzable tannins	1	0.43243	60.82	0.0001
Condensed X hydrolyzable	1	0.16086	22.63	0.0001

TABLE 3. MOST SIGNIFICANT TANNIN CLASSES IN STEPWISE REGRESSION ON FOLIN-DENIS ABSORBANCE VALUES[a]

Source of polyphenol	Variable	R^2	F	P
Red oak (*Quercus rubra*)	HT	0.9983	5881.8	0.0001
Black oak (*Quercus velutina*)	CT + HT	0.9972	3235.4	0.0001
Scarlet oak (*Quercus coccinea*)	HT	0.9990	9760.8	0.0001
Chestnut oak (*Quercus prinus*)	CT + HT	0.9936	1398.8	0.0001
White oak (*Quercus alba*)	HT	0.9979	4678.6	0.0001
Sugar maple (*Acer saccharinum*)	CT	0.9980	2443.4	0.0001
Red maple (*Acer rubrum*)	CT + HT	0.9864	436.0	0.0001
Striped maple (*Acer pensylvanicum*)	CT	0.9916	354.5	0.0003
White birch (*Betula papyrifera*)	CT	0.9982	505.6	0.0001
Ironwood (*Ostrya virginiana*)	CT	0.9913	682.0	0.000
American ash (*Fraxinus americana*)	CT	0.9849	196.2	0.0008
Black cherry (*Prunus serotina*)	CT	0.9942	510.8	0.0002
Flowering dogwood (*Cornus florida*)	CT + HT	0.9988	4241.8	0.0001
Witch hazel (*Hamamelis virginiana*)	CT	0.9812	209.3	0.0001
Viburnum (*Viburnum acerifolium*)	CT	0.9893	555.9	0.0001
Blueberry (*Vaccinium angustifolium*)	CT	0.9873	311.2	0.0001

[a]No other classes contributed more than 2% of the variation in Folin absorbance. CT = condensed tannins, HT = hydrolyzable tannins in foliage of 14 forest tree and 2 shrub species collected in July 1998.

Oaks. Relationships between FA absorbance and tannin concentrations were strongly and significantly linear, as expected, for all oak species on each of three dates (R^2 values ranged from 0.960 to 0.999, all $P = 0.0001$). However, even within the genus *Quercus* and within subgenera, there were differences among the slopes and intercepts of standard curves [slopes ranged from 0.019 to 0.029 (Figure 2), intercepts from 0.004 to 0.021. GLM analysis revealed strong statistical interactions between polyphenol source (species) and date, and between species and date at all but the lowest concentration (Table 4). Specific pairwise comparisons revealed differences among species in their absorbance in the FA (Tukey's studentized range test $P < 0.05$). The relationship (slope) between polyphenol concentration and FA absorbance differed among the oak species, differed within species among dates, and shifted among species from date to date (Figure 2). In four of the five species, May values were highest and July values lowest. In some cases, the *same amount* of purified tannins from May and July leaves produced a twofold difference in FA values. These seasonal and intrageneric differences are as large in magnitude as the interfamilial differences in Figure 1.

Seasonal shifts in FA values within oak species are likely due to changing ratios of polyphenol types (Feeny, 1968; Hatano et al., 1992). Both condensed and hydrolyzable tannins contributed significantly to FA results, with significant

TABLE 4. GLM ANALYSIS OF EFFECTS OF SPECIES, SAMPLING DATE, AND THEIR INTERACTIONS ON LEVELS OF FOLIN-DENIS REACTIVE PHENOLICS FOR FIVE OAK SPECIES AT THREE SAMPLING DATES AND FOUR CONCENTRATIONS OF PURIFIED POLYPHENOLS

Source	*df*	Type III SS	*F*	*P*
Concentration = 2 mg/ml				
Species	4	0.000313	33.18	0.0001
Date	2	0.000070	1.48	0.2444
Species × date	8	0.001334	7.07	0.0001
Concentration = 8 mg/ml				
Species	4	0.014429	99.50	0.0001
Date	2	0.004474	61.71	0.0001
Species × date	8	0.007983	27.53	0.0001
Concentration = 14 mg/ml				
Species	4	0.033520	86.50	0.0001
Date	2	0.009317	48.08	0.0001
Species × date	8	0.022376	28.87	0.0001
Concentration = 20 mg/ml				
Species	4	0.068371	404.81	0.0001
Date	2	0.021463	254.16	0.0001
Species × date	8	0.040830	121.17	0.0001

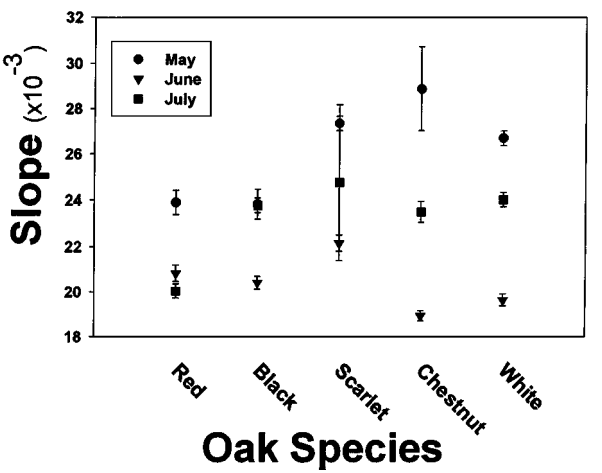


FIG. 2. Slopes and 95% confidence intervals for linear regressions of absorbance as a function of concentration (milligrams purified polyphenol per milliliter) in the Folin-Denis assay of total phenolics in foliage of five oak species collected in May, June, and July 1994.

TABLE 5. GLM ANALYSIS OF EFFECTS OF SAMPLING DATE, CONDENSED TANNIN LEVELS, HYDROLYZABLE TANNIN LEVELS, AND THEIR INTERACTIONS ON LEVELS OF FOLIN-DENIS REACTIVE PHENOLICS FOR FIVE OAK SPECIES

Source	*df*	Type III SS	*F*	*P*
Date	2	0.00968	1.18	0.3093
Condensed tannins	1	0.23741	57.98	0.0001
Hydrolyzable tannins	1	0.10400	25.40	0.0001
Date × condensed tannins	2	0.05333	6.51	0.0019
Date × hydrolyzable tannins	2	0.22242	27.16	0.0001
Condensed X hydrolyzable	1	0.00041	1.01	0.3162

interactions with date (Table 5). Thus, the impact of each class of tannin on FA absorbance changed with date (Table 6).

Commercial Standards. Although relationships between FA absorbance and known concentrations of three commercial standards were strongly and significantly linear, as expected, they too differed dramatically in the slopes and intercepts of their standard curves (Figure 1). Quebracho (a condensed tannin mixture from

TABLE 6. MOST SIGNIFICANT TANNIN CLASSES IN STEPWISE REGRESSION ON FOLIN-DENIS ABSORBANCE VALUES[a]

Source of polyphenol	Variable	R^2	*F*	*P*
Red oak (*Quercus rubra*)				
May	CT	0.9977	2552.44	0.0001
June	CT	0.9987	2266.02	0.0001
July	CT	0.9983	4021.44	0.0001
Black oak (*Quercus velutina*)				
May	CT	0.9811	363.29	0.0001
June	CT + HT	0.9915	817.81	0.0001
July	HT	0.9770	170.03	0.0002
Scarlet oak (*Quercus coccinea*)				
May	CT + HT	0.9972	2124.10	0.0001
June	CT	0.9954	1518.23	0.0001
July	HT	0.9690	218.64	0.0001
Chestnut oak (*Quercus prinus*)				
May	CT	0.9916	236.01	0.0042
June	CT + HT	0.9896	668.57	0.0001
July	CT	0.9746	191.70	0.0001
White oak (*Quercus alba*)				
May	CT	0.9866	444.13	0.0001
June	CT + HT	0.9833	412.06	0.0001
July	CT + HT	0.9930	988.277	0.0001

[a]No other classes contributed more than 2% of the variance in Folin absorbance. CT = condensed tannins, HT = hydrolyzable tannins in foliage of 5 oak species collected in May, June, July 1995.

Schinopsis spp., Anacardiaceae) had a much lower slope than that of all the plant species except American ash (AA) and substantially lower slope than the two hydrolyzable tannin standards. Sigma's T-0125 tannic acid, a mixture of gallotannins and monomers extracted from *Rhus* (Anacardiaceae) (Figure 1), produced slopes and intercepts significantly lower than Sigma's T-8406 product (tara tannin, a mixture of gallotannins from *Caesalpinia spinosa*, Leguminosae). Thus, none of the commonly used commercial standards better approximated the FA slopes and intercepts of the plants examined here, and in most cases were substantially out of range.

To compare results using a commercial standard with those using a standard purified from each plant species (self standard), we calculated the ratio of FA values obtained from the standard curves of the commercial standard and from those of purified tannin from each species, for a hypothetical absorbance of 0.2 (Figure 3). This ratio reflects the degree of over- or underestimation of the tannin content of each species by three commercial standards. Since the slopes of the commercial standards differed, none over- or underestimated the tannin content of all species to the same degree, producing widely varying errors of up to twofold in

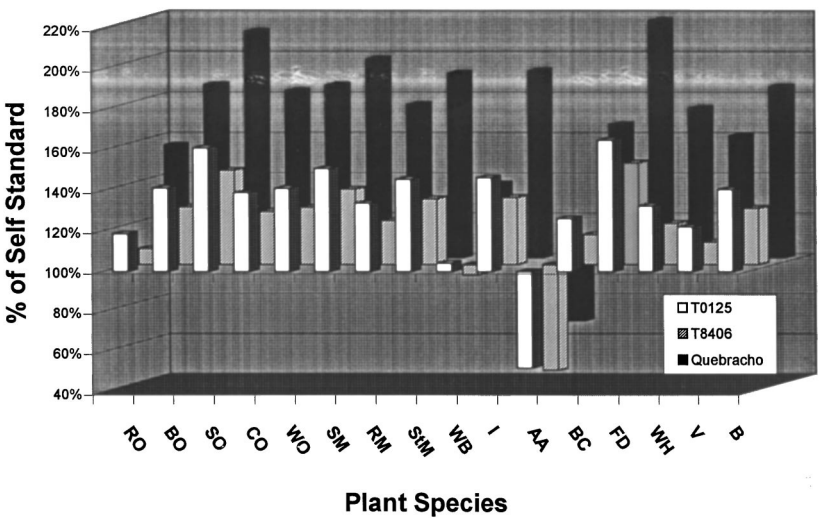


FIG. 3. The effect of choice of standard on Folin-Denis estimates of total phenolics in leaf extracts. Bars represent positive and negative deviations from "true," concentrations (as in Fig. 4) calculated using self standards, obtained by (as in Fig. 4) using three commercial standards. RO = red oak, BO = black oak, SO = scarlet oak, CO = chestnut oak, WO = white oak, SM = sugar maple, RM = red maple, StM = striped maple, WB = white birch, I = Ironwood, AA = American ash, BC = black cherry, FA = flowering dogwood, WH = witch hazel, V = viburnum, B = blueberry.

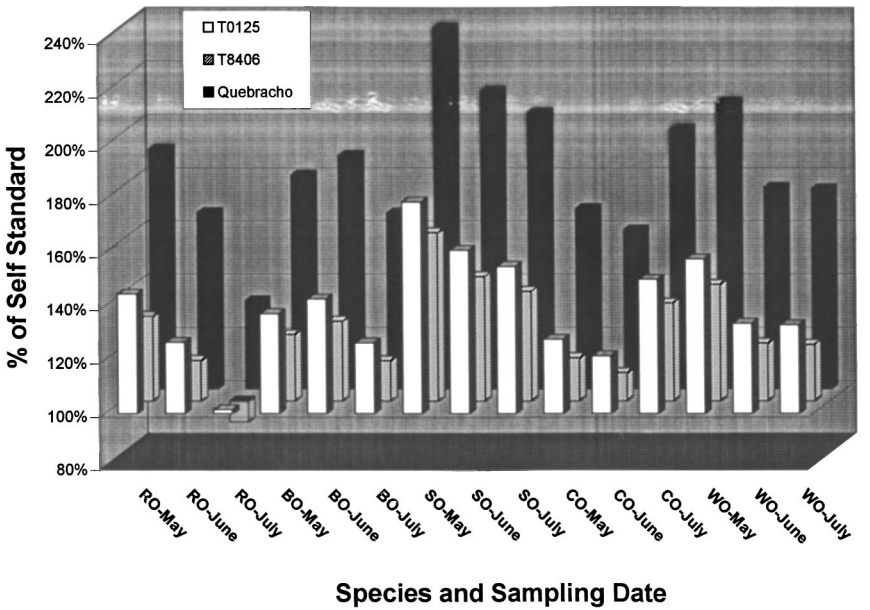


FIG. 4. The effect of choice of standard and seasonal variation on Folin-Denis estimates of total phenolics in leaf extracts. Bars represent positive and negative deviations from "true" concentrations, calculated using self standards, obtained by using three commercial standards. RO = red oak, BO = black oak, SO = scarlet oak, CO = chestnut oak, WO = white oak.

the estimates of polyphenol concentrations (Figure 3). All three standards consistently overestimated tannin content in all but ironwood (I), and to a lesser extent white birch (WB), the one species that had a lower slope than the commercial standards. In a few cases, commercial standards came close to approximating tannin levels in some species. Both tannic acid sources estimated WB within 5%, and the tara tannic acid (Sigma T-8406) came within 10% for red oak (RO) and viburnum (V). Even seasonal variation in polyphenol composition is sufficient to invalidate the use of commercial standards to compare FA values within a species across sampling dates (Figure 4). In five oak species, the same amount of polyphenols produced significantly different FA values at different times of the season (Table 4), and none of the commercial standards reflected this variation (Figure 4).

DISCUSSION

Our study examined the utility of the Folin-Denis assay (FA) to compare the levels of polymeric polyphenols (tannins) in leaves of 16 woody plant species at a

single sampling date and of five closely related tree species at three sampling dates within one season. We found that there was dramatic variation among species in the reactivity of the *same amount* of purified tannins in the FA assay, even among related species, and even among sampling dates of the same species. This variation was associated with differences in the relative amounts of hydrolyzable and condensed tannins in the tannin mixtures and probably also reflected variation in a potentially large number of other phenolics.

Our study was a conservative test of the limitations of the FA because it was designed to minimize structural differences among tannin sources. First, we compared primarily tannins, and not total phenolics, which reduced the potentially great variation among species in the amounts and structures of simple, nontannin phenolics. For oaks, this approach restricted analyses to less than one third of the phenolic species likely to be present (Knopps and Jensen, 1980; Appel and Schultz, unpublished data). Second, we included a comparison of five related species of oaks, known to have structurally related and less seasonally variable tannin chemistries than some other tree species [e.g., *Liquidambar* (Hatano et al., 1992; Appel and Schultz, unpublished data)]. As is true for other classes of secondary metabolites, the more distantly related two plant species are, the greater the potential difference between them in phenolic composition; Folin-type assays should be less reliable as these differences grow greater. However, our conservative estimates indicate that even congeneric comparisons with FA using a single standard produce errors likely to be biologically significant, and intraspecific comparisons through time may require separate standards. This would also be true for other redox-based assays like the Prussian blue (Price and Butler, 1977), and to a lesser extent, for more structurally specific assays, such as the butanol HCl proanthocyanidin assay for condensed tannins (Hagerman and Butler, 1989) and the potassium iodate method (Schultz and Baldwin, 1982) for hydrolyzable tannins.

These observations are not new. Over 30 years ago, Swain and Goldstein (1964) described 10-fold variation in FA estimates of phenolic content among 20 different equimolar samples of purified phenolics. Feeny's (1968, 1969) widely cited papers on the tannin chemistry and ecology of English oak indicated clearly that phenolic composition changes seasonally within a single plant. Caveats about Folin methods have appeared sporadically since then (Martin and Martin, 1982; Mole and Waterman, 1987; Wisdom et al., 1987; Hagerman and Butler, 1989; Waterman and Mole, 1994), but with little evident effect: every ecological paper we could find in two major journals during 1994–1999 in which phenolics were "quantified" used Folin methods without appropriate standards. In the same search, we could not find any phenolic quantification (including condensed tannins, hydrolyzable tannins, etc.) in which appropriate standards were used.

The complete phenolic composition of most plants is unknown, and the relationships among phenolic concentration, phenolic composition, reducing power, and FA absorbance are unknown and unpredictable for most species. Hence, one

can say very little *a priori* about the likely relationship between composition or concentration and FA absorbance within or between species. Failing to do so can lead to serious over- or underestimates of total concentrations or amounts of phenolic fractions, as shown in this study.

Although the relationships between actual purified phenolic concentrations and FA absorbance for all but three species we examined had slopes between 0.019 and 0.026, these differences are biologically significant. For example, constitutive and induced levels of tannins in oaks differ to a similar degree and are related to herbivore performance (Schultz and Baldwin, 1982; Rossiter et al., 1988; Hunter and Schultz, 1993). Levels of tannins in flowering dogwood vary in similar magnitudes as a function of microsite and are related to levels of herbivory (Dudt and Shure, 1994). These biologically significant differences in tannin content would go undetected if inappropriate standards were used.

The magnitude of seasonal differences estimated—or mistaken—with the FA are also biologically significant (Schultz et al., 1982; Schultz, 1983) but would go undetected with inappropriate standards. Temporal differences in FA reactivity of phenolic extracts from the same species and tissues clearly make the use of a single standard across the season inappropriate, even though the standard may be from the same trees at an earlier or later date.

The widespread use of commercial standards only exacerbates the problem. Our results indicate that the use of commercial standards often fails to yield results similar to those obtained with self standards comprising phenolics extracted and purified from the material under study. Using these commercial standards can over- or underestimate presumed actual concentrations by as much as twofold in the species examined and may not even retain the rankings among species or between dates. These deviations from reality presumably arise from structural differences between the standards and the material actually being assayed in the plant extract, yielding different impacts on the FA reagent. For example, different plant species produce condensed tannins containing different mixtures of anthocyanidins and catechins, hydrolyzable tannins containing different mixtures of gallotannins and ellagitannins, and hybrid molecules containing gallotannins and flavonoids (gallocatechins). As a result, standards containing only a single structure or structural group (e.g., tannic acid or quebracho) may be irrelevant or misleading.

Commercially available standards with the same name ("tannic acid") can produced significantly different results. This is not surprising, given that some are extracted from plants in different families. We have also found measurable variation among batches of the same product, as have others (Hagerman and Butler, 1989), but purification of standards reduces much of this batch-to-batch variation (Appel and Schultz, unpublished data).

Purifying self standards from the same leaf material to be used in the FA assay resolves this problem because it provides a standard curve that allows one to interpret sample absorbances in the same currency in which they exist in the

leaf. Rather than using a commercial standard that may over- or underestimate the reducing power of one's extract, self standards quantify exactly what is present in the leaf. If one wishes to compare results with previously collected data lacking a self standard, one can simply generate a second standard curve with the previous standard.

Purifying self standards is neither complex nor expensive and would greatly improve the ecological inferences arising from any study of plant phenolics. Ideally, one would develop separate self standards for each sample that might have a different phenolic composition, e.g., different tissues, tissues of different ages, plants of different ages, samples from different dates, and possibly from different treatments if treatments are expected to change composition. This is clearly not feasible for large numbers of different sources of samples or for limited amounts of samples. However, one can create standards that are a mixture of sample material, reflecting the diversity of phenolics present within a population, treatment, etc., to which individual samples are compared. Valid comparative results can be obtained using mixtures of tissues from potentially differing sources as long as attention is given to the proportions of different tissue sources used. Modern microtiter plate readers and the ability to store standard curves facilitate this approach. The FA is still in widespread use by ecologists to quantify the relative and absolute amounts of phenolics produced in response to environmental variables or correlated with biological activity, but failure to use self standards can result in under- or overestimates of up to twofold, depending on the species compared and the commercial standard selected (see Figure 3), even for intraspecific comparisons (see Figure 4). Many influential papers on plant herbivore interactions, as well as some of our own, have based conclusions, in part, on variation within the range we find is generated by using inappropriate standards (Coley, 1983; Rossiter et al., 1988; Denslow et al., 1990; Shure and Wilson, 1993; Dudt and Shure, 1994; Shure et al., 1998). We find that differences in such studies probably need to exceed 100% to be biologically meaningful and account for error introduced by the use of inappropropriate standards for the FA reagent.

Biological activity can be estimated reliably by the Folin-type redox assay when it is known to depend directly or indirectly on phenolic oxidation. This is the case in many ecological settings (Appel, 1993). In our own studies (e.g., Schultz, 1983; Rossiter et al., 1988; Hunter and Schultz, 1993; Appel and Schultz, 1994) and some others (e.g., Dearing, 1997), FA values have been consistent correlates of the biological activity of plant phenolics for this reason.

It is clear that the Folin assays cannot be used to determine absolute amounts of phenolics to test hypotheses about plant investment in phenolics (e.g., Coley et al., 1985; Herms and Mattson, 1992; Shure and Wilson, 1993; Dudt and Shure, 1994; Shure et al., 1998). As we have demonstrated, there is no absolute relationship between FA values and the amount of phenolics in a plant extract. Interspecific comparisons, differences in tissue age or condition, and the use of a single commercial

standard make estimates of amounts impossible to obtain with this reagent. The only truly reliable and accurate way to estimate amounts of "total phenolics" or phenolic classes is to extract, purify, and weigh them, but this is not feasible for large numbers of small samples. Estimates may be obtained with the FA and self standards, but their approximation of reality should be determined by extracting and weighing a subset of samples. Even extensive extraction is unlikely to recover bound polyphenols and provide a true measure of 'total phenolic' mass.

We suggest that Folin reagents can be useful in ecological studies as indicators of redox-based biological activity and as indicators of relative amounts of phenolics if appropriate standards are used, but never as indicators of absolute concentration or mass and never for interspecific or temporal comparisons using a common standard. We recommend that investigators considering the use of Folin-type assays do the following: (1) Decide whether the goal is to quantify the *amount* or *activity* of phenolics. (2) If measuring amount is the goal, then the most accurate approach is to purify and weigh total phenolics, although extraction is unlikely to be complete. Colorimetric assays can be made semiquantitative by using purified self standards. (3) If measuring *activity* is the goal, use purified plant phenolics for standards and measure the samples' redox potential with the FA. (4) Always make separate colorimetric standards for each plant species. (5) Within species, design standards or standard mixtures to reflect the range of variation encountered within the desired unit of comparison, i.e., include subsamples of the sources in appropriate proportions. (6) When reporting results, be specific about assay limitations.

Acknowledgments—Grants USDA-NEPIAP 91-00392, USDA NCRIG 91-00926 (HMA and JCS) and NSF DEB 9318073 (JCS) supported this research. Helpful comments on earlier drafts were made by Brian Rehill, Tom Arnold, German Avila, and anonymous reviewers.

REFERENCES

APPEL, H. M. 1993. Phenolics in ecological interactions: The importance of oxidation. *J. Chem. Ecol.* 19:1521–1552.

APPEL, H. M., and SCHULTZ, J. C. 1994. Oak tannins reduce effectiveness of thuricide (*Bacillus thuringiensis*) in the gypsy moth (Lepidoptera: Lymantriidae). *J. Econ. Entomol.* 87:1736–1742.

BATE-SMITH, E. C. 1977. Astringent tannins of *Acer* species. *Phytochemistry* 16:1421–1426.

COLEY, P. D. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 53:209–233.

COLEY, P. D., BRYANT, J. P. and CHAPIN, F. S. III. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

DEARING, D. 1997. The manipulation of plant toxins by a food-hoarding herbivore, *Ochotona princeps*. *Ecology* 78:774–781.

DENSLOW, J. S., SCHULTZ, J. C., VITOUSEK, P. M., and STRAIN, B. R. 1990. Growth responses of tropical shrubs to treefall gap environments. *Ecology* 71:165–179.

DUDT, J. F., and SHURE, D. J. 1994. The influence of light and nutrients on foliar phenolics and insect herbivory. *Ecology* 75:86–98.

FEENY, P. P. 1968. Seasonal changes in the oak leaf. Tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–581.

FEENY, P. P. 1969. Inhibitory effect of oak leaf tannins on the hydrolysis of proteins by trypsin. *Phytochemistry* 8:2119–2126.

FEENY, P. P. 1976. Plant apparency and chemical defense, pp. 1–40, *in* J. W. Wallace and R. L. Mansell (eds.). *Biochemical Interactions Between Plants and Insects*. Plenum Press, New York.

FOLIN, O., and DENIS, W. 1915. A colorimetric method for the determination of phenols (and phenol derivatives) in urine. *J. Biol. Chem.* 22:305–308.

HAGERMAN, A. E., and BUTLER, L. G. 1989. Choosing appropriate methods and standards for assaying tannins. *J. Chem. Ecol.* 15:1795–1810.

HAGERMAN, A. E., and KLUCHER, K. M. 1986. Tannin-protein interactions, pp. 67–76., *in* V. Cody, E. Middleton, and J. Harborne (eds.). Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure Activity Relationships. Alan R. Liss, New York.

HATANO, T., OKONOGI, A. and OKUDA, T. 1992. Oligomeric hydrolyzable tannins from *Liquidambar formosana* and spectral analysis of the orientation of valoneoyl groups in their molecules, pp. 195–207, *in* R. W. Hemingway and P. E. Laks (eds.). Plant Polyphenols. Plenum Press, New York.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: To grow or defend. *Q. Rev. Biol.* 67:283–335.

HUNTER, M. D., and SCHULTZ, J. C. 1993. Induced plant defenses breached? Phytochemical induction protects an herbivore from disease. *Oecologia* 94:195–203.

KARBAN, R., and BALDWIN, I. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago.

KNOPPS, J. F., and JENSEN, R. J. 1980. Morphological and phenolic variation in a three-species community of red oaks. *Bull. Torrees Bot. Club* 107:418–428.

MARTIN, M. M., and MARTIN, J. L. 1982. Tannin assays in ecological studies: Lack of correlation between phenolics, proanthocyanidins and protein-precipitating constituents in mature foliage of six oak species. *Oecologia* 54:205–211.

MOLE, S., and WATERMAN, P. G. 1987. A critical analysis of techniques for measuring tannins in ecological studies.. I. Techniques for chemically defining tannins. *Oecologia* 72:137–147.

PRICE, M. P., and BUTLER, L. G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of *Sorghum* grain. *J. Agric. Food Chem.* 25:1268–1273.

RHOADES, D. F. 1983. Herbivore population dynamics and plant chemistry, pp. 155–200, *in* R. F. Denno and M. S. McClure, (eds.). *Herbivore Population Dynamics and Plant Chemistry*. Academic Press, Orlando, Florida.

RHOADES, D. F., and CATES, R. G. 1976. Toward a general theory of plant antiherbivore chemistry. *Recent Adv. Phytochem.* 10:168–174.

ROSSITER, M. C., SCHULTZ, J. C., and BALDWIN, I. T. 1988. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. *Ecology* 69:267–277.

SAS Institute. 1996. SAS/STAT User's Guide: Statistics, Version 6, 4th ed. SAS Institute, Cary, North Carolina.

SCALBERT, A., and HASLAM, E. 1987. Polyphenols and chemical defence of the leaves of *Quercus robur*. *Phytochem* 26:3191–3195.

SCHULTZ, J. C. 1983. Habitat selection and foraging tactics of caterpillars in heterogeneous trees, pp. 61–90, *in*: R. F. Denno and M. S. McClure (eds.). Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York.

SCHULTZ, J. C., 1988. Plant responses induced by herbivores. *Trends Ecol. Evol.* 3:45–49.

SCHULTZ, J. C., and BALDWIN, I. T. 1982. Oak leaf quality declines in response to defoliation by gypsy moth larvae. *Science* 217:149–151.

SCHULTZ, J. C., NOTHNAGLE, P. J., and B*aldwin*, I. T. 1982. Individual and seasonal variation in leaf quality of two northern hardwoods tree species. *Am. J. Bot.* 69:753–659.

SHURE, D. J. and WILSON, L. A. 1993. Patch-size effects on plant phenolics in successional opening of the southern Appalachians. *Ecology* 74:55–67.

SHURE, D. J., MOORESIDE, P. D., and OGLE, S. M. 1998. Rainfall effects on plant-herbivore processes in an upland oak forest. *Ecology* 79:604–617.

SWAIN, T., and HILLIS, W. E. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sc. Food Agric.* 10:63–68.

SWAIN, T., and GOLDSTEIN, J. L. 1964. *in* J. B. Pridham (ed.). The quantitative analysis of phenolic compounds, pp. 131–145, *Methods in Polyphenol Chemistry*. Pergamon Press, Oxford, England.

VAN ALSTYNE, K. L. 1995. Comparison of three methods for quantifying brown algal polyphenolic compounds. *J. Chem. Ecol.* 21:45–58.

WATERMAN, P. G., and MOLE, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, London.

WISDOM, C., GONZALEZ-COLOMA, S. A., and RUNDEL, P. W. 1987. Ecological tannin assays. Evaluation of proanthocyanidins, protein binding assays and protein precipitating potential. *Oecologia* 72:395–401.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

TESTING THE EFFECTS OF DRYING METHODS ON WILLOW FLAVONOIDS, TANNINS, AND SALICYLATES

RIITTA JULKUNEN-TIITTO* and SINIKKA SORSA

University of Joensuu
Department of Biology
Box 111 FIN-80101 Joensuu, Finland

(Received August 31, 2000; accepted December 14, 2000)

Abstract—In this study, we compared the effects of several preservation methods on the secondary phenolics of the mature leaves of purple willow (*Salix purpurea* L., Salicaceae) with results obtained with fresh leaf analyses. Conventional freeze-drying, in which the leaves were first frozen with liquid nitrogen and then placed in a freeze-dryer, produced substantial qualitative and quantitative changes in purple willow flavonoids and salicylates. Modified freeze-drying, in which leaves were put into a freeze-dryer without being prefrozen, gave concentrations that, for most secondary components, were comparable with those found in fresh leaves. Reducing the freeze-dryer chamber temperature hindered the decomposition of phenolics in prefrozen leaves and in leaves dried without prefreezing. Heat drying induced substantial changes in the composition of all phenolics, except for apigenin-7-glucoside. Vacuum drying at room temperature gave the highest concentrations for nearly all phenolics, while room-drying with desiccation gave results that were comparable with those obtained by fresh leaf analyses.

Key Words—Drying tests, phytochemicals, flavonoids, tannins, salicylates, leaves, willow, *Salix*.

INTRODUCTION

One problem in phytochemical research is the difficulty of obtaining valid results due to the high chemical and biochemical lability of many of the phytochemicals found in plants. In order to obtain accurate results for concentrations of secondary products, it is important to use a suitable method of prehandling the fresh plant material. This is especially critical in ecophysiological and ecobiochemical research, where a small group of components or even a single component may determine

*To whom correspondence should be addressed. e-mail: rjt@joensuu.fi

0098-0331/01/0400-0779$19.50/0

the primary biological or chemical response of plant tissues to biotic factors such as herbivores and pathogens and abiotic factors such as nutrition and UV radiation (e.g., Tahvanainen et al., 1985; Hakulinen et al., 1995; Julkunen-Tiitto, 1996; Juntheikki et al., 1996; Lavola et al., 1998). Phytochemical preservation is also essential in herbal drug production and chemotaxonomical research (e.g., Julkunen-Tiitto, 1989; Julkunen-Tiitto and Meier, 1992).

Salicaceous plants produce a wide variety of lower molecular mass phenolics, mainly glycosylated flavonoids and salicylates, together with high concentrations of condensed tannins. During the last 30 years these have been studied intensively by European and American researchers investigating defense functions, ontogeny, phenology, chemotaxonomy, herbal medication, etc. (e.g., Thieme, 1965; Pearl and Darling, 1970; Egloff, 1982; Bryant et al., 1987; Meier et al., 1988; Julkunen-Tiitto, 1989; Shao, 1991). The occurrence of these bioactive components is fairly species-specific and plant-part specific, and their relative proportions in specific tissues may change substantially as the plant matures (Bryant and Julkunen-Tiitto, 1995).

Most of the flavonoids are considered to be fairly stable under different prehandling and extraction conditions (e.g., Markham, 1982; Keinänen and Julkunen-Tiitto, 1996), while several higher molecular mass salicylates, such as salicortin and tremulacin, are known to be labile and prone to decomposition during sample preservation and extraction (e.g., Thieme, 1965; Meier et al., 1988; Julkunen-Tiitto, 1989). Neutral and alkaline conditions, certain organic solvents, and being soaked in water readily result in lower molecular mass salicylates (e.g., Julkunen-Tiitto, 1985; Julkunen-Tiitto and Meier, 1992). It has been reported that conventional freeze-drying induces deleterious changes in the leaves of *Populus* and *Salix species* (Lindroth and Pajutee, 1987; Julkunen-Tiitto and Tahvanainen, 1989; Orians, 1995). Vacuum-drying (Orians, 1995) and conventional freeze-drying (prefreezing with liquid nitrogen) at low temperature (Lindroth and Koss, 1996) are previously recommended methods of preserving *Populus tremuloides*, *Salix sericea*, and *S. eriocephala* leaves. Unfortunately, vacuum-drying has been found to alter the carbohydrate content in the leaves of *Populus tremuloides*, with the result that the decrease in starch content is reflected by an increase in percent leaf dry mass for glucose (Lindroth and Koss, 1996). Oven-drying mature willow leaves at low temperature (48°C) gives practically no qualitative or quantitative differences in salicylates compared those found in fresh leaves (Julkunen-Tiitto and Tahvanainen, 1989). As expected, tissue destruction before oven-drying induces deleterious changes in willow salicylates (see Lindroth and Pajutee, 1987)

In this study, the appropriateness of different plant preservation methods was studied in order to find a faster and safer method of preserving the most labile phytochemicals. The mature leaves of purple willow (*Salix purpurea* L.) were used because purple willow contains the two most labile salicylates, tremulacin and

TABLE 1. TREATMENTS USED FOR PURPLE WILLOW (*S. purpurea*) PHENOLICS

Test	Method	Drying chamber temperature (°C)	Time (hr)
I	Immediate fresh analyses (test control)		
II	Room drying, without desiccation	+22	12
III	Room drying, desiccation with silicagel	+22	12
IV	Room drying, desiccation with molecular sieve	+22	12
V	Vacuum drying with a freeze-dryer, cooler off	+22	16
VI	Freeze-drying, prefreezing with liquid nitrogen	+18	16
VII	Freeze-drying, without prefreezing	+18	16
VIII	Freeze-drying, prefreezing with liquid nitrogen	−30	72
IX	Freeze-drying, without prefreezing	−30	72
X	Air drying (heat)	+60	12
XI	Air drying (heat)	+90	12

salicortin, together with a small amount of salicin and tremuloidin. It also contains partners of three different flavonoid groups: flavanones, flavones, and flavan-3-ol, the lability of which has not been thoroughly studied previously.

METHODS AND MATERIALS

Leaf Material. Leaves were collected from an ornamental purple willow (*S. purpurea* L.) clone growing on the campus of the University of Joensuu, Finland. Fully expanded, mature leaves were detached from their shoots, put into a plastic bag, and placed in a Styrox container, where they were kept at $<4^{\circ}$C. The leaves were taken to the laboratory within 5 min and randomly separated into batches of 20 for the prehandling tests. We tested 10 different drying methods (Table 1). Immediate fresh analyses were carried out as a control treatment. In method II the leaves were dried on the laboratory table. In methods III and IV, which used drying media, the fresh leaves (2.9 g) were processed in desiccators each containing 300 g of drying medium (20 leaves, one leaf fresh wt $= 0.143 \pm 0.011$). Well-ventilated ovens were used for air drying in methods X and XI.

Chemical Analyses. Eight mature leaves from each preparation method were analyzed separately for willow flavonoids, condensed tannins, and salicylates. In order to ensure complete recovery of the soluble compounds, care was taken not to overload the extraction solvent. Three to four milligrams of leaf tissues, cut so as to avoid main veins, were extracted three times with 400 μl of the solvent. Methanol was used for flavonoids and salicylates. This extraction procedure gave more than 98% recovery for the components. Soluble condensed tannins were extracted with 70% acetone (as above) and quantified by oxidative depolymerization

TABLE 2. CONDITIONS USED IN HPLC GRADIENT ELUTION FOR *S. purpurea* PHENOLICS[a]

Time (min)	Solvent A (%)	Solvent B (%)
Initial	100	0
5	100	0
10	80	20
20	70	30
30	65	35
40	50	50
45	50	50
Rinsing	0	100
Equilibration	100	0

[a]Solvent A = aqueous 1.5% tetrahydrofuran + 0.25% orthophosphoric acid in DAD and acetic acid in MS; solvent B = 100% methanol.

to anthocyanidins in acid butanol, as described by Porter et al. (1986). The extraction residue was hydrolyzed with acid butanol for insoluble tannins. The sum of soluble and insoluble condensed tannins was quantified using Sephadex LH20 purified tannin from *S. purpurea* leaves (see Hagerman, 1995). The residual water was measured by drying the leaves for 12 hr at 105°C. The results are expressed as milligrams per gram dry weight at 105°C. Flavonoids and salicylates were quantified by HPLC-DAD as reported by Julkunen-Tiitto et al. (1996). HPLC-MS was used to identify the components. The gradient used is described in Table 2. HPLC/API-ES (positive ions) conditions were as follows: the column was Hypersil Rp C-18, 2 mm ID, 10 cm long; the ES fragmentor voltage was 80–120, depending on the components; the flow rate was 0.4 ml/min.

The quantification of components was determined as follows: salicin, salicyl alcohol, tremulacin, eriodictyol-7-, naringenin-7-, luteolin-7-, apigenin-7-glucoside, and luteolin were based on commercial standards; salicortin and tremuloidin were based on purified standards obtained from Prof. Beat Meier, ETH, Zurich, Switzerland. The derivatives of eriodictyol, naringenin, and luteolin were based on corresponding flavonoid-7-glucoside. HPLC/API-ES produced the following molecular weights: eriodictyol-7 glucoside, 473 (M + 23); eriodictyol-glycoside 2, 922 (M + 23, tentatively identified as dieriodictyol-monoglucoside); naringenin-7-glucoside, 457 (M + 23); luteolin-7-glucoside, 471 (M + 23); luteolin-glycoside 2, 557 (M + 23, tentatively identified as diacetylderivative of luteolin-monoglucoside); luteolin-glycoside 3, 499 (M + 23, tentatively identified as methylderivative of luteolin-glucuronide); apigenin-7-glucoside, 455 (M + 23); (+)-catechin, 291 (M + 1); salicin, 309 (M + 23); salicortin, 447 (M + 23); tremuloidin, 413 (M + 23); and tremulacin, 551 (M + 23). In addition, two unknown salicylates (871, M + 23) were detected in very tiny amounts (results not shown). These were tentatively identified as disalicortins.

RESULTS AND DISCUSSION

In this study mature, fully expanded purple willow (*S. purpurea*) leaves were used to test the effect of 10 preservation methods on flavonoids, condensed tannins, and salicylates. During heat-drying the leaves turned brown or blackish, indicating kinone formation and advanced decomposition of the phenolics. After all the other drying methods the leaves were still green or, after freeze-drying with prefreezing, slightly pale green. The residual water content of the dried material varied between methods. In methods III and IV it was $4.80 \pm 0.11\%$ and $3.02 \pm 0.08\%$, respectively, while in methods II and V–IX the mean residual water was $6.65 \pm 0.31\%$.

We found four flavanones (naringenin-7-glucoside, eriodictyol-7-glucoside, and two unknown eriodictyol-glycosides), six flavones (apigenin-7-glucoside, luteolin-7 glucoside, luteolin-glycosides 1-3 and luteolin), two flavan-3-ols [(+)-catechin and condensed tannins], and four salicylates (salicortin, tremulacin, salicin, and tremuloidin) and compared the concentrations with those found in the fresh leaf extracts. We found that the concentrations of individual phytochemicals were significantly affected by the prehandling method (Figures 1–3). Aglycons identified as decomposition products of the corresponding glycosides included luteolin, naringenin, salicyl alcohol, and 6-OH-oxocyclohexenone, depending on the preservation method.

The most abundant flavonoids in purple willow leaves are flavanones, eriodictyol-7-glucoside (more than 3% dry wt), and naringenin 7-glucoside (about 0.4% dry wt) (Figure 1) (Meier, 1988; Shao, 1991). Both flavanones (except for minor eriodictyol-glycoside 2) were significantly reduced by conventional freeze-drying (method VI). In this method, the prefrozen samples were put into an external drying chamber normally used for drying solid material. The temperature inside the chamber was 18°C. For some flavanones, room-drying without desiccation (method II) and freeze-drying at a low temperature (method VIII) also gave concentrations that were lower, although not significantly so. Air drying at an elevated temperature (method X and XI) was deleterious to leaf flavanones: there was a greater than 50% reduction in the concentration of naringenin-7-glucoside and a greater than 60% reduction in that of eriodictyol glycosides. All the other methods tested gave concentrations of flavanones that were similar to those found in the fresh leaf analyses.

The responses of flavones, luteolin glycosides, and apigenin-7-glucoside to conventional freeze-drying (method VI) were similar to those of flavanones (Figure 2). The amounts of luteolin-7-glucoside and apigenin-7-glucoside were reduced by more than 30% compared with those found in the fresh leaf analyses. The concentrations of apigenin-7-glucoside, luteolin-7-glucoside, and two luteolin-derivatives were also reduced by method VII (freeze-dying without prefreezing). Room drying (method II) also caused a moderate reduction in the amounts of luteolin-7-glucoside and its two derivatives (1 and 2). Desiccation at

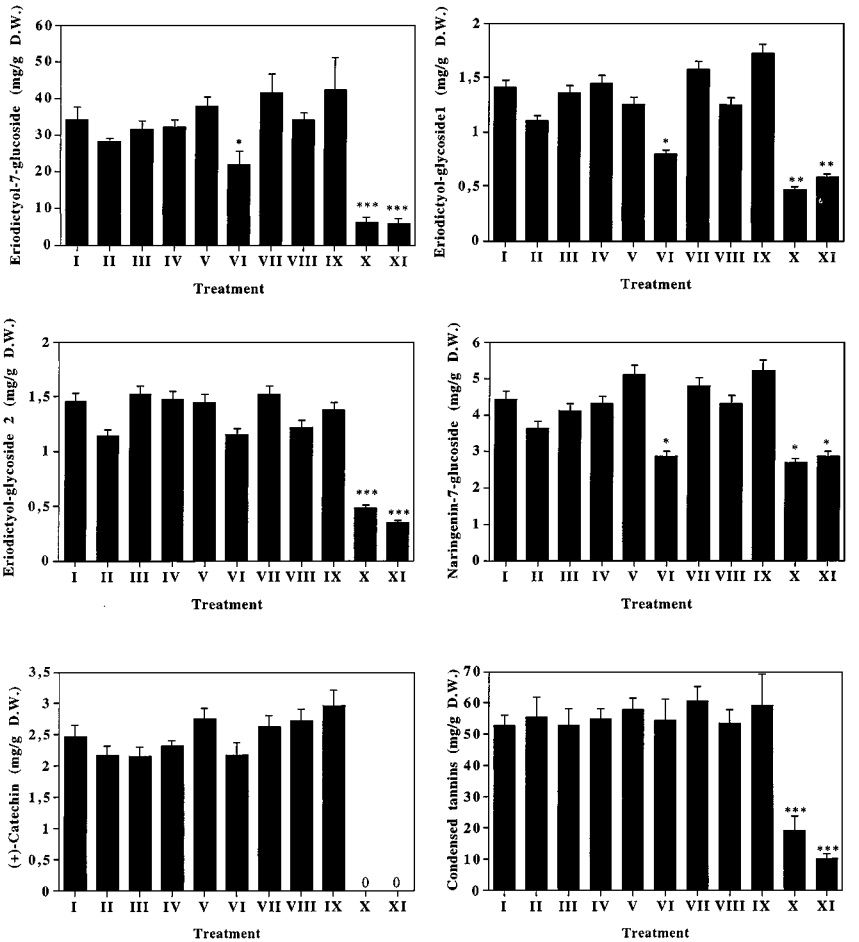


FIG. 1. The effects of prehandling methods on flavanones and flavan-3-ols in mature leaves of purple willow (*S. purpurea*). The results are expressed as means ± SE from eight separate leaves. Multiple comparisions were done using the Dunnett *t* test, with method I as the control ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

room temperature (methods III and IV) enhanced the yield of apigenin-7-glucoside as compared to the control level (method I). Heat drying at 60°C and 90°C (methods X and XI) was too high for most luteolins, but apigenin-7-glucoside remained fairly stable. Our results show that the flavanone glucosides and flavone glucosides of purple willow leaves are very sensitive to preservation methods compared with flavonol glycosides, which are the most abundant flavonoids in birch (*Betula pendula* Roth) leaves. The concentrations of quercetin and myricetin glycosides

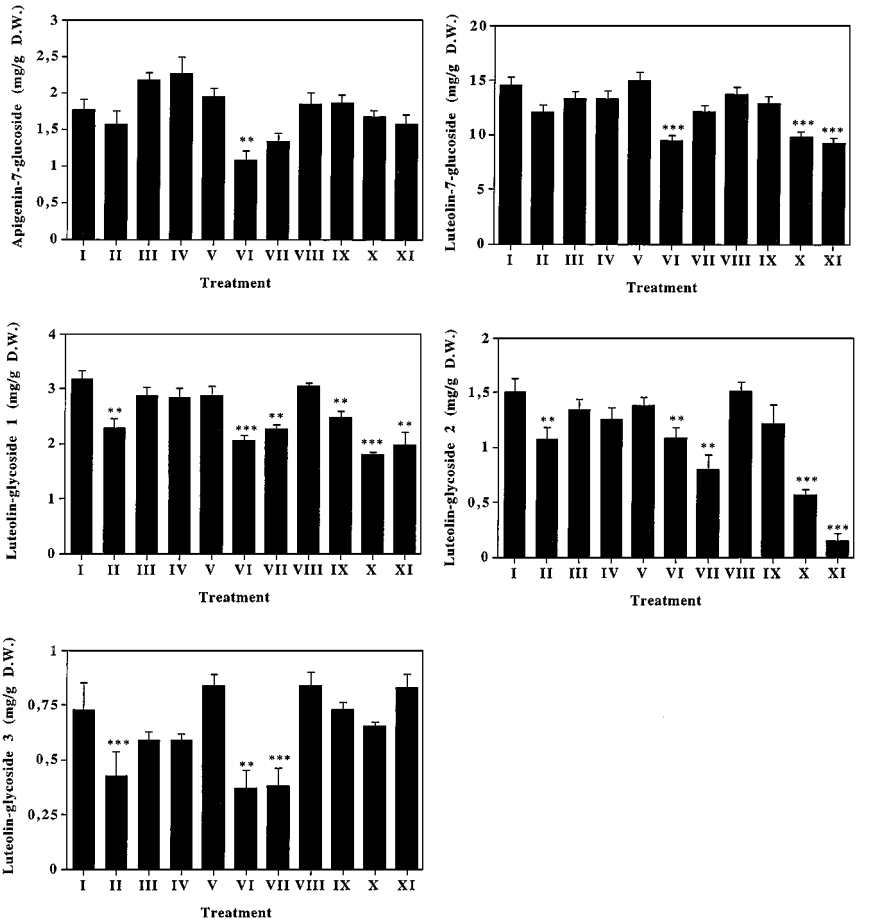


FIG. 2. The effects of prehandling methods on flavones in mature leaves of purple willow (*S. purpurea*). The results are expressed as means ± SE from eight separate leaves. Multiple comparisions were done using the Dunnett *t* test with method I as the control (** $P < 0.01$; *** $P < 0.001$).

were significantly higher in leaves dried at 80°C than in leaves dried at 40°C or 20°C (Keinänen and Julkunen-Tiitto, 1996). Conventional freeze-drying (prefreezing at −18°C) yielded higher concentrations of those flavonols compared with those found when the leaves were dried without prefreezing.

The third flavonoid group in purple willow is flavan-3-ols, comprising (+)-catechin (0.25% dry wt) and condensed tannins (about 5–6% dry wt) (Figure 1). The concentrations of (+)-catechin were similar for all methods except for both

heat-drying methods (X and XI). Heat drying induced strong decomposition, tight binding with other components, or both for (+)-catechin, leaving no traces at all of (+)-catechin after treatment. This result is very different from those obtained from mature birch (*Betula pendula* Roth) leaves in our laboratory, where a <20% decrease in (+)-catechin was found after drying at 80°C (Keinänen and Julkunen-Tiitto, 1996). Except for the heat drying methods (methods X and XI), none of the methods used resulted in a significant reduction of condensed tannins (oligomeric and polymeric flavan-3-ols). Hagerman (1988) recommends freeze-drying for condensed tannins, while Orians (1995) and Lindroth and Koss (1996) prefer vacuum drying. Increasing the drying temperature to 60°C (method X) lowered the condensed tannin amount by >60%, and using 90°C for drying (method XI) gave only 21% of the tannin amount compared with that found in the fresh analyses. The effect of heat drying on purple willow condensed tannins was similar to that found in *Salix* cv. *aquatica* and *S. phylicifolia* leaves (Julkunen-Tiitto, 1985). On the other hand, heat drying had a less severe effect on the condensed tannins found in *Salix eriocephala, S. sericea*, and their hybrid compared with freeze-dried leaves by Orians (1995).

The highest concentrations of the most abundant salicylates, salicortin and tremulacin, were obtained from vacuum-dried leaves (method V) (Figure 3). This difference in secondary products may be due to changes in other metabolites, such as volatiles induced by vacuum drying. Lindroth and Koss (1996) have reported that, compared with freeze-drying, vacuum drying using *Populus tremuloides* leaves increased hexoses and reduced the starch level by 38%. The salicylate results with vacuum drying are consistent with those obtained from *Salix sericea* (Orians, 1995) and *Populus tremuloides* leaves (Lindroth and Koss, 1996).

We found that conventional freeze-drying in an external drying chamber (material kept frozen, method VI) induced completely artifactual concentrations of the main salicylates (Figure 3) (Julkunen Tiitto and Tahvanainen, 1989). The high amounts of decomposition products, such as salicyl alcohol (10,07 $\pm$ 1.16 mg/g dry wt) and 6-OH cyclohexenone (not quantified), as well as significantly increased salicin and tremuloidin confirm the unsuitability of method VI. Freeze-drying is too slow and, although most of them may be frozen, some thin leaves may thaw sufficiently during water sublimation to induce interconversion among the compounds by chemical or even biochemical decomposition. Freeze-drying, without prefreezing, in an external drying chamber (method VII) proved to be a far better method, but it still reduced the concentrations of salicortin and tremulacin compared with those found in the fresh analyses. This indicates that not only thawing, as suggested by Lindroth and Koss (1996), but also prefreezing matters in the freeze-drying process. Method VIII, which is comparable to VI except that chamber temperature in the freeze-dryer during sublimation was -30°C, led to a small, but nonsignificant loss of the most abundant salicylates. In contrast, method IX (comparable to method VII), which used the same freeze-drying temperature but

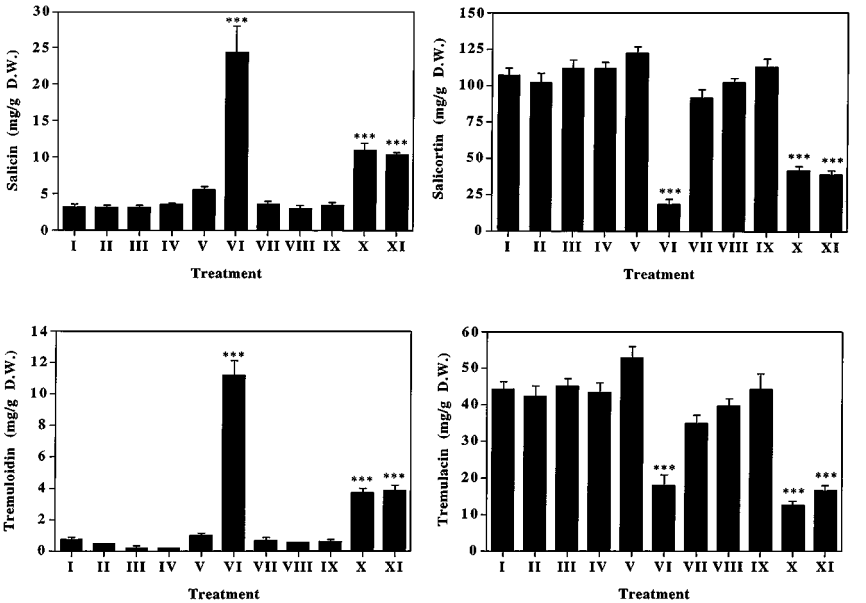


FIG. 3. The effects of prehandling methods on salicylates in mature leaves of purple willow (*S. purpurea*). The results are expressed as means ± SE from eight separate leaves. Multiple comparisions were done using the Dunnett *t* test with method I as the control (*** $P < 0.001$).

did not prefreeze the leaves, gave amounts that were quite similar to those found in the fresh leaf analyses. Thus, if one wants to use a freeze-dryer to preserve salicylate material, prefreezing with liquid nitrogen or at −20°C should be avoided or the temperature in a drying chamber must be low (less than −20°C).

Air drying at room temperature (method II) (Figure 3) and at 45°C (Julkunen-Tiitto and Tahvanainen, 1989) and the use of drying media (silica gel in method III and molecular sieves in method IV) proved to be safe and suitable drying techniques for labile *S. purpurea* leaf salicylates. The results show levels for salicortin and tremulacin similar to those found in the fresh analyses. The effect of using silica gel and molecular sieves is to speed up the drying process compared with the air drying at room temperature. They are also cheap and easy to use in the field, so that samples can be treated on the way to the laboratory. Increasing the drying temperature to 60°C induced very significant losses of salicortin and tremulacin, increased the levels of salicin and tremuloidin, and possibly caused them to decompose further. Interestingly, similar air drying at 60°C reduced the concentrations of salicortin and 2′-*O*-cinnamoylsalicortin in *S. sericea* leaves only slightly (Orians, 1995).

Salicortin, which contains a carboxyl ester bond, decomposed much more easily under the drying conditions than tremulacin, which also contains a benzoyl

ester bond. Freeze-drying method VI reduced the amount of salicortin by more than 83%, while that of tremulacin was reduced by about 60%. Thus, it may be that the benzoyl ester stabilizes the compound under these drying conditions while the carboxyl esters are very labile. Decomposition of salicortin and tremulacin produce salicin and tremuloidin, respectively, as their first decomposition products. Tremuloidin further decomposes to salicin. In this study, salicin and tremuloidin were detected in low amounts in fresh leaves. So far, we do not know if salicin is a precursor of salicortin and tremulacin, but earlier phenological and ontogenetic screening of willow leaves in our laboratory indicates that salicin is a real component and not simply induced by decomposition of higher molecular mass salicylates. This is consistent with the results of Meier (1988) and Shao (1991), who have reported 0.1–1.6% of salicin in Central European *S. purpurea* leaves, and Thieme and Benecke (1970), who detected 1–2% of salicin in *P. tremuloides* leaves. Our results disagree with the findings of Orians (1995) and Lindroth and Koss (1996), who did not detect salicin in *S. sericea* and *P. tremuloides* leaves.

In our study we used fully expanded, mature, undamaged leaves that had a moderately low water content ($59.22 \pm 0.34\%$). We found that fresh analyses should be made whenever possible. However, if analytical procedures necessitate sample preservation, then desiccation at room temperature and freeze-drying at low temperature, preferably without prefreezing, are the best methods for mature willow leaves.

Acknowledgments—This research was supported by the Academy of Finland (grant 45591). We thank also Mr. Kenneth Meany for helping to improve the English language.

REFERENCES

BRYANT, J. P., CLAUSEN, T. P., REICHARDT, P. B., MCCARTHY, M. C., and WERNER, R. A. 1987. Effect of nitrogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix (Choristoneura conflictana (Walker). *Oecologia (Berlin)* 73:513–517.

BRYANT, J., and JULKUNEN-TIITTO, R. 1995. Ontogenetic development of chemical defense by seedling resin birch: Energy cost of defense production. *J. Chem. Ecol.* 21:883–896.

EGLOFF, C. 1982. Phenolglycoside einheimischer Salix-Arten. Ph D thesis. Zurich.

HAGERMAN, A. 1988. Extraction of tannin from fresh and preserved leaves. *J. Chem. Ecol.* 14:453–461.

HAGERMAN, A. 1995. Tannin analysis. Department of Chemistry, Miami University, Oxford, Ohio.

HAKULINEN, J., JULKUNEN-TIITTO, R., and TAHVANAINEN, J. 1995. Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism? *Trees* 9:235–240.

JULKUNEN-TIITTO, R. 1985. Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics. *J. Agric. Food. Chem.* 33:213–217.

JULKUNEN-TIITTO, R. 1989. Distribution of certain phenolics in *Salix* species (Salicaceae). University of Joensuu, Publications in Sciences, No. 15.

JULKUNEN-TIITTO, R., and MEIER, B. 1992. Enzymatic decomposition of salicin and its derivatives obtained from Salicaceae species. *J. Nat. Prod.* 55:1204–1212.

JULKUNEN-TIITTO, R., and TAHVANAINEN, J. 1989. The effect of the sample preparation method on extractable phenolics of Salicaceae species. *Planta Med.* 55:55–58.

JULKUNEN-TIITTO, R., ROUSI, M., BRYANT, J., SORSA, S., SIKANEN, H., and KEINÄNEN, M. 1996. Chemical diversity of Betulaceae species: Comparison of phenolics and terpenoids in Northern birch species. *Trees* 11:16–22.

JUNTHEIKKI, M. -R., JULKUNEN-TIITTO, R., and HAGERMAN, A. 1996. Salivary tannin-binding proteins in root vole (*Microtus oeconomus*). *Biochem. Syst. Ecol.* 24:25–35.

KEINÄNEN, M., and JULKUNEN-TIITTO, R. 1996. Effect of sample preparation method on birch (*Betula pendula* Roth) leaf phenolics. *J. Agric. Food Chem.* 44:2724–2727.

LAVOLA, A., JULKUNEN-TIITTO, R., APHALO, P., DE LA ROSA, T., and LEHTO, T. 1998. The effect of UV-B radiation on UV-absorbing secondary metabolites in birch seedlings grown under simulated forest soil. *New Phytol.* 137:617–621.

LINDROTH, R. L., and PAJUTEE, M. S. 1987. Chemical analysis of phenolic glycosides: art, fact, and artifacts. *Oecologia* 74:144–148.

LINDROTH, R. L., and KOSS, P. A. 1996. Preservation of Salicaceae leaves for phytochemical analyses: Further assessment. *J. Chem Ecol.* 22:765–771.

MARKHAM, K. R. 1982. Techniques of Flavonoid Identification. Academic Press, London, pp. 15–16.

MEIER, B. 1988. Analytik, chromatographisches Verhalten und potentielle Wirksamkeit der Inhaltsstoffe salicylathaltiger Arzneipflanzen Mitteleuropas. Habilitationschrift, ETH, Zürich.

MEIER, B., JULKUNEN-TIITTO, R., TAHVANAINEN, J., and STICHER, O., 1988. Comparative high-performance liquid and gas–liquid chromatographic determination of phenolic glucosides in Salicaceae species. *J. Chromatogr.* 442:175–186.

ORIANS, C. M. 1995. Preserving leaves for tannin and phenolic glycoside analyses: A comparison of methods using three willow taxa. *J. Chem. Ecol.* 21:1235–1243.

PEARL, I. A., and DARLING, S. F. 1970. Phenolic extractives of *Salix purpurea* bark. *Phytochemistry* 9:1277–1281.

PORTER, L. J., HRSTICH, L. N., and CHAN, B. G. 1986. The conversion of proanthocyanidins and prodelphinidins to cyanidins ans delphinidins. *Phytochemistry* 25:223–230.

SHAO, Y. 1991. Phytochemischer Atlas der Schweizer Weiden. Dissertation ETH. Nro. 9532. Zurich.

TAHVANAINEN, J., JULKUNEN-TIITTO, R., and KETTUNEN, J. 1985. Phenolic glycosides govern the food selection pattern of willow feeding leaf beetles. *Oecologia* 67:52–56.

THIEME, H. 1965. Die Phenolglycoside der Salicaceen. *Pharmazie* 20:570–574.

THIEME, H., and BENECKE, R. 1970. Die Phenolglycoside der Salicaceen. 7. Mitteilung: Über die glycosidführungen einheimischer bzw. in Mitteleuropa kultivierten *Populus*-Arten. *Pharmazie* 25:780–788.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

(*E*4,*Z*9)-TETRADECADIENAL, A SEX PHEROMONE FOR THREE NORTH AMERICAN MOTH SPECIES IN THE GENUS *Saturnia*

J. STEVEN MCELFRESH,[1] JOCELYN G. MILLAR,[1,]* and DANIEL RUBINOFF[2]

[1]*Department of Entomology*
University of California
Riverside California 92521

[2]*Division of Insect Biology*
Department of Environmental Science, Policy & Management
University of California, Berkeley California 94720

(Received July 10, 2000; accepted December 14, 2000)

Abstract—The lepidopteran genus *Saturnia* has three representatives in North America, *S. walterorum*, *S. mendocino*, and *S. albofasciata*. (*E*4,*Z*9)-Tetradecadienal (*E*4,*Z*9–14 : Ald) was identified as a sex pheromone component for all three species by combinations of coupled gas chromatography–electroantennogram detection (GC-EAD), GC–mass spectrometry (MS), and field trials. In field trials, all three species were strongly attracted to (*E*4,*Z*9–14 : Ald) as a single component. Small amounts of (*Z*)-9-tetradecenal (*Z*9–14 : Ald) also were found in extracts of all three species, but blends of this compound with *E*4,*Z*9–14 : Ald were no more attractive to male moths than *E*4,*Z*9–14 : Ald alone. Extracts of pheromone glands of female *S. walterorum* occasionally contained a third, trace compound eliciting responses from male antennae in GC-EAD experiments, but this compound was not identified. It is suggested that the three species can use the same, single component as a sex attractant because the flight period of *S. albofasciata* (fall) is different than that of the other two species (spring), whereas the geographic distributions of *S. mendocino* and *S. walterorum* overlap over only small portions of their ranges. Furthermore, the latter two species readily hybridize, so there may be minimal fitness cost to cross-attraction.

Key Words—(4*E*,9*Z*)-tetradecadienal, (*Z*)-9-tetradecenal, *Saturnia* spp., pheromone.

*To whom correspondence should be addressed. e-mail: jocelyn.millar@ucr.edu

0098-0331/01/0400-0791$19.50/0

INTRODUCTION

The genus *Saturnia* (Lepidoptera: Saturniidae) is represented by three species in the New World, *Saturnia mendocino* Behrens, *S. walterorum* Hogue and Johnson, and *S. albofasciata* (Johnson), all of whose distributions and biologies have been described by Tuskes et al. (1996). All three species are univoltine. *S. mendocino* and *S. walterorum* are sister species that are found in both montane and coastal chaparral plant communities, whereas *S. albofasciata* is restricted to montane chaparral. *S. mendocino* ranges from central California to Oregon and is found in both the coastal and Sierra Nevada mountain ranges, whereas *S. walterorum* is found from southern California to northern Baja California, Mexico. The ranges of the latter two species overlap in San Luis Obispo and Monterey counties of California. The range of *S. albofasciata* spans that of the other two species, and it is found at higher elevations from northern California to northern Baja California, Mexico. Adults of *S. mendocino* and *S. walterorum* emerge from February through May and are diurnally active. In contrast, *S. albofasciata* flies from mid-October to mid-November, with males being active in the late afternoon until dusk, and females ovipositing just after sunset. Eggs of all three species are laid singly or in small clusters on the larval hosts, which include manzanita (*Arctostaphylos* spp. Adanson) and madrone (*Arbutus menziesii* Pursh) for *S. mendocino*, manzanita (*Arctostaphylos* spp.) and laurel sumac (*Malosma laurina* Nuttall) for *S. walterorum*, and several *Ceanothus* spp. L. and *Cercocarpos betuloides* Nuttall for *S. albofasciata* (Tuskes et al., 1996). Eggs of *S. mendocino* and *S. walterorum* hatch in approximately three weeks, whereas those of *S. albofasciata* overwinter and hatch the following spring. Larvae of all three species develop through the spring, spin silk cocoons on or near the host, and subsequently emerge in the fall of that year for *S. albofasciata*, or in the spring of the following year for the other two species.

In conjunction with an ongoing study of sex attractant pheromones of saturniid moth species in the western United States, we undertook a study of the sex pheromone chemistry of these three *Saturnia* spp. We describe here the identification and field testing of (*E*4,*Z*9)-tetradecadienal (*E*4,*Z*9–14 : Ald) as a female-produced sex pheromone component for all three species.

METHODS AND MATERIALS

Insects

Pupae of *S. mendocino* were obtained from a culture maintained by one of the authors (D.R.) at the Division of Insect Biology, University of California, Berkeley, California. This culture had been outcrossed frequently by mating

laboratory-reared females with wild males collected from several northern California counties, so as to maintain the vigor of the laboratory colony. Specimens of *S. walterorum* were obtained from C. Conlan and were from cultures that originated in San Diego County, California. Larvae of both species were reared on cut bouquets of natural hosts in water or in nylon sleeves directly upon living plants. Cocoons were kept outdoors in screen cages (approx. 30 cm square) in the shade until adult emergence. Freshly emerged adults were separated by sex and were either used immediately for preparation of pheromone gland extracts (females) or for electroantennograms (males' antennae), or were held at 4–10°C in glassine envelopes for up to one week. Storing at cool temperatures was necessary because the nonfeeding adult moths typically live less than one week.

A few pupae of *S. albofasciata* were obtained from C. Conlan, and these produced a single female and several males. Further males used in GC-EAD studies were collected from field sites, using *E*4,*Z*9–14 : Ald lures to attract them (see below).

Extraction of Sex Pheromone Glands

Sex pheromone glands were removed from 1- to 4-day-old calling virgin females. The gland was extruded by applying gentle pressure to the abdomen to evert the ovipositor, and the terminal abdominal segments were excised into a 0.25-ml conical glass vial insert containing ~30 μl pentane. Care was taken to avoid contamination of the samples with the dark-colored material that was abundant in the abdomen, which may be used to glue eggs to the host plant. The glands were extracted for 2–20 min, and the extract was transferred to a clean insert, with two rinses with 1 drop of pentane. Extracts were concentrated by passive evaporation as needed prior to analysis. If not used immediately, extracts were stored at −20°C. Approximately 20 extracts of female *S. walterorum* were obtained, whereas fewer than 10 *S. mendocino* female extracts and only a single *S. albofasciata* extract were available.

Gas Chromatography

GC analyses were conducted with Hewlett-Packard 5890A or 5890 Series II gas chromatographs (H-P = Agilent, Palo Alto, California), with helium carrier gas, in splitless mode. Columns and programs used to analyze female extracts of *S. walterorum* included DB-225 (J&W Scientific, Folsom, California; 30 m × 0.26 mm ID, 0.25-μm film, 100°C/0 min, 15°C/min to 130°C/0 min, 3°C/min to 175°C/0 min, then 20°C/min to 220°C for 60 min), DB-WAX (J&W Scientific; 30 m × 0.32 mm ID, 0.25-μm film, 100°C for 1 min, 5°C/min to 240°C for 10 or more min), DB-5 (J&W Scientific; 30 m × 0.25 mm ID, 0.25-μm film, 100°C for

1 min, 5°C/min to 275°C for 60 min). *S. mendocino* extracts were analyzed on the DB-225 column as described above. Extracts also were analyzed by coupled GC-EAD (see below). Tentative identifications were made by comparison of retention times of compounds in extracts to those of synthetic standards.

Coupled Gas Chromatography–Electroantennography (GC-EAD)

Antennae from male *S. mendocino* ($N = 3$) were stimulated with standards of the *E* and *Z* isomers of each of the monounsaturated tetraenals with the double bond in positions 3–12. One-microliter aliquots containing approx. 50 ng of a given standard in hexane were injected in random order onto a DB-5 column (30 m × 0.32 mm, 0.25-μm film) in split mode with a 20 : 1 split ratio, run isothermally at 200°C. Antennal response peak heights were measured to the nearest 0.5 mm, then analyzed using two-way analysis of variance of the log transformed data followed by a Student-Neumann-Keuls means separation test. Because these standards were the products of microscale oxidations and partial purifications, their concentrations varied. Concentrations of individual samples were adjusted to keep the amount of each compound injected within a twofold range, but no further compensation was made for variability in amounts injected because antennal responses were relatively insensitive to variations in the quantity injected. For example, halving the amount of *Z*9–14 : Ald injected resulted in only a 27% reduction in antennal response.

Aliquots of pheromone gland extracts from females and synthetic standards were analyzed by splitless coupled GC-EAD, using an H-P 5890 series II GC equipped with a DB-5 column (30 m × 0.32 mm ID, 0.25-μm film, 100°C/1 min, 5°C/min to 150°C/5 or 10 min, then 25°C/min to 275°C/30 min for *S. walterorum* and *S. mendocino*, and 100°C/1 min, then 10°C/min to 275°C/25 min for *S. albofasciata*), using an injector temperature of 250°C. The column effluent was split equally with a press-fit Y-connector (J&W Scientific) into two branches of 0.25-mm-ID, uncoated fused silica tubing, with one branch going to the GC flame ionization detector (FID), and the other being directed through a heated transfer line (275°C) to a 15-mm-diameter glass stimulus delivery tube. The capillary effluent was diluted with humidified air (400 ml/min) and passed over the male moth antennal preparation (see below). The moth antenna was suspended between glass capillary electrodes filled with modified Locke's saline (7.5 g NaCl, 0.21 $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ in 1 liter distilled water) (Humason, 1972) or Kaissling's saline (Kaissling and Thorson, 1980), with electrical contact to the custom-built amplifier made with AgCl-coated silver wires. The amplifier and FID outputs were simultaneously recorded on a matched pair of H-P 3394 recording integrators.

Male antennae were removed using fine forceps to firmly hold the scape and pull the antenna free of the head, without crushing the scape. The terminal rami and tip of the antenna were removed so that the end of the antenna could be placed into

the saline-filled recording electrode. The antenna was positioned on the amplifier mount at the end of the delivery tube such that the plane of the antennal branches was perpendicular to the air flow from the stimulus delivery tube. A single antenna preparation could be used for several hours with periodic additions of saline.

Field Trials

Pherocon 1C sticky traps (Trécé Inc., Salinas, California) were used in all trials, with 11-mm red rubber septum lures (Wheaton Scientific, Millville, New Jersey), loaded with heptane solutions (100 μl) of test compounds, plus 2 drops of 10 mg/ml butylated hydroxytoluene (BHT) in heptane as an antioxidant. Lures were kept in 20-ml glass vials in a freezer or an ice-chest when not in use. Traps were placed in areas where host plants were common. Traps were spaced approximately 8 m apart and blocks were 600 m or more apart. Traps were suspended from shrubs at heights of 0.5–1.5 m above ground. Trapped moths were counted at irregular intervals of 3–11 days for *S. mendocino* and *S. walterorum*, with traps left in the field for nearly one month in total.

S. mendocino field trials were conducted in the western Sierra Nevada mountains near Hams Station, Amador County, California in April 1997 using 10 blocks of treatments and controls. A second *S. mendocino* trial, comparing a blend of *E*4,*Z*9–14 : Ald and *Z*9–14 : Ald (100 : 10 μg) to 100 μg *E*4,*Z*9–14 : Ald alone, was conducted in Del Puerto Canyon, Stanislaus County, California in March 1998. *S. walterorum* field trials were conducted in late March to April 1997 with five blocks of traps placed in the vicinity of Viejas grade near Descanso, and the Laguna Mountains along Highway S1 between Monument peak and Kwaaymii Point, San Diego County, California. *S. albofasciata* field trials were conducted in November 1998 near Thomas Mountain, Riverside County, California. A single block of traps was used, consisting of three traps baited with 100 μg *E*4,*Z*9–14 : Ald and three traps baited with blank controls. Traps were left in place for one day, after which time the treatment trap bottoms were completely covered with male moths and so further testing was discontinued.

For the purposes of statistical analysis, a replicate consisted of a single block, and counts for a particular treatment in a given block were pooled to eliminate day effects. Trap catches were analyzed using two way analysis of variance of the square root $(x + 0.5)$ transformed counts.

Synthesis of Authentic Standards

Standards of tetradecenals with the double bonds at carbons 3–12 were prepared by oxidation of samples of the corresponding alcohols, obtained from the Research Institute for Plant Protection, Wageningen, The Netherlands, with excess pyridinium dichromate in methylene chloride for 2 hr (Corey and Suggs, 1979).

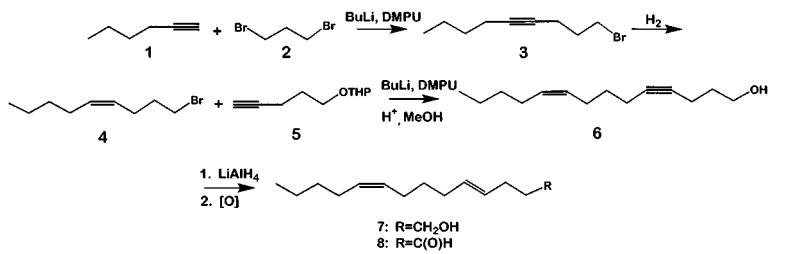
The reaction mixtures were diluted with 10 volumes of pentane, then vortexed for 30 sec. The precipitate was allowed to settle, and the supernatant solutions were filtered through 2.5-cm plugs of Celite. The resulting solutions were used immediately for electroantennogram studies as described above.

4,9-Tetradecadienals

Proton NMR spectra were taken on a General Electric QE-300 instrument (300 MHz), as $CDCl_3$ solutions. Electron impact mass spectra (70 eV) were taken with a Hewlett-Packard 5970B mass selective detector interfaced to a 5890 gas chromatograph fitted with a DB-5MS column (20 m × 0.2 mm ID, J&W Scientific). Mass spectra are reported as *m*/*z* (abundance). All reactions were carried out in oven-dried glassware. THF was purified by distillation from sodium benzophenone ketyl under nitrogen. Unless otherwise specified, worked up reaction mixtures were dried with anhydrous Na_2SO_4 and concentrated by rotary evaporation under reduced pressure. Flash chromatography (Still et al., 1978) and vacuum liquid chromatography (Coll and Bowden, 1986) were carried out with 230 to 400-mesh silica gel (Aldrich Chemical Co.).

Synthesis of (E4,Z9)-Tetradecadienal (Scheme 1)

*1-Bromonon-4-yne **(3)***. Butyllithium (42.5 ml of 2.5 M solution in hexanes, 110 mmol) was added dropwise over ∼20 min to an icebath-cooled solution of 1-hexyne **1** (8.2 g, 100 mmol) in THF (100 ml) under Ar. The mixture was stirred 20 min, then warmed to room temperature, and 1,3-dibromopropane **2** (12.2 ml, 120 mmol) was added dropwise. The mixture was warmed to 55°C for 24 hr, then cooled and quenched with water (250 ml). The mixture was extracted with hexane, and the hexane extracts were washed with brine, dried, and concentrated, yielding a mixture of unreacted starting material, the desired bromide **3**, and the alkadiyne from coupling at both ends of 1,3-dibromopropane (16 : 66 : 12 by GC). The residue was slowly Kugelrohr distilled, yielding 20.11 g of a purified monobromide fraction, oven temperature 55–60°C (0.4 mm Hg). NMR: δ 3.54



SCHEME 1

(t, 2H, $J = 6.9$ Hz), 2.34 (tt, 2H, $J = 6.7$, 2.1 Hz), 2.15 (tt, 2H, $J = 6.7$, 2.3 Hz), 2.02 (quint, 2H, $J = 6.6$ Hz), 1.52–1.35 (m, 4H), 0.91 (t, 3H, $J = 6.9$ Hz). MS: *m*/*z* 202/204 (1/1, M^+), 174/176 (8/7), 132/134 (31/30), 123 (5), 107 (8), 95 (67), 81 (99), 79 (58), 77 (49), 67 (78), 53 (69), 41 (100).

*(*Z*)-1-Bromo-4-nonene **(4)***. Cyclohexene (8.3 ml, 82 mmol) was added dropwise to BH_3-dimethylsulfide complex (4.0 ml, 40 mmol) in 50 ml of THF at 0°C under Ar. The mixture was warmed to room temperature for 2 hr, then cooled to 0°C again, followed by dropwise addition of 4.06 g (20 mmol) of bromoalkyne **3** to the white slurry. The mixture was warmed to room temperature, stirred 2 hr, then cooled in an icebath while glacial acetic acid (16 ml) was added. The mixture was stirred overnight, then cooled in an icebath during sequential slow addition of 5 M NaOH (60 ml) and 30% H_2O_2 (16 ml; Caution: Exothermic). The resulting mixture was diluted with water, extracted twice with hexane, and the hexane extracts were washed with brine, dried, and concentrated. The more volatile fraction of the residue (∼4 g) was removed by careful Kugelrohr distillation, oven temperature 30–35°, ∼0.4 mm Hg. The remaining liquid (∼6 g) was purified by vacuum flash chromatography in a 150-ml sintered glass funnel, eluting with hexane. The purified bromoalkene was Kugelrohr distilled (oven temperature ∼50°C, 0.4 mm Hg), yielding 2.22 g of **4** as a colorless oil. The yield was not optimized, and the volatile fraction removed before chromatography contained approx. 25% of the bromoalkene. NMR: δ 5.49–5.62 (m, 2H), 3.42 (t, 2H, $J = 6.5$ Hz), 2.20 (br. quart, 2H, $J = 7.3$ Hz), 2.1–2.0 (m, 2H), 1.92 (quint, 2H, $J = 6.9$ Hz), 1.4–1.28 (m, 4H). MS: *m*/*z* 204/206 (4/4, M+), 162/164 (4/4), 148/150 (9/9), 135 (4), 109 (4), 95 (9), 83 (20), 81 (21), 69 (46), 55 (78), 41 (100).

*(*Z*)-9-Tetradecen-4-yn-1-ol **(6)***. Butyllithium (6.4 ml of a 2.5 M solution in hexanes; 16 mmol) was added to a solution of THP-protected pentynol **5** (2.54 g, 15 mmol) in THF (25 ml) at −10°C, under Ar. The solution was stirred 20 min, followed by sequential addition of 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU, 8 ml) and bromoalkene **4** (2.05 g, 10 mmol). The reaction was warmed to room temperature and stirred overnight, then poured into water and extracted with hexane. The hexane extract was washed with dilute HCl, water, and brine; dried; and concentrated. The residue was taken up in 25 ml MeOH, and *p*-toluenesulfonic acid was added until the solution became acidic to moistened litmus paper. The mixture was stirred at room temperature until the transacetalization reaction was complete (∼2 hr), the reaction was quenched by addition of 1 g $NaHCO_3$, and the solvent was removed on a rotary evaporator. The residue was partitioned between hexane and water, and the hexane extract was washed with brine, dried, and pumped under vacuum (0.1 mm Hg) to remove the THP methyl ether byproduct. The crude enynol **6** (96% pure by GC) was used without further purification. NMR: δ 5.46–5.28 (m, 2H), 3.77 (t, 2H, $J = 6.1$ Hz), 2.29 (tt, 2H, $J = 6.8$, 2.3 Hz), 2.19–1.95 (m, 6H), 1.75 (quint, 2H, $J = 6.4$ Hz), 1.54 (quint, 2H, $J = 7.3$ Hz), 1.39–1.25 (m, 4H), 0.91 (t, 3H, $J = 6.9$ Hz). MS: *m*/*z* 164 (2), 149

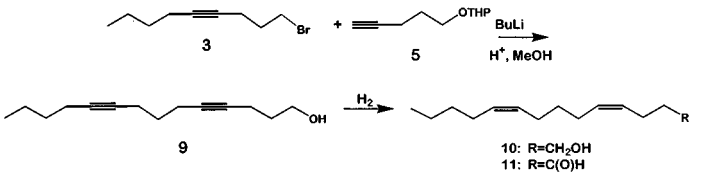
(8), 137 (16), 121 (17), 119 (18), 108 (44), 107 (21), 105 (22), 93 (49), 91 (50), 79 (61), 67 (59), 55 (65), 41 (100).

*(*E*4,*Z*9) -Tetradecadienol **(7)***. $LiAlH_4$ (2 g, 53 mmol) was added in portions to an ice-cooled mixture of dry diglyme (20 ml) and THF (2.5 ml) under Ar. When the vigourous foaming had subsided, 2 g of the crude enynol **6** (∼9.6 mmol) in 5 ml diglyme was added to the thick slurry. When the foaming had subsided, the mixture was heated to 110°C overnight. The next morning, the mixture was cooled to 0°C, then hexane (150 ml) was added, followed sequentially by cautious dropwise addition of 2.1 ml water (Foams!), 1.6 ml 20% NaOH, and 7.5 ml water. The mixture was stirred for 15 min to allow the precipitate to granulate, then the hexane layer was decanted, and the residue was rinsed several times with aliquots of hexane. The combined hexane solutions were washed several times with water and once with brine, then dried and concentrated. The residue was purifed by flash chromatography (2.5 × 25 cm), eluting with a stepwise gradient of 15–25% EtOAc in hexane. The purifed product was Kugelrohr distilled (bp ∼80°C at 0.1 mm Hg), yielding 1.1 g of (*E*4,*Z*9)-tetradecadienol **7**. NMR: δ 5.48–5.41 (m, 2H), 5.41–5.32 (m, 2H), 3.66 (t, 2H, $J = 6.5$ Hz), 2.12–1.94 (m, 8H), 1.64 (quint, 2H, $J = 6.9$ Hz), 1.40 (quint, 2H, $J = 7.5$ Hz), 1.36–1.25 (m, 4H), 0.91 (t, 3H, $J = 6.9$ Hz). MS: *m*/*z* 210 (trace, M^+), 138 (5), 135 (4), 121 (6), 110 (9), 95 (22), 82 (25), 81 (50), 79 (59), 67 (61), 55 (62), 41 (100).

*(*E*4,*Z*9)-Tetradecadienal **(8)***. Dienol **7** (0.42 g, 2 mmol) was added to a slurry of pyridinium dichromate (2.26 g, 6 mmol) and powdered 3Å molecular sieve (2 g) in CH_2Cl_2 (12 ml) (Herscovici et al., 1982). The mixture was stirred vigourously for 30 min, then diluted with 50 ml hexane. After stirring for 5 min, the mixture was filtered, decolorized with ∼2 cc decolorizing charcoal, filtered again, and concentrated. A few milliliters of toluene were added to the residue, which was concentrated again to azeotrope out traces of pyridine. The clear liquid was then Kugelrohr distilled (oven temperature 80–105°C, 0.1 mm Hg), yielding 325 mg of aldehyde **8**, >98% pure by GC. The aldehyde was diluted with hexane to a concentration of 20 mg/ml, butylated hydroxytoluene was added (∼10 mg), and the solution was stored at −20°C under N_2. NMR: δ 9.77 (t, 1H, $J = 1.2$ Hz), 5.54–5.28 (m, 4H), 2.50 (tt, 2H, $J = 6.9$,1.2 Hz), 2.34 (br. quart., 2H, $J = 6.5$ Hz), 2.0 (m, 6H), 1.46–1.28 (m, 6H), 0.91 (t, 3H, $J = 7.0$ Hz). MS: *m*/*z* 208 (trace, M^+), 164 (3), 151 (2), 135 (3), 121 (4), 110 (10), 95 (19), 82 (33), 81 (34), 67 (60), 55 (71), 41 (100).

*Synthesis of (*Z*4,*Z*9)-Tetradecadienal **(11)** (Scheme 2)*

*4,9-Tetradecadiynol **(9)***. Butyllithium (10 ml of 2.5 M solution in hexanes, 25 mmol) was added dropwise to an icebath-cooled solution of 4-pentyn-1-ol THP ether **5** (4.2 g, 25 mmol) in 25 ml THF under Ar. The mixture was stirred 10 min, then bromoalkyne **3** (5.1 g, 25 mmol) was added. The mixture was warmed to



SCHEME 2

55°C and stirred overnight. GC analysis showed considerable quantities of starting material remaining. However, further heating resulted in no change in the product–starting material ratio so the mixture was worked up by quenching with water and extracting with hexane. The hexane extracts were washed with brine, dried, and concentrated. The residue was transacetalated by treatment with MeOH and PTSA as described above. The crude diynol **9** (3 g) was purified by vacuum flash chromatography in a 150-ml sintered glass funnel, eluting with 20% EtOAc in hexanes. The purified alcohol was Kugelrohr distilled (oven temperature ∼100–120°C, 0.1 mm Hg), yielding 2.1 g of clear oil. NMR: δ 3.76 (t, 2H, $J = 6.2$ Hz), 2.33–2.22 (m, 6H), 2.14 (tt, 2H, $J = 7.0$, 2.1 Hz), 1.74 (quint, 2H, $J = 6.3$ Hz), 1.67 (quint, 2H, $J = 7.0$ Hz), 1.52–1.33 (m, 4H), 0.91 (t, 3H, $J = 7.1$ Hz). MS: *m*/*z* 177 (3), 163 (35), 150 (11), 135 (16), 131 (14), 119 (27), 117 (29), 105 (40), 91 (100), 79 (57), 67 (41), 55 (45), 41 (94).

*(Z4,Z9)-Tetradecadienol **(10)***. $Ni(OAc)_2 \cdot 4H_2O$ (150 mg, 0.6 mmol) was dissolved in 20 ml 95% EtOH (Brown and Ahuja, 1973). The solution was purged with Ar, then 0.6 ml of a filtered 1 M solution of $NaBH_4$ (prepared by dissolving 0.2 g $NaBH_4$ in 4.75 ml EtOH and 0.25 ml 2 M aq. NaOH) was added by syringe. After bubbling ceased, the reaction vessel was flushed with H_2 and maintained under H_2 for the duration of the reduction. Ethylene diamine (0.1 ml) was added, followed 5 min later by addition of diynol (1.04 g, 5 mmol). The mixture was stirred 2 hr, monitoring by GC, then poured into 300 ml of 0.2 M HCl and extracted with hexane. The hexane extracts were washed with water and brine, dried, concentrated and Kugelrohr distilled (oven temperature ∼110–120°C, 0.1 mm Hg), yielding 0.85 g of dienol **10**. NMR: δ 5.48-5.33 (m, 4H), 3.67 (t, 2H, $J = 6.5$ Hz), 2.12 (br. quart, 2H, $J = 7.5$ Hz), 2.10–1.98 (m, 6H), 1.64 (quint, 2H, $J = 7.0$ Hz), 1.42 (quint, 2H, $J = 7.6$ Hz), 1.38–1.28 (m, 4H), 0.91 (t, 3H, $J = 6.8$ Hz). MS: *m*/*z* 210 (trace, M^+), 138 (5), 135 (4), 121 (8), 110 (8), 95 (27), 82 (27), 81 (49), 79 (58), 67 (65), 55 (70), 41 (100).

(*Z4,Z9*)-*Tetradecadienal **(11)***. (*Z*4,*Z*9)-tetradecadienol **10** (20 mg) was oxidized to the aldehyde as described above for the *E*4,*Z*9-isomer. The crude aldehyde **11** was purified by passage through a short column of silica gel in a disposable pipet, eluting with ether. NMR: δ 9.78 (t, 1H, $J = 1.4$ Hz), 5.49–5.29 (m, 4H), 2.50 (tt, 2H, $J = 7.3$,1.3 Hz), 2.38 (br quart, 2H, $J = 7.0$ Hz), 2.11–1.99 (m, 6H), 1.48–1.20 (m, 6H), 0.91 (t, 3H, J = 7.0 Hz).

Scrambling of Double Bonds of (Z4,Z9)-Tetradecadienol (Sonnet, 1974). (*Z*4,*Z*9)-tetradecadienol **10** (0.21 g, 1 mmol) was mixed with 52 μl 2M aq. $NaNO_2$ and 36 μl 6M HNO_3 in a heavy-wall conical bottomed vial, and the mixture was flushed with Ar, sealed, and heated 45 min at 75°C with stirring. The cooled mixture was extracted with hexane, and the hexane solution was washed with dilute $NaHCO_3$, water, and brine; simultaneously dried and decolorized with a mixture of charcoal and anhyd. Na_2SO_4; filtered; and concentrated. The crude mixture was used in the next step.

4,9-Tetradecadienal Isomer Mixture. The mixture of 4,9-tetradecadienol isomers was oxidized as described above for (*E*4,*Z*9)-tetradecadienal. In GC analyses of the two pure isomers and the scrambled mixture, all four isomers were distinguishable on the DB-5 and DB-Wax columns using a 5°C/min temperature program. The isomers were identified by comparison with the two authentic standards (*Z*4,*Z*9 and *E*4,*Z*9), and on the assumption that the most abundant isomer in the scrambled mixture would be the most thermodynamically stable *E*4,*E*9-isomer. By elimination, the fourth isomer was *Z*4,*E*9–14 : Ald.

RESULTS

Pheromone gland extracts of females contained only a single compound that elicited large and consistent responses from male antennae in coupled GC-EAD experiments, for all three species (Figure 1). The compound was the same for all

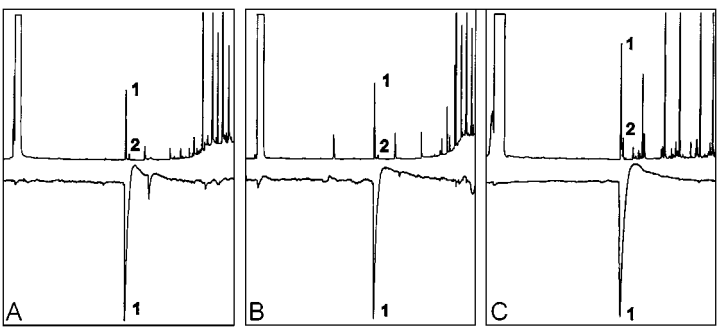


FIG. 1. Coupled gas chromatogram–electroantennograms of pheromone gland extracts from single females and antennae from males of : (A) *Saturnia walterorum*, (B) *S. mendocino*, and (C) *S. albofasciata*. Column: DB-5 (30 m × 0.32 mm, 0.25-μm film; program for *S. walterorum*, 100°C for 0 min, 15°C/min to 275°C for 25 min; for *S. mendocino*, 100°C for 1 min, 15°C/min to 150°C for 5 min, then 25°C/min to 275°C for 40 min; for *S. albofasciata*, 100°C for 1 min, 10°C/min to a final temperature of 275°C for 25 min. The male *S. albofasciata* was field collected using a lure containing *E*4,*Z*9–14 : Ald at Thomas Mountain, Riverside County, California.

three species, based on identical retention times on polar and nonpolar columns. On a nonpolar DB-5 column, the retention time was slightly shorter than that of a *Z*9–14 : Ald standard. Furthermore, during preliminary trials to determine the retention characteristics of the antennal stimulatory compound on various GC columns, it was discovered that the *Z*9–14 : Ald standard elicited strong responses from male moth antennae, providing circumstantial evidence that the insect-produced compound was probably a 14-carbon aldehyde, possibly with a double bond in the 9 position. The fact that the retention time of the insect-produced compound was slightly shorter than that of *Z*9–14 : Ald eliminated the possibility that the compound might be an α, β-unsaturated aldehyde or a conjugated dienal, because both of these structural units result in significant increases in retention times on DB-5 columns versus a monoenal (Marques et al., 2000). The mass spectrum also suggested a C_{14} dienal, with a small molecular ion at *m*/*z* 208, a small ion at *m*/*z* 190 from loss of water from the M^+ ion, and abundant ions at *m*/*z* 67 (100%), 81 (70%), 95 (43%), and 110 (30%), characteristic of unsaturated hydrocarbon fragment ions.

Further GC-EAD studies were carried out with the complete series of (*Z*)- and (*E*)-tetradecenal isomers with double bonds in the C_3 to C_{12} position, using *S. mendocino* as the test species. These experiments provided compelling evidence for a *Z* double bond at C_9, with male moth antennae being strongly stimulated by the *Z*9–14 : Ald standard (Figure 2). However, the slightly shorter retention time of the insect-produced compound (10.32 min) versus the *Z*9–14 : Ald standard (10.62 min) on the nonpolar DB-5 column, and the slightly longer retention time (11.35 min) versus *Z*9–14 : Ald (11.19 min) on a polar DB-225 column suggested the presence of a second, unconjugated double bond. The most likely position and geometry appeared to be *E*4, based on the GC-EAD trials with tetradecenal standards (Figure 2), but the antennal responses to *E*4-14 : Ald were much smaller than those to the *Z*9–14 : Ald standard. On the assumption that alkenes with double bonds in the 7, 8, 10, and 11 positions could be excluded because this would result in conjugated dienes with significantly longer retention times than the monoenes; a second analysis of treatments 3, 4, 5, 6, and 12 (Figure 2) showed that response to *E*4–14 : Ald was significantly larger than the responses elicited by the other possible isomers with double bonds at positions 3, 5, 6, and 12.

Comparison of the retention times of authentic *E*4,*Z*9–14 : Ald on polar and nonpolar GC columns with those of the other three stereoisomers confirmed that *E*4,*Z*9–14 : Ald was the isomer in the female extracts of all three species. On DB-5 all four isomers were resolved using a 5°C/min ramp (*ZZ* = 15.47, *ZE* = 15.51, *EZ* = 15.59, and *EE* = 15.72 min), and an exact retention time match with *E*4,*Z*9–14 : Ald was obtained upon coinjection of standards and an *S. walterorum* female extract. On DB-Wax, all four isomers also were resolved (*ZE* = 10.89, *ZZ* = 10.94, *EZ* = 11.02, and *EE* = 11.11 min), and the retention time of the insect-produced compound exactly matched that of *E*4,*Z*9–14 : Ald. Final proof of

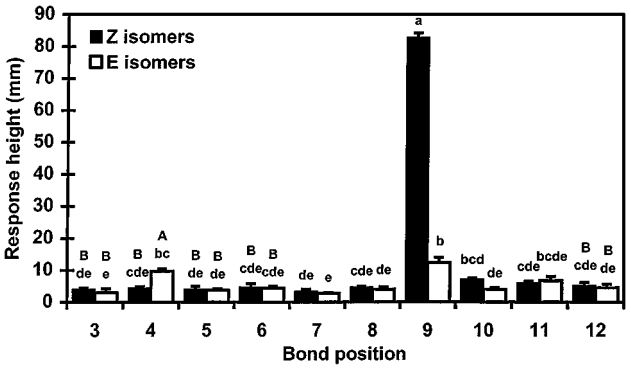


FIG. 2. Mean (±SE) electroantennographic responses from antennae of three male *S. mendocino* to monounsaturated tetradecenals. Data were analyzed first by two-way ANOVA of all data points ($\log_{10} x + 1$ transformed) (bond position effect, $F = 20.55$, $df = 19,38$, $P < 0.0001$; for male effect $F = 9.91$, $df = 2,38$, $P = 0.0003$) followed by a Student-Neuman-Kuels means separation test ($\alpha = 0.05$); bars surmounted by different lowercase letters were significantly different. Second, the data were reanalyzed, assuming that one double bond was at C-9, and that the compound was not conjugated or an allene, so that only the C-3–C-6 and the C-12 positions were included in the two-way ANOVA (for bond position effect $F = 13.4$, $df = 8,26$, $P < 0.0001$; for male effect $F = 26$, $df = 2,26$, $P = 0.0001$) and Student-Neuman-Kuels means separation test ($\alpha = 0.05$, bars surmounted by different uppercase letters are significantly different).

the structure was obtained from match-up of the electron impact mass spectrum of the compound with that of the standard and by the strong responses of male moths to *E*4,*Z*9–14 : Ald in field trials.

In addition to the dienal, pheromone gland extracts of all three species contained small amounts of *Z*9–14 : Ald (compound 2 in Figure 1A–C). The antennal responses to this compound were not resolved from those of the slightly earlier eluting *E*4,*Z*9–14 : Ald. The identification was confirmed by matching of retention times on three GC columns, and matching of the mass spectra with that of an authentic standard. Although the molecular ion at *m*/*z* 210 was not seen, there was a significant ion at *m*/*z* 192 from loss of water from the parent ion. Furthermore, match-up of retention times on both DB-5 and DB-Wax columns excluded all other monoene aldehyde positional and geometrical isomers from consideration (Marques et al., 2000). The relative amount of *Z*9–14 : Ald as a percentage of *E*4,*Z*9–14 : Ald for *S. walterorum* was 9.7 ± 2.6 % (mean of eight single-female extracts) and 10.9% for a four-female composite extract, whereas the amounts found in single extracts of *S. mendocino* and *S. albofasciata* females was 9.3% and 19.5%, respectively.

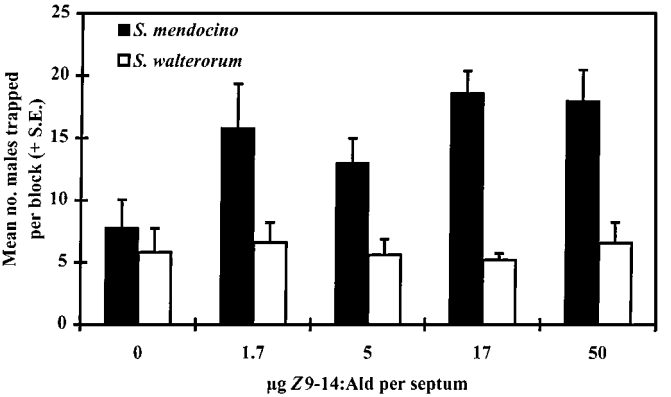


FIG. 3. Results of *S. mendocino* and *S. walterorum* field trials using blends of *E*4,*Z*9–14 : Ald (100 μg) and *Z*9–14 : Ald as trap lures. *S. mendocino* field trials were conducted in Amador County, California from April 27 to May 10, 1997, with a total of 381 moths trapped. Two-way ANOVA for *Z*9–14 : Ald dose effect, $F = 2.733$, $df = 4,16$, $P = 0.0633$, for block effect, $F = 0.909$, $df = 4,16$; $P = 0.4824$. *S. walterorum* field trials conducted in San Diego County, California from March 27 to April 24, 1997, total of 149 moths trapped. Two-way ANOVA for *Z*9–14:Ald dose effect, $F = 0.128$, $df = 4,16$; $P = 0.9702$, for block effect, $F = 4.203$, $df = 4,16$; $P = 0.0163$.

A second EAD response was seen sporadically to a trace component in *S. walterorum* extracts. This compound was not identified, but comparisons of its retention time on a DB-5 column with those of standards determined that it was possibly *E*4,*Z*9–14 : OH or a geometrical isomer, a compound that might be expected to be present as a precursor to *E*4,*Z*9–14 : Ald.

Field Trials. Sticky traps baited with synthetic *E*4,*Z*9–14 : Ald (100 μg) were attractive to male *S. mendocino* and *S. walterorum* moths (Figure 3). For both species, addition of *Z*9–14 : Ald did not significantly increase the attractiveness of blends relative to the diene alone. These results were corroborated for *S. mendocino* in a subsequent field trial in Del Puerto Canyon, in which there was no difference in attractiveness between baits containing *E*4,*Z*9–14 : Ald alone and baits with a 10 : 1 blend of *E*4,*Z*9–14 : Ald + *Z*9–14 : Ald (2-way ANOVA, for blend effect: $F = 0.0101$, $df = 1,9$, $P = 0.9221$, for block effect $F = 1.2018$, $df = 9,9$, $P = 0.3944$, based on totals of 100 moths in the treatment with *Z*9–14 : Ald, and 90 moths in the treatment without *Z*9–14 : Ald). Thus, the available data suggest that *Z*9–14 : Ald is not a pheromone component for these two species.

In a single field trial with *S. albofasciata*, three traps baited with *E*4,*Z*9–14 : Ald (100 μg) caught a total of 151 moths in one night whereas the control traps caught no moths. This number may underestimate the attractiveness of the lures because the sticky trap surfaces were completely covered with moths. In addition,

males showed no hesitation in approaching lures, and, when in close proximity, were observed to initiate copulatory attempts with lures, further suggesting that the pheromone consists of a single component.

DISCUSSION

There have been few reports of 14-carbon 4,9-diene compounds as lepidopteran pheromone components, and even in those few reports, details have been sketchy. For example, Bestmann et al. (1987a) identified *E*4,*Z*9–14 : Ac in pheromone gland extracts from the silk moth *Antheraea pernyi*, and located a large-spiking antennal cell that was stimulated by this compound. However, the effects of this compound on male moth behavior were not reported. A second study by Bestmann et al. (1987b) examining the effects of differing functional groups on the responses of single receptor cells used both *E*4,*Z*9–14 : Ac and *E*4,*Z*9–14 : Ald, but in neither case were the syntheses of the compounds reported. Tomida et al. (1987) prepared all four 4,9–14 : OH isomers and tested the formate derivatives in laboratory bioassays as mimics of E6,Z11-16 : Ald, a sex pheromone component of the eri-silk moth *Philosamia cynthia ricini*; no synthetic details were given. Other syntheses of *E*4,*Z*9–14 : OH (Sharma et al., 1990; Du et al., 1994) and Z4,*Z*9–14 : OH (Ducoux et al., 1992) have been published, but there have been no other reports of biological activity associated with any C_{14} 4,9-dienes as lepidopteran pheromone components.

The numbers of moths of each of the three *Saturnia* species available for study varied. Pheromone gland extracts were obtained from 21 female *S. walterorum* and five female *S. mendocino*, providing sufficient material for GC and GC-EAD analyses on several columns and GC-MS studies. In contrast, only a single extract from a female *S. albofasciata* was obtained, due to the relative scarcity of this species and difficulties in rearing it. This extract provided only sufficient material for GC-EAD analysis on a DB-5 column. Nevertheless, given that the retention time exactly matched that of *E*4,*Z*9–14 : Ald and that all four 4,9–14 : Ald stereoisomers were separable under the conditions used, there can be little doubt that *E*4,*Z*9–14 : Ald is indeed a sex pheromone component for this species.

All three species appear to use the same, single compound as their pheromone, leading in theory to the possibility of interspecific interference in the pheromone channels. However, the flight period of *S. albofasciata* is in the fall, whereas the other two species fly in the spring, precluding any interference between *S. albofasciata* and the other two species. Furthermore, experimental manipulation of *S. walterorum* to encourage unnatural fall emergence of adults by exposing pupae to an artificially lengthened cold period (November of one year until October of the next) resulted in *S. walterorum* males that were attracted to and attempted to mate with *S. albofasciata* females and vice versa (K. Wolfe and C. Conlan, personal

communication, 2000), despite gross differences in size. No copulations occurred due to the physical incompatibility of the two species. Nevertheless, these results suggest that separation in time provides an effective barrier to interspecific mating between these two species, allowing them to share the same sex pheromone with no fitness cost. For the other two species, only small parts of their ranges overlap, and the two species are closely related and known to hybridize (Tuskes and Collins, 1981), so the costs of cross-attraction may be minimal. Overall, it is clear that the pheromone chemistry is highly conserved within the North American members of the genus.

Furthermore, there are obvious parallels with the pheromone chemistry of other members of the Saturniinae subfamily. For example, the pheromone blends of *Antherea pernyi* Guérin-Méneville (Bestmann et al., 1987a) and the congeneric *A. polyphemus* Cramer (Kochansky et al., 1975) are reported to consist of *E*6,*Z*11–16 : Al and the corresponding acetate, both of which contain the same diene motif as *E*4,*Z*9–14 : Ald, but with two more carbons in the chain. *E*4,*Z*9–14 : Ac also was reported from *A. pernyi* gland extracts. In another genus and tribe, the pheromone blend of *Samia cynthia ricini* Donovan consists of *E*4,*Z*6,*Z*11–16 : Ald and *E*4,*Z*6,*Z*11–16 : Ac, again with two of the three double bonds in the same position relative to the nonfunctionalized end of the chain. Finally, Bestmann et al. (1982) mentioned that *E*6,*Z*11–16 : Ac was attractive to *Eudia* (= *Saturnia*) *pavonia*, but provided no further details.

In conclusion, we have presented spectroscopic, retention time, and field trapping data that indicate that the three North American *Saturnia* species share the same sex pheromone component, *E*4,*Z*9–14 : Ald. Possible negative effects of cross-attraction between species are largely avoided by temporal and geographic barriers that keep the species apart in space and time.

Acknowledgments—We thank Christopher Conlan and Kirby Wolfe for their generous donations of insects and for sharing unpublished information used in this study.

REFERENCES

BESTMANN, H.-J., KOSCHATSKY, K. H., and VOSTROWSKY, O. 1982. Stereoselektive Synthesen von 1,5- und 1,6-Alkadienen als Sexualpheromone und Pheromonanaloge. *Liebigs Ann. Chem.* 1982:1478–1498.

BESTMANN, H.-J., ATTYGALLE, A. B., BROSCHE, T., ERLER, J., PLATZ, H., SCHWARZ, J., VOSTROWSKY, O., WU, C.-H., KAISSLING, K. E., and CHEN, T.-M. 1987a. Identification of three sex pheromone components of the female saturniid moth *Antheraea pernyi* (Lepidoptera: Saturniidae). *Z. Naturforsch.* 42c:631–636.

BESTMANN, H.-J., WU, C.-H., DOHLA, B., LI, K., and KAISSLING, K.-E. 1987b. Functional group recognition of pheromone molecules by sensory cells of *Antheraea polyphemus* and *Antheraea pernyi* (Lepidoptera: Saturniidae). *Z. Naturforsch.* 42c:435–441.

BROWN, C. A., and AHUJA, V. K. 1973. P-2 nickel catalyst with ethylenediamine, a novel system for highly stereospecific reduction of alkynes to cis-olefins. *J. Chem. Soc. Chem. Commun.* 1973:553–554.

COLL, J. C., and BOWDEN. B. F. 1986. The application of vacuum liquid chromatography to the separation of terpene mixtures. *J. Nat. Prod.* 49:934–936.

COREY, E. J., and SUGGS, J. W. 1975. Pyridinium chlorochromate. An efficient reagent for oxidation of primary and secondary alcohols to carbonyl compounds. *Tetrahedron Lett.* 1975:2647–2650.

DU, G., NG, C.-S., and PRESTWICH, G. D. 1994. Odorant binding by a pheromone binding protein: Active site mapping by photoaffinity labelling. *Biochemistry* 33:4812–4819.

DUCOUX, J.-P., LE MENEZ, P., KUNESCH, N., KUNESCH, G., and WENKERT, E. 1992. An efficient and stereoselective synthesis of insect pheromones by way of nickel-catalyzed Grignard reactions. Syntheses of gossyplure and pheromones of *Eudia pavonia* and *Drosophila melanogaster. Tetrahedron* 48:6403–6412.

HERSCOVICI, J., EGRON, M.-J., and ANTONAKIS, K. 1982. New oxidative systems for alcohols: molecular sieves with chromium-(VI) reagents. *J. Chem Soc. Perkin Trans. I* 1982:1967–1973.

HUMASON, G. L. 1972. Animal Tissue Techniques, 3 ed. W. H. Freeman and Company. San Francisco.

KAISSLING, K.-E., and THORSON, J. 1980. Insect olfactory sensilla: Structural, chemical and electrical aspects of the functional organization, pp. 261–282, D. B. Sattelle, L. M. Hall, and J. G. Hildebrand (eds.), *in* Receptors for Neurotransmitters, Hormones, and Pheromones in Insects, Elsevier/North Holland Biomedical Press, New York.

KOCHANSKY, J., TETTE, J., TASCHENBERG, E. F., CARDÉ, R. T., KAISSLING, K.-E., and ROELOFS, W. 1975. Sex pheromone of the moth *Antheraea polyphemus. J. Insect. Physiol.* 21:1977–1985.

MARQUES, F. A., MCELFRESH, J. S., and MILLAR, J. G. 2000. Kovats retention indexes of monounsaturated C_{12}, C_{14}, and C_{16} alcohols, acetates, and aldehydes commonly found in lepidopteran pheromone blends. *J. Braz. Chem. Soc.* 11:592–599.

SHARMA, S., SABHARWAL, A., VOHRA, N., SHARMA, M. L., VIG, O. P., and KAD, G. L. 1990. Syntheses of the sex pheromone components of wild silk moths, fruit fly, melon fly, and olive fly. *J. Indian Chem. Soc.* 67:31–33.

SONNET, P. E. 1974. A practical synthesis of the sex pheromone of the pink bollworn. *J. Org. Chem.* 39:3793–3794.

STILL, W. C., KAHN, M., and MITRA, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43:2923–2925.

TOMIDA, I., IWAKOSHI, K., KONO, S., TAKAHASHI, T., MATSUNAGA, T., and KAYAHARA, H. 1987. Flutter-stimulating activities on male Eri-silk moths, *Philosamia cynthia ricini*, for four geometric isomers of 4,9-tetradecadienyl formate. *Agric. Biol. Chem.* 51:939–940.

TUSKES, P. M., and COLLINS, M. M. 1981. Hybridization of *Saturnia mendocino* and S. *walterorum*, and phylogenetic notes on Saturnia and Agapema (Saturniidae). *J. Lepid. Soc.* 35:1–21.

TUSKES, P. M., TUTTLE, J. P., and COLLINS, M. M. 1996. The Wild Silk Moths of North America: A Natural History of the Saturniidae of the United States and Canada. Cornell University Press, Ithaca, New York.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

CAN SIMULTANEOUS INHIBITION OF SEEDLING GROWTH AND STIMULATION OF RHIZOSPHERE BACTERIAL POPULATIONS PROVIDE EVIDENCE FOR PHYTOTOXIN TRANSFER FROM PLANT RESIDUES IN THE BULK SOIL TO THE RHIZOSPHERE OF SENSITIVE SPECIES?

KAREN STAMAN,[1] UDO BLUM,[1,]* FRANK LOUWS,[2] and DOMINIQUE ROBERTSON[1]

[1]*Department of Botany*

[2]*Department of Plant Pathology*
North Carolina State University
Raleigh, North Carolina 27695

(Received July 20, 2000; accepted December 13, 2000)

Abstract—In order to demonstrate that allelopathic interactions are occurring, one must, among other things, demonstrate that putative phytotoxins move from plant residues on or in the soil, the source, through the bulk soil to the root surface, a sink, by way of the rhizosphere. We hypothesized that the incorporation of phytotoxic plant residues into the soil would result in a simultaneous inhibition of seedling growth and a stimulation of the rhizosphere bacterial community that could utilize the putative phytotoxins as a sole carbon source. If true and consistently expressed, such a relationship would provide a means of establishing the transfer of phytotoxins from residue in the soil to the rhizosphere of a sensitive species under field conditions. Presently, direct evidence for such transfer is lacking. To test this hypothesis, cucumber seedlings were grown in soil containing various concentrations of wheat or sunflower tissue. Both tissue types contain phenolic acids, which have been implicated as allelopathic phytotoxins. The level of phytotoxicity of the plant tissues was determined by the inhibition of pigweed seedling emergence and cucumber seedling leaf area expansion. The stimulation of cucumber seedling rhizosphere bacterial communities was determined by the plate dilution frequency technique using a medium containing phenolic acids as the sole carbon source. When sunflower tissue was incorporated into autoclaved (to reduce the initial microbial populations) soil, a simultaneous inhibition of cucumber seedling growth and stimulation of the community of phenolic acid utilizing rhizosphere bacteria occurred. Thus, it was possible to observe simultaneous inhibition of cucumber seedlings and

*To whom correspondence should be addressed.

0098-0331/01/0400-0807$19.50/0

stimulation of phenolic acid utilizing rhizosphere bacteria, and therefore provide indirect evidence of phenolic acid transfer from plant residues in the soil to the root surface. However, the simultaneous responses were not sufficiently consistent to be used as a field screening tool but were dependent upon the levels of phenolic acids and the bulk soil and rhizosphere microbial populations present in the soil. It is possible that this screening procedure may be useful for phytotoxins that are more unique than phenolic acids.

Key Words—Allelopathy, bulk-soil and rhizosphere bacteria, *Cucumis sativus, Helianthus annuus, Triticum aestivum, Amaranthus retroflexus,* phenolic acid mixtures, chlorogenic acid, phytotoxicity.

INTRODUCTION

In order to establish that inhibitory plant–plant allelopathic interactions are operative in the field, one must demonstrate, among other things, that potential organic phytotoxins released into the soil by living plants (e.g., root exudates, leachates) or plant residues contact the roots of a sensitive plant (Willis, 1985; Blum et al., 1999). Because of the lack of direct access to roots and the dynamic nature of field soil processes (e.g., sorption, oxidation, microbial interconversion, polymerization, and leaching of phytotoxins in soils), direct tracing of the movement of phytotoxins from living plants or plant residues to the roots of another plant in the soil is extremely difficult, if not impossible. Establishing that such transfer actually occurs under field conditions is an essential step in demonstrating the existence and importance of allelopathic interactions. At present, no definitive data on direct transfer of phytotoxins between living roots of one plant (and/or plant residue) to another plant root system have been obtained under field conditions. The objective of this research was to determine if evidence for such a transfer could be obtained.

Research by Blum and Shafer (1988), Shafer and Blum (1991), and Blum et al. (2000) suggested such a means. They observed that phenolic acid enrichments of soils inhibited cucumber seedling growth and stimulated both bulk soil and rhizosphere microorganisms. Of particular interest was the observed increase of bacteria in the rhizosphere that could utilize phenolic acids as a sole carbon source. Assuming that phytotoxic levels of phenolic acids released from plant residues in the field would also increase phenolic acid-utilizing bacteria in the rhizosphere of sensitive species, such increases could provide the first evidence for the transfer of phytotoxic concentrations of phenolic acids from the bulk soil to the rhizosphere. We, therefore, hypothesized that phenolic acids released at phytotoxic levels from living plants or plant residues should simultaneously inhibit the growth of sensitive plant species and stimulate phenolic acid utilizing bacteria within the rhizosphere of sensitive species.

We initially chose to test this hypothesis in the laboratory using field grown wheat shoot and root tissue and sunflower leaf tissue, since a variety of potential

phytotoxins, including phenolic acids, have been identified for wheat (Chou and Patrick, 1976; Liebl and Worsham, 1983; Barnes et al., 1986; Blum et al., 1991) and sunflower (Armstrong et al., 1970, 1971; Hall et al., 1982, 1983; Macias et al., 1993, 1998, 1999). Among the phenolic acids that have been identified for these species are ferulic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid, and chlorogenic acid. Since wheat and sunflower tissue phytotoxicity has been characterized by inhibition of several weed species, including pigweed, pigweed seedling emergence was used to characterize wheat and sunflower tissue phytotoxicity (Blum et al., 1997; Lehman and Blum, 1997). Simultaneous inhibition of growth and stimulation of rhizosphere bacteria, however, was determined for cucumber seedlings to be consistent with earlier observations of Shafer and Blum (1991) and Blum et al. (2000).

METHODS AND MATERIALS

Plant Tissue and Soil Materials. Living wheat (*Triticum aestivum* L. Southern States 555) roots and shoots were collected on May 15, 1997, and June 8 and 9, 1998, from experimental plots at the Lake Wheeler Field Laboratory, approximately 5 km south of North Carolina State University. Roots and shoots were immediately separated, and the remaining soil was removed from freeze-dried roots with a fine bristle brush. Sunflower leaves (*Helianthus annuus* cv. Black Oil) were collected in July 1999 from plots 20 miles west of North Carolina State University. Freeze-dried plant tissue samples were stored in closed plastic bags at room temperature in the dark. On May 7, 1998, Cecil A_p horizon soil material (pH 5.06, total C = 2.24%, and total N = 0.21%) was collected from plots adjacent to the wheat plots. This soil was sieved (2.5-mm mesh), air dried, and stored at room temperature in the laboratory. Prior to each experiment, wheat shoot, wheat root, and/or sunflower leaf blade tissue was ground in a Wiley mill (20-mesh screen). A range of 0–10 mg/g soil of the plant tissue collected in 1997 and/or 1998 was incorporated into a 150 g mixture of washed river sand and the Cecil A_p soil (2:1 sand to soil ratio, w/w).

Pigweed Seedling Emergence. Plant tissue–soil mixtures (150 g/Petri dish) were placed into 100 × 25-mm Petri dishes. Petri dishes were used to provide sufficient surface area for planting the pigweed seeds. The dishes were seeded just below the soil surface with 50 pigweed seeds (*Amaranthus retroflexus* L.; Azland Seed Service, Leland, Mississippi), and the loosely covered Petri dishes were placed under a light bank (140 μmol/m^2/sec PAR supplied by cool white florescent bulbs, 12-hr photoperiod, room temperature). All Petri dishes were watered to 0.093 g H_2O/g soil each day.

Emerging pigweed seedlings were counted each day and removed from the Petri dishes. Lehman and Blum (1997) had previously determined that total

seedling emergence over six days was adequate for characterizing the phytotoxicity of wheat tissue, thus, the experiments were terminated after six days and cumulative (days 1–6) seedling emergence was calculated.

Phenolic Acid Utilizing-Bacteria in Bulk Soil. Petri dishes or cup systems (see below) containing soil and/or plant tissue–soil mixtures were placed under a light bank (140 μmol/m^2/sec PAR supplied by cool white florescent bulbs, 12-hr photoperiod, room temperature) and supplied with water to bring the water content of the soil to 0.093 g H_2O/g soil each day. Soil in Petri dishes or cup systems was subsampled to determine soil moisture (100°C) and in the presence of soil-borne bacterial populations that could utilize phenolic acids as a sole carbon source. Bacterial populations were determined on day 6 by the plate-dilution frequency technique (Harris and Sommers, 1968). Approximately 20 g of a given soil sample plus 30 ml of a solution containing 8.5 g/liter NaCl and 0.1% agar were shaken on a wrist action shaker for 30 min. The resulting soil suspensions were diluted (10-fold series) to 1×10^{-9} or 1×10^{-10} using the aqueous agar–NaCl solution. Colony forming units (CFU) were determined on an agar (15g/liter) medium composed of mineral salts (1 g/liter KH_2PO_4, 0.4 g/liter $MgSO_4 \cdot 7H_2O$, 0.13 g/liter $CaCl_2 \cdot 2H_2O$, 0.1 g/liter NaCl, 0.01 g/liter $FeCl_3$, 0.5 g/liter KNO_3), Delvocid (0.05g/liter natamycin, an antimycotic; G B Fermentation Industries, Inc. Charlotte, North Carolina), and a 0.5 mM phenolic acid mixture composed of 0.071 mM caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, sinapic, syringic, and vanillic acids, or a 0.5 mM phenolic acid mixture composed of 0.125 mM ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids. Inoculated plates were incubated in the dark in closed plastic bags at 30°C. CFU were determined after six or seven days of bacterial growth.

Cucumber Seedling Growth and Phenolic Acid-Utilizing Bacterial Populations in the Rhizosphere. Cucumber seeds (*Cucumis sativus* cv. Early Green Cluster; Wyatt Quarles Seed Company, Raleigh, North Carolina) were germinated in the dark at 30°C in a tray containing vermiculite and distilled water. After 48 hr, seedlings were transferred to a light bank (140 μmol/m^2/sec PAR supplied by cool white florescent bulbs, 12-hr photoperiod, room temperature). After 48 hr in the light bank, seedlings were transplanted into cups containing 150 g of an autoclaved or nonautoclaved soil–sand mixture (Cecil Ap; 1 : 2 w/w) and 0–10 mg/g soil wheat or sunflower tissue collected in 1998 or 1999. For the autoclaved soil mixture, batches (approximately 3 kg) of the soil–sand mixture were autoclaved once for 30 min at 16–18 psi and 121°C in a 29 × 17-cm steel tray before placing 150 g into each cup. The autoclaving procedure was used not to sterilize the soil but to reduce the initial microbial populations. The initial log CFU per gram of soil for the autoclaved and nonautoclaved soil was 3.47 ± 0.02 (2945 actual CFU per gram soil) and 3.59 ± 0.01 (3912 actual CFU per gram soil), respectively.

In addition, soil–sand mixtures without plant tissue added were treated with 0.6 μmol/g soil (days 5, 7, 9, 11) of chlorogenic acid (a phenolic acid, pH 5) or

a phenolic acid mixture (equimolar solution of ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids, pH 5). The seedling cup systems (Blum et al., 1989) were also supplied with 7 ml double-strength Hoagland's solution (pH 5.0) on days 4, 6, 8, 10, 12, 14, and 16. Water (controls) or phenolic acids were added on alternate days. Systems were harvested on day 6, 7, 8, 10, 12, or 17. Water content of soil was brought up to 0.167 g H_2O/g soil each day.

Leaf areas for seedlings were nondestructively measured to calculate absolute rates of seedling leaf expansion over days 9–11, 9–12, 11–13, 13–15, and/or 15–17 (Blum et al., 1985). For each harvest at the termination of the experiment, shoot and root dry weights (100°C) were determined. Prior to dry weight determinations, the root systems were dipped three times in sterile deionized water to dislodge larger soil particles. Root systems with remaining rhizosphere soil were transferred to bottles containing 99 ml of a sterile solution composed of 8.5 g/liter NaCl, 0.1% aqueous agar, and 1 g of sand. Bottles were shaken on a platform shaker for 30 min. The resulting suspension of rhizosphere soil was diluted and assayed by the plate-dilution frequency technique (Harris and Sommers, 1968). Colony forming units (CFU) were determined on mineral agar medium (see previous section, "Phenolic Acid-Utilizing Bacteria in Bulk Soil" for details) containing either a 0.5 mM phenolic acid mixture composed of equimolar 0.125 mM ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids, or 0.5 mM chlorogenic acid (all at pH 5). The root systems were subsequently recovered from the dilution bottles to determine their dry weights. Shoots and roots were dried in an oven at 100°C. Then CFU per gram of root dry weight, seedling leaf area, and shoot and root dry weights were determined.

Plant Tissue Analysis. The phenolic acid content of the tissue was determined by the polyvinylpyrrolidone (PVP) method (Anderson and Todd, 1968). Tissue extracts were obtained by autoclaving 200 mg of ground tissue (Wiley mill, 60 mesh screen) in 50 ml deionized water. Extracts were filtered prior to phenolic acid analysis (Whatman No. 1) (Lehman and Blum, 1997).

Data Analyses. All data were subjected to analysis of variance and/or regression analysis using the Statistical Analysis Systems of JMP (SAS Institute, Cary, North Carolina). Means comparisons were made by the Tukey-Kramer HSD test. Alpha was set at 0.05 for all analyses. Analyses of CFU of bacterial populations that could utilize phenolic acids as a sole carbon source were all based on log CFU per gram soil or log CFU per gram root dry weight (Harris and Sommers, 1968; Loper et al., 1984). All experiments were organized in the light bank in a randomized complete block design. Calculations were as follows: Absolute rates of seedling leaf expansion (AGRSL) = [leaf area on day 11 (or 13, 15, 17) − leaf area on day 9 (or 11, 12, 15, respectively)]/2 or (leaf area on day 12 − leaf area on day 9)/3; % Inhibition of cumulative pigweed emergence or AGRSL = [(individual treatment unit − control mean)/control mean] ∗ 100; and % stimulation of CFU = [(individual treatment unit − control mean)/control mean] ∗ 100.

Experimental Objectives and Designs. The objectives for each experiment are provided in the Results section. The specific design for each experiment is provided in Table 1.

RESULTS

For comparative purposes, percent changes from the control are presented for cumulative pigweed seedling emergence, absolute rates of seedling leaf expansion (ARGSL), and shoot and root dry weights. For the sake of clarity, inhibitory effects are presented as negative values, while stimulatory effects are presented as positive values. Both soil and rhizosphere bacterial colony forming units (CFU) are presented as log CFU and actual CFU. However, all statistical analyses of CFU for bulk-soil and rhizosphere bacteria were based on log CFU (Harris and Sommers, 1968; Loper et al., 1984). The initial log CFU per gram soil for the autoclaved and non-autoclaved soil was 3.47 ± 0.02 (2945 actual CFU per gram soil) and 3.59 ± 0.01 (3912 actual CFU per gram soil), respectively. Finally, for all regression models the tissue concentrations added to soil are in milligrams per gram of soil and time is in days.

Experiment 1

The purpose of experiment 1 was to determine the effects of soil-incorporated wheat shoot and root tissue on pigweed seedling emergence (experiment 1a) and on bulk-soil bacterial populations that can utilize phenolic acids as a sole carbon source (experiment 1b). Wheat shoot and root tissues collected during May 1997 and June 1998 significantly reduced pigweed seedling emergence when these tissues ranging from 0 to 10 mg/g soil were incorporated into a Cecil A_p substrate (Figure 1). The main treatment effects of tissue type (shoot vs. root tissue), tissue concentration, and the interactions between tissue type $\times$ concentration were significantly different for pigweed emergence. The effects of collection time (1997 vs. 1998) of wheat tissues on seedling emergence, however, were not significantly different. The cumulative emergence of pigweed seedlings (over days 1–6) in soils without tissue incorporated (i.e., control; mean $\pm$ SE) was 27.5 ± 0.91 out of 50, or 55%.

The percent change in cumulative pigweed seedling emergence became more negative (i.e., more inhibitory) in a concave manner with increasing concentrations of wheat shoot tissue (Figure 1; % change in emergence $= 0 - [18.59 * \text{concentration}] + [1.08 * \text{concentration}^2]$; $P < 0.001$; $R^2 = 0.91$) and became more negative (i.e., more inhibitory) in a linear manner with increasing concentrations of wheat root tissue (Figure 1; % change in emergence $= 0.16 - [2.10 * \text{concentration}]$; $P = 0.009$; $R^2 = 0.27$). Soil-incorporated shoot tissues (-78% change for 10 mg/g soil) were more inhibitory to cumulative seedling emergence than root tissues (-21% change for 10 mg/g soil).

TABLE 1. EXPERIMENTAL DESIGNS

Exp.	System	Bioassay species	Autoclaved soil	Tissues and/ or phenolic acids	Concentration in soil	Treatment day[a]	Harvest day	C source of bacterial media	Rep
1a	Petri dish	Pigweed	no	wheat shoot,[b] wheat root	0, 5, 10 mg/g	1	1–6[c]		8
1b	Petri dish	Bulk soil bacteria	no	wheat shoot, wheat root	0, 5, 10 mg/g	1	6	7 phenolic acid mixture	8
2	Cup	Cucumber[d] and rhizosphere bacteria	no	wheat shoot	0, 2.5, 5, 10 mg/g	4	17	4 phenolic acid mixture	5
3	Cup	Cucumber and rhizosphere bacteria	yes[e]	wheat shoot, phenolic acids	0, 5 mg/g 0.6 μmol/g[f]	4 5, 7, 9, 11	6, 7, 8, 10, 12	4 phenolic acid mixture	5/day
4a	Petri dish	Pigweed	no	sunflower leaf	0, 2.5, 5, 10 mg/g	1	1–6		7
4b	Cup	Bulk soil bacteria	no	sunflower leaf	0, 2.5, 5, 10 mg/g	1	6	4 phenolic acid mixture	7
4c	Cup	Cucumber and rhizosphere bacteria	no	sunflower leaf	0, 2.5, 5, 10 mg/g	4	17	4 phenolic acid mixture	9
5	Cup	Cucumber and rhizosphere bacteria	no	sunflower leaf	0, 5 mg/g	4	12	4 phenolic acid mixture	14
6	Cup	Cucumber and rhizosphere bacteria	yes	sunflower leaf, chlorogenic acid	0, 5 mg/g 0.6 μmol/g	4 5, 7, 9, 11	6, 7, 8, 10, 12	4 phenolic acid mixture chlorogenic acid	5/day

[a]Hoagland's solution was added every other day beginning with day 4 for all cucumber seedling experiments.
[b]All wheat shoot experiments used tissue collected in 1998 except 1a, which used tissue collected in 1997 and 1998. All sunflower experiments used leaf tissue collected in 1999. The sunflower petioles were removed, and only the leaf blade tissue was incorporated into the soil.
[c]Emerging seedlings were determined each day up to day 6 and summed to obtain a cumulative value for pigweed emergence.
[d]The following were determined for cucumber seedlings: shoot and root dry weight, and absolute leaf area on days 9, 11, 12, 13, 15, and/or 17.
[e]Soils were autoclaved only once to reduce soil-borne bacteria.
[f]Phenolic acid mixture and chlorogenic acid treatments were given on days 5, 7, 9, and 11.

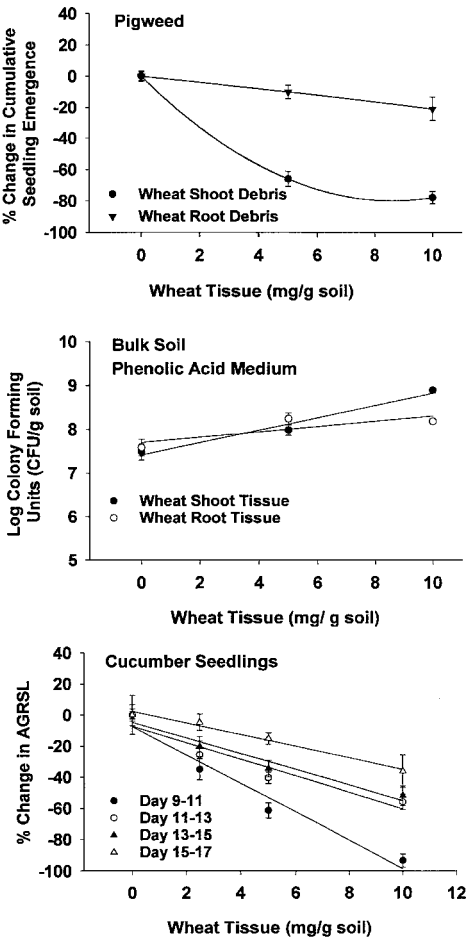


FIG. 1. Percent change in cumulative pigweed seedling emergence (Experiment 1a), log colony forming units of bulk soil bacteria (Experiment 1b) and percent change in cucumber seedling absolute growth rates of seedling leaf expansion (AGRSL) (Experiment 2) in the presence of soil-incorporated wheat shoot or root tissue. For the sake of clarity, inhibitory effects are presented as negative numbers while stimulatory effects are presented as positive numbers. Data in figures are means ± SE.

Wheat shoot and root tissues collected during June 1998 significantly stimulated the bulk-soil bacteria that could utilize phenolic acids as a sole carbon source when these tissues, ranging from 0 to 10 mg/g soil, were incorporated into the Cecil A_p substrate. Only the main treatment effects of concentration and the interactions between tissue type × concentration were significantly different.

Log bacterial colony forming units (Figure 1; log CFU on day 6) per gram of soil (bulk soil) increased linearly with increasing concentrations for both soil-incorporated shoot (log CFU $= 7.41 + [0.14 * \text{concentration}]$; $P < 0.001$; $R^2 = 0.84$) and root tissues (Figure 1; log CFU $= 7.70 + [0.06 * \text{concentration}]$; $P = 0.024$; $R^2 = 0.41$). The stimulatory effects of 10 mg/g soil-incorporated wheat shoot tissues were significantly greater (+2412% change in actual CFU per gram soil for 10 mg/g soil) than wheat root tissue (+298% change in actual CFU per gram soil for 10 mg/g soil). However, there were no significant differences in log CFU per gram of soil for incorporated wheat shoot and root tissue at 5 mg/g soil when compared to each other. The mean ± SE for CFU per gram for soils without tissue incorporated (i.e., control) was 7.58 ± 0.19 log CFU per gram of soil (3.8×10^7 actual CFU per gram soil).

Experiment 2

The purpose of experiment 2 was to determine the effects of soil-incorporated wheat shoot tissue on cucumber seedlings and on rhizosphere bacteria that can utilize phenolic acids as a sole carbon source. Soil-incorporated wheat shoot tissue (0–10 mg/g soil) collected during June 1998 significantly inhibited absolute rates of cucumber seedling leaf expansion (AGRSL) for seedlings measured over days 9–11, 11–13, 13–15, and 15–17 (Table 2, Figure 1). Percent changes for these intervals were −99%, −61%, −55%, and −35% for the 10 mg/g soil, respectively. Control AGRSL (mean ± SE) were 4.61 ± 0.53, 8.38 ± 0.57, 11.97 ± 0.46, and 13.56 ± 0.33 (cm^2/day), respectively.

Shoot dry weight determined on day 17 was significantly reduced (% change in shoot dry weight $= -9.25 - [4.64 * \text{concentration}]$; $P < 0.001$; $R^2 = 0.64$;

TABLE 2. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR PERCENT CHANGE IN AGRSL OF CUCUMBER SEEDLINGS GROWN IN PRESENCE OF SOIL-INCORPORATED WHEAT (EXPERIMENT 2) OR SUNFLOWER SHOOT TISSUE (EXPERIMENT 4C)

Growth period (days)	Intercept	Linear	Quadratic	R^2	P
Wheat shoot tissue					
9–11	−7.58	−9.12		0.80	<0.001
11–13	−7.21	−5.34		0.66	<0.001
13–15	−4.69	−5.05		0.71	<0.001
15–17	2.29	−3.75		0.54	<0.001
Sunflower shoot tissue					
9–11	2.07	−7.84		0.77	<0.001
11–13	5.85	−6.57		0.60	<0.001
13–15	12.33	−6.74		0.55	<0.001
15–17	0.58	+7.17	−1.19	0.64	<0.001

−56% change for 10 mg/g soil) by wheat shoot tissue. The soil-incorporated shoot wheat tissue had no effect on root dry weight or the rhizosphere bacteria of cucumber seedlings that could utilize phenolic acids as a sole carbon source. Shoot and root dry weights (mean ± SE) of cucumber seedlings and rhizosphere log CFU per gram root dry weight (day 17) for soils without incorporated tissue (i.e., control) were 248.88 ± 16.93 mg, 35.34 ± 3.79 mg, and 10.71 ± 0.41 log CFU per gram root (5.13×10^{10} actual CFU per gram root), respectively.

Experiment 3

The purpose of experiment 3 was to determine how cucumber seedlings and rhizosphere bacteria that can utilize phenolic acids as a sole carbon source respond over time (transplanted day 4; harvested days 6–12) in autoclaved (to reduce initial microbial populations) soil to soil-incorporated wheat shoot tissue or multiple soil applications of a phenolic acid mixture.

Soil-Incorporated Wheat Tissue. Wheat tissue (5 mg/g soil) had no negative effect on AGRSL (days 9–12 only measurement) and shoot and root dry weights. However, shoot and root dry weights increased over time (days 6–12) in an exponential manner: (a) shoot dry weight (mg) = 92.35 − [22.24 ∗ time] + [1.64 ∗ time^2]; $P < 0.001$; $R^2 = 0.88$, and (b) root dry weight (mg) = 16.59 − [3.60 ∗ time] + [0.27 ∗ time^2]; $P < 0.001$; $R^2 = 0.75$. Control mean ± SE for AGRSL (days 9–12) was 5.26 ± 0.24 cm^2/day. Control means ± SE for shoot and root dry weights for days 6–12 ranged from 16.46 ± 1.27 to 65.24 ± 3.59 mg, and from 4.26 ± 0.72 to 11.88 ± 1.06 mg, respectively.

The main treatment effects of time (days 6–12), soil-incorporated wheat shoot tissue (0 or 5 mg/g soil), and the interaction between incorporated shoot tissue and time significantly impacted log CFU per gram root of rhizosphere bacteria that utilize phenolic acids as a sole carbon source. The log CFU per gram cucumber root dry weight increased linearly over time for both soil with incorporated wheat shoot tissue (Figure 2; log CFU per gram root = 10.80 + [0.15 ∗ time]; $P = 0.004$; $R^2 = 0.30$) and without (Figure 2; log CFU per gram root = 10.23 + [0.16 ∗ time]; $P = 0.001$; $R^2 = 0.48$). Based on means comparisons, the log CFU per gram root were significantly higher than the controls only on days 7 (+496% change in actual CFU) and 12 (+333% change in actual CFU). The log CFU/per gram root for soil without incorporated wheat shoot tissue (i.e., control) on days 6, 7, 8, 10, and 12 was 11.04 ± 0.07, 11.56 ± 0.16, 11.38 ± 0.06, 12.16 ± 0.28, and 11.99 ± 0.07, respectively. Mean actual values ranged from 1.10×10^{11} to 1.44×10^{12} CFU per gram root.

Multiple Application of Phenolic Acid Mixture. The four-phenolic acid mixture (0.6 μmole/g soil) significantly impacted cucumber AGRSL (−24% ± 8 change; days 9–12) only when an outlier from the phenolic acid treatment was removed. No significant effects of the phenolic acid mixture were observed for

shoot and root dry weights over the 4 to 12-day period. However, both shoot (mg $= 51.12 - [12.34 * \text{time}] + [1.10 * \text{time}^2]$; $P < 0.001$; $R^2 = 0.88$) and root dry weight (mg $= -3.13 + [1.16 * \text{time}]$; $P < 0.001$; $R^2 = 0.69$ increased in an exponential and linear manner, respectively, with time. Control mean $\pm$ SE for AGRSL (days 9–12) was 5.26 ± 0.24 cm^2/day. Control means $\pm$ SE for shoot and root dry weights for days 6–12 ranged from 16.46 ± 1.27 to 65.24 ± 3.59 mg, and from 4.26 ± 0.72 to 11.88 ± 1.06 mg, respectively.

The main treatment effects of time (days 6–12), phenolic acid mixture (0 or 0.6 μmol/g soil), and the interaction between time and phenolic acid mixture significantly impacted log CFU per gram root dry weight of rhizosphere bacteria that utilize phenolic acids as a sole carbon source. The log CFU per gram root of the controls (no phenolic acid treatments) increased in a linear manner over time (Figure 2; log CFU per gram root $= 10.23 + [0.0163 * \text{time}]$; $P = 0.001$; $R^2 = 0.48$), while the log CFU per gram root increased in an asymptotic manner when the phenolic acid mixture (0.06 μmol/g soil) was applied to the soil (log CFU per gram root $= 4.21 + [1.50 * \text{time}] - [0.06 * \text{time}^2]$; $P < 0.001$; $R^2 = 0.83$). Based on means comparisons, the log CFU per gram root values were significantly higher than the controls on days 8 (+626% change in actual CFU per gram root) and 12 (+1029% change in actual CFU per gram root). The values of log

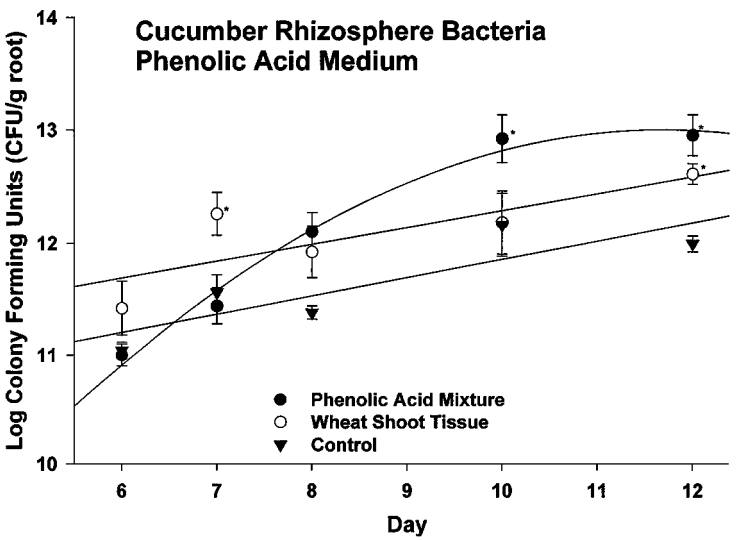


FIG. 2. Log colony-forming units of rhizosphere bacteria based on phenolic acid medium on days 6, 7, 8, 10, and 12 (Experiment 3) for cucumber seedlings treated with 0.6 μmol/g soil of a phenolic acid mixture or 5 mg/g soil wheat shoot tissue. Data in figures are means $\pm$ SE. Asterisks indicate significant differences between control and treatments.

CFU per gram root for soil without phenolic acid treatment (i.e., control) on days 6, 7, 8, 10, and 12 were 11.04 ± 0.07, 11.56 ± 0.16, 11.38 ± 0.06, 12.16 ± 0.28, and 11.99 ± 0.07, respectively. Mean actual CFU per gram root ranged from 1.10×10^{11} to 1.44×10^{12}.

Experiment 4

The purpose of experiment 4 was to determine the effects of soil-incorporated sunflower leaf tissue on pigweed seedling emergence (experiment 4a), bulk-soil bacteria that can utilize phenolic acids as a sole carbon source (experiment 4b), and cucumber seedlings and rhizosphere bacteria (day 17) that can utilize phenolic acids as a sole carbon source (experiment 4c). The incorporation of 0–10 mg/g soil of sunflower leaf tissue collected in 1999 to the Cecil A_p substrate significantly reduced (days 1–6) cumulative pigweed seedling emergence (Figure 3; % change in emergence $= -0.13 - [8.05 * \text{concentration}]$; $P < 0.001$; $R^2 = 0.70$; -81% change for 10 mg/g soil). Control mean $\pm$ SE of cumulative pigweed seedling emergence was 21.43 ± 2.27 out of 50 or 43%.

Sunflower leaf tissue significantly stimulated the bulk-soil bacteria that could utilize phenolic acids as a sole carbon source when these tissues ranging from 0 to 10 mg/g soil were incorporated into the Cecil A_p substrate. Log bacterial CFU (Figure 3; log CFU on day 6) per gram of soil (bulk soil) increased in a convex manner with increasing concentrations of soil-incorporated sunflower leaf tissue (log CFU $= 6.80 + [0.07 * \text{concentration}] - [0.004 * \text{concentration}^2]$; $P < 0.001$; $R^2 = 0.70$. Mean $\pm$ SE for CFU per gram for soils without tissue incorporated (i.e., control) was 6.80 ± 0.02 log CFU per gram soil (6.4×10^6 actual CFU per gram soil).

Cucumber AGRSL were inhibited by sunflower leaf tissue over days 9–11, 11–13, 13–15, and 15–17 (Table 2, Figure 3). Percent changes for these intervals were -76%, -60%, -55%, and -47% for the 10 mg/g soil treatment, respectively. Control means $\pm$ SE for AGRSL over days 9–11, 11–13, 13–15, and 15–17 were 6.79 ± 0.22, 8.94 ± 0.74, 11.63 ± 0.88, and 12.75 ± 0.64 (cm^2/day), respectively.

Shoot (% change $= 6.78 - [6.01 * \text{concentration}]$; $P < 0.001$; $R^2 = 0.61$; -53% change for 10 mg/g soil) and root dry weights (% change $= 0.063 - [6.31 * \text{concentration}]$; $P = 0.001$; $R^2 = 0.59$; -63% change for 10 mg/g soil) determined on day 17 were also significantly reduced by sunflower leaf tissue. Means $\pm$ SE (day 17) for shoot and root dry weights were 219.54 ± 12.83 and 38.58 ± 2.82 mg, respectively. Soil-incorporated sunflower leaf tissue, however, did not modify the cucumber seedling rhizosphere bacteria (day 17) that could utilize phenolic acids as a sole carbon source (log CFU per gram root dry weight $= 11.69 \pm 0.15$, actual CFU per gram root $= 4.87 \times 10^{11}$).

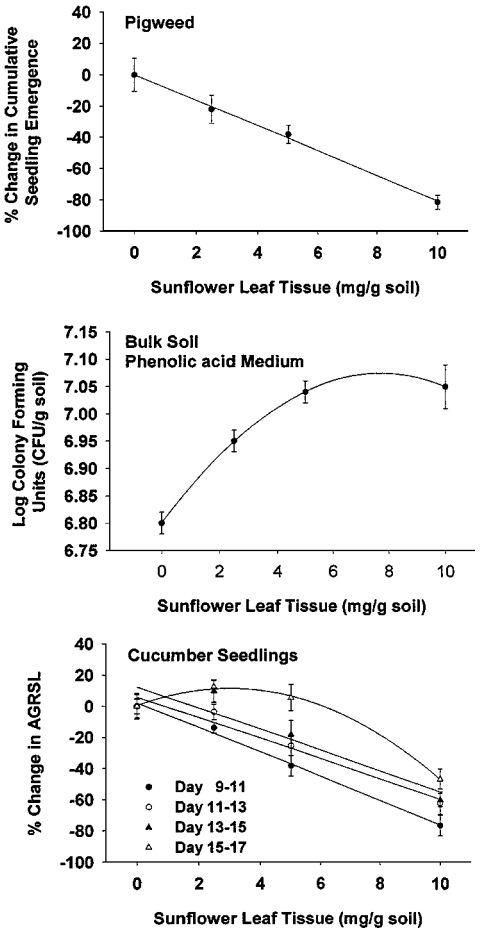


FIG. 3. Percent change in cumulative pigweed seedling emergence (Experiment 4a) log colony forming units of bulk soil bacteria (Experiment 4b) and cucumber seedling absolute growth rates for seedling leaf expansion (AGRSL; Experiment 4c) in the presence of soil-incorporated sunflower leaf tissue. For the sake of clarity, inhibitory effects are presented as negative numbers while stimulatory effects are presented as positive numbers. Data in figures are means ± SE.

Experiment 5

The purpose of experiment 5 was to determine the effects of soil-incorporated sunflower leaf tissue on cucumber seedlings and rhizosphere bacteria (day 12) that can utilize phenolic acids as a sole carbon source. The incorporation of 5 mg/g soil of sunflower leaf tissue to the Cecil A_p substrate significantly inhibited

AGRSL ($-25\% \pm 3$ change; days 9–12), shoot ($-27\% \pm 3$ change; day 12) and root dry weights ($-36\% \pm 6$ change; day 12) when compared to the control (no soil-incorporated sunflower leaf tissue). Mean control values $\pm$ SE for AGRSL and shoot and root dry weight were 6.42 ± 0.23 cm^2/day, 80.01 ± 4.01 mg, and 14.31 ± 1.23 mg, respectively. However, cucumber seedling rhizosphere bacteria (control $= 11.56 \pm 0.13$ log CFU per gram root dry weight, 3.63×10^{11} actual CFU per gram root) that could utilize phenolic acids as a sole carbon source were not changed in the presence of soil-incorporated sunflower leaf tissue.

Experiment 6

The purpose of experiment 6 was to determine how cucumber seedlings and rhizosphere bacteria that can utilize phenolic acids as a sole carbon source respond over time (days 6–12) in autoclaved (to reduce initial microbial populations) soil to soil-incorporated sunflower leaf tissue or multiple applications of chlorogenic acid.

Soil-Incorporated Sunflower Leaf Tissue. Soil-incorporated sunflower leaf tissue (5 mg/g soil) significantly reduced AGRSL ($-52\% \pm 11$ change), which was only determined over days 9–12. Control mean $\pm$ SE for AGRSL was 4.83 ± 0.51 cm^2/day.

The main treatment effects of time (days 6–12), soil-incorporated sunflower leaf tissue (0 or 5 mg/g soil), and the interaction between time and tissue significantly impacted shoot and root dry weights. Cucumber seedling shoot dry weight significantly increased over time in the presence ($\text{mg} = -5.85 + [3.54 * \text{time}]$; $P < 0.001$; $R^2 = 0.67$) and absence ($\text{mg} = -33.66 + [7.87 * \text{time}]$; $P < 0.001$; $R^2 = 0.88$) of soil-incorporated sunflower leaf tissue. Cucumber seedling root dry weight did not increase over time in the presence of soil-incorporated sunflower leaf tissue, while in the absence of soil-incorporated sunflower leaf tissue root dry weight increased in a linear manner with time ($\text{mg} = -1.03 + [0.88 * \text{time}]$; $P < 0.001$; $R^2 = 0.57$).

Based on means comparisons, soil-incorporated sunflower leaf tissue had no effect on shoot and root dry weight from days 6 to 8. On day 10, soil-incorporated sunflower leaf tissue significantly reduced shoot dry weight ($-27\% \pm 7$ change), but not root dry weight. On day 12, soil-incorporated sunflower leaf tissue significantly reduced shoot ($-43\% \pm 6$ change) and root dry weight ($-48\% \pm 10$ change) of cucumber seedlings. Control means $\pm$ SE (days 6–12) for shoot and root dry weight ranged from 18.14 ± 1.96 to 64.54 ± 3.76 and 4.78 ± 0.35 to 10.16 ± 1.48 (mg), respectively.

For rhizosphere bacteria grown on medium with chlorogenic acid as a sole carbon source, the main treatment effects of time (days 6–12) and incorporated sunflower leaf tissue (0 or 5 mg/g soil) and the interaction between time and tissue significantly impacted the log CFU per gram root dry weight. Rhizosphere bacteria

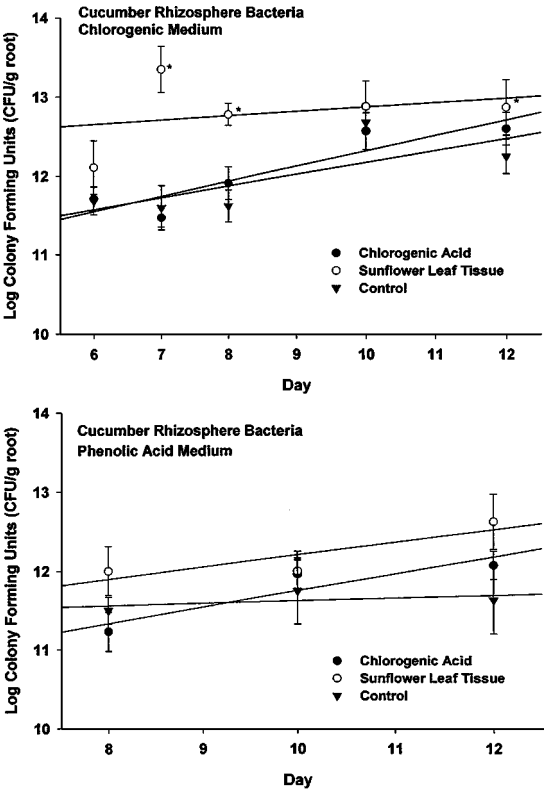


FIG. 4. Log colony forming units of cucumber rhizosphere bacteria based on chlorogenic medium or phenolic acid medium on days 6, 7, 8, 10, and 12 or days 8, 10, and 12, respectively (Experiment 6). Cucumber seedling systems were treated with 0.6 μmol/g soil chlorogenic acid or 5 mg/g soil sunflower leaf tissue. Data in figures are means $\pm$ Standard errors. Asterisks indicate significant differences between control and treatments.

were harvested on days 6, 7, 8, 10, and 12. Log CFU per gram root of chlorogenic acid utilizing rhizosphere bacteria for the soil-incorporated sunflower leaf tissue treatment did not change with time. However, log CFU per gram root of the control (no tissue incorporated) significantly increased in a linear manner (Figure 4; log CFU per gram root $= 10.69 + [0.15 * \text{time}]$; $P = 0.005$; $R^2 = 0.31$).

Based on means comparisons, incorporated sunflower leaf tissue significantly stimulated log CFU per gram root of rhizosphere bacteria grown on media with chlorogenic acid as a sole carbon source on days 7 (+4798% change of actual CFU per gram root), 8 (+1095% change of actual CFU per gram root), and 12 (+586% change of actual CFU per gram root) when compared to their respective controls

(Figure 4). Means ± SE for control log CFU per gram root for days 6, 7, 8, 10, and 12 were 11.68 ± 0.17, 11.60 ± 0.28, 11.62 ± 0.20, 12.68 ± 0.12, 12.25 ± 0.22 log CFU, respectively. Mean actual CFU per gram root ranged from 3.98×10^{11} to 4.79×10^{12}.

For rhizosphere bacteria grown on medium with phenolic acids as a sole carbon source, the main treatment effect of soil-incorporated sunflower tissue (5 mg/g soil) significantly increased the log CFU per gram root of rhizosphere bacteria (+279% change in actual CFU per gram root) when compared to the control (11.63 ± 0.10 log CFU per gram root; 4.27×10^{11} actual CFU per gram root). There was no significant change in log CFU per gram root over time (days 8, 10, and 12) for control and tissue treatments (Figure 4).

Multiple Application of Chlorogenic Acid. Multiple applications of chlorogenic acid (0.6 μmol/g soil) had no effect on cucumber AGRSL (control; days 9–12 = 4.83 ± 0.51 cm^2/day) or shoot or root dry weights. However, time (days 6–12) significantly impacted shoot dry weight in an exponential manner (mg = $64.03 - [14.85 * \text{time}] + [1.24 * \text{time}^2]$; $P < 0.001$; $R^2 = 0.89$) and root dry weight in a linear manner (mg = $-0.17 + [0.78 * \text{time}]$; $P < 0.001$; $R^2 = 0.58$). Mean ± SE (days 6–12) for control shoot and root dry weight ranged from 18.14 ± 1.96 to 64.54 ± 3.76 and 4.78 ± 0.35 to 10.16 ± 1.48 (mg), respectively.

For rhizosphere bacteria grown on medium with chlorogenic acid as a sole carbon source, the multiple applications of 0.6 μmol/g soil of chlorogenic acid had no effect on the log CFU per gram root dry weight when compared to the control (no applications of chlorogenic acid). The log CFU per gram root of rhizosphere bacteria for control and treatment increased over time (Figure 4; log CFU per gram root = $10.39 + [0.19 * \text{time}]$; $P < 0.001$; $R^2 = 0.50$). The log CFU per gram root ranged from 11.53 ± 0.15 (3.39×10^{11} actual CFU per gram root) to 12.62 ± 0.12 (4.17×10^{12} actual CFU per gram root).

For rhizosphere bacteria grown on medium with phenolic acids as a sole carbon source, the multiple applications of chlorogenic acid had no effect on the log CFU per gram root of phenolic acid utilizing bacteria in the rhizosphere of cucumber seedlings. However, the rhizosphere log CFU per gram root dry weight of cucumber seedlings for both control and treatment increased (log CFU = $10.47 + [0.12 * \text{time}]$; $P = 0.025$; $R^2 = 0.17$) with time (days 8, 10, and 12). The log CFU per gram root ranged from 11.35 ± 0.15 (2.2×10^{11} actual CFU per gram root) to 11.85 ± 0.15 (7.1×10^{11} actual CFU per gram root).

DISCUSSION

We hypothesized that soil-incorporated wheat or sunflower tissue would simultaneously inhibit cucumber seedling absolute rates of seedling leaf expansion (AGRSL) and stimulate the log colony forming units (CFU) per gram root

of phenolic acid utilizing rhizosphere bacteria, and therefore provide indirect evidence of phytotoxin transfer from residues in the bulk soil to the rhizosphere of a sensitive species. In nonautoclaved soil, soil-incorporated sunflower or wheat tissue inhibited cucumber seedling AGRSL but had no effect on the log CFU per gram root of phenolic acid utilizing bacteria. In autoclaved soil (−25% reduction in initial microbial populations), soil-incorporated wheat tissue significantly stimulated the log CFU per gram root of phenolic acid utilizing bacteria, but had no effect on cucumber seedling AGRSL. However, soil-incorporated sunflower tissue did simultaneously inhibit cucumber seedling AGRSL and stimulate log CFU per gram root of phenolic acid utilizing rhizosphere bacteria.

Allelopathic cover crop residues that will provide consistent year-to-year weed control and help reduce the use of preemergence herbicides have been eagerly sought by farmers, weed scientists, regulators, and environmentalists. However, the complex nature of the soil environment, the intensity of biological activity and associated chemical transformation within soils (e.g., sorption, oxidation, microbial interconversions, polymerization, and leaching), and the lack of direct access to roots in the field have prevented researchers from actually documenting the transfer and efficacy of phytotoxins produced by cover crop residues, such as wheat residues, under field conditions. At present, no definitive data on direct transfer of phytotoxins (i.e., substances that inhibit germination and/or seedling growth at field concentrations) from putative cover crop residues in or on the soil to roots of sensitive weed or crop species have been obtained under field conditions. The objective of this research was to determine if evidence for such transfer could be obtained by other than direct means, since evidence for direct transfer has eluded researchers so far.

Wheat residues have been observed to be phytotoxic to a variety of weeds, including pigweed, prickly sida, and morning-glory (Lehman and Blum, 1997; Blum et al., 1997). Potential allelopathic compounds identified in living and decomposing tissue of small grain cover crops, such as wheat, include phenolic acids (Liebl and Worsham, 1983; Blum et al., 1991), organic acids (Patrick, 1971; Lynch, 1977; Tang and Waiss, 1978; Shilling et al., 1985), hydroxamic acids and related compounds (Willard and Penner, 1976; Niemeyer et al., 1989), and volatiles (Buttery et al., 1985; Bradow, 1991). However, phenolic acids, in particular cinnamic (e.g., ferulic acid, *p*-coumaric acid) and benzoic acid (e.g., p-hydroxybenzoic acid, vanillic acid) derivatives, have been the most frequently cited allelopathic agents for wheat residues.

It has also been observed that phenolic acids, individually or in mixtures, applied to soil–microbe–plant systems not only inhibit germination and seedling growth, but also stimulate bulk soil and rhizosphere bacteria that can utilize phenolic acids as a sole carbon source (Blum and Shafer, 1988; Shafer and Blum, 1991; Blum et al., 2000). More importantly, Blum et al. (2000) noted that there was an inverse relationship between phytotoxicity and rhizosphere bacterial populations

that could utilize phenolic acids as a sole carbon source when cucumber seedlings growing in Cecil A_p soil were treated with a phenolic acid mixtures.

Phenolic acids released from wheat residues, however, do not enter soils in isolation. In fact, leachates from such debris are not only composed of a variety of phenolic acids, but also of other organic molecules and inorganic ions. Such complex mixtures of promoters, inhibitors, ions, and carbon sources will influence plant growth and associated microbial populations in a variety of ways. For example, the presence of carbohydrates, amino acids, or other organic molecules can increase the inhibitory activity of phenolic acids in soils; as the addition of noninhibitory levels of glucose or inhibitory levels of methionine to soil has been shown to increase the inhibitory activity of *p*-coumaric acid on morning-glory seedling biomass production (Blum et al., 1993; Pue et al., 1995). Additionally, Pue et al. (1995) and Blum et al. (2000) observed that phenolic acid utilization by soil microbes was reduced in the presence of other more readily available carbon sources. This induction and/or selection of phenolic acid–utilizing bacteria in bulk soil and in the rhizosphere of cucumber seedlings was not dramatically impacted by the presence of another carbon source, such as glucose. However, no matter how complex such debris leachates may be, if phenolic acids are to have a phytotoxic effect, they must pass through the rhizosphere where they will, depending on the rate of their movement and the nature of the rhizosphere bacterial populations, lead to various levels of selection and/or induction of phenolic acid–utilizing bacteria.

If the observations made for phenolic acid treatments could be demonstrated for wheat residues or other plant residues high in phenolic acids, such as sunflower (Armstrong et al., 1970, 1971; Hall et al., 1982, 1983; Macias et al., 1993, 1998, 1999), then the simultaneous inhibition of seedling growth and stimulation of rhizosphere bacteria that can utilize phenolic acids as a sole carbon source could provide indirect evidence for the transfer of phenolic acids released from soil debris particles to the rhizosphere and root surface of sensitive species.

Before field trials were initiated, however, the potential of the simultaneous inhibition of plant growth and stimulation of phenolic acid–utilizing rhizosphere bacteria was tested for wheat and sunflower tissue in the laboratory. Since the phytotoxicity of wheat and sunflower tissue varies with growth conditions and age of plants (Koeppe et al., 1970; Armstrong et al., 1970, 1971; del Moral, 1972; Lehman and Blum, 1997), we utilized pigweed seedling emergence to determine if phytotoxicity was similar to past experiments. The levels of phytotoxicity for wheat and sunflower tissue observed for pigweed seedling emergence were consistent with those observed by Lehman and Blum (1997) and Hall et al. (1982, 1983), respectively. However, Hall et al. (1983) observed that total phenolic acid content in sunflower tissue did not account for a significant amount of inhibition of pigweed plant biomass unless the N, P, K content of the sunflower tissue was included in the response model. Total phenolic acid and N added to soil by sunflower tissue

were negatively correlated with pigweed plant biomass, whereas P and K were positively correlated.

Total phenolic acid content ± SE for wheat shoot and root tissue and sunflower leaf tissue determined by Folin and Ciocateau's phenol reagent were 98 ± 5, 75 ± 0.6, and 465 ± 67 μg/g as ferulic acid equivalents, respectively. The inhibition of pigweed seedling emergence for 5 mg/g soil-incorporated wheat shoot and root tissue and sunflower leaf tissue were −66%, −10%, and −40%, respectively. The unexpected pattern of ferulic acid equivalence and percent inhibition was very likely a result of both qualitative and quantitative tissue differences in the phenolic acids present (e.g., for wheat cinnamic and benzoic acid derivatives and sunflower chlorogenic acids, isochlorogenic acids, the coumarins scopolin and ayapin, cinnamic acid derivatives, and benzoic acid derivatives) (Wilson and Rice, 1968; Koeppe et al., 1970; Macias et al., 1999), differences in color development for different phenolic acids (Blum et al., 1991), and the presence of a number of other phytotoxins (see Macias et al., 1999) and nontoxins (Gerig and Blum, 1991; Blum et al., 2000).

We chose to use the cucumber bioassay system used by Shafer and Blum (1991) and Blum et al. (2000) to test the effects of wheat shoot and sunflower leaf tissues on cucumber seedling absolute rates of leaf expansion and rhizosphere bacteria that could utilize phenolic acids as a sole carbon source. For comparative purposes we discuss only the results for 5 mg/g tissue soil treatments and the 0.6 μmol/g soil phenolic acid treatments.

The inhibition of AGRSL by 5 mg/g soil-incorporated wheat shoot and sunflower leaf tissue in both autoclaved and nonautoclaved soil ranged from −0% to −53% and from −25% to −52%, respectively. Such variation in inhibition by similar concentrations of plant tissue samples is not uncommon and results from differences in tissue chemistries and bioassay environments. Recall that new tissue samples were ground for each experiment. The inhibition of AGRSL by the 0.6 μmol/g soil four-phenolic acid mixture (primary phenolic acids in wheat) and the 0.6 μmol/g soil chlorogenic acid (primary phenolic acid in sunflower) treatments in autoclaved (to reduce initial microbial populations) soil were −24% and 0%, respectively. This effect of the phenolic acid mixture is consistent with Blum et al. (2000), who found that the 0.6 μmol/g soil phenolic acid mixture reduced cucumber seedling AGRSL by 20–23%. The absence of a chlorogenic acid effect on seedling growth, in the presence of nutrient solution treatments, has also been observed by Hall et al. (1983) for pigweed seedlings. The stimulation of rhizosphere bacteria that utilize phenolic acids as a sole carbon source in both autoclaved and nonautoclaved soil was just as variable from one tissue experiment to another as that of the inhibition of cucumber seedling AGRSL. Stimulation of rhizosphere bacteria ranged from 0% to +496% when 5 mg/g wheat shoot tissue was incorporated into soil and ranged from +0% to +4798% when 5 mg/g sunflower leaf tissue was incorporated into soil. The stimulation of rhizosphere bacteria over days 5–11

ranged from +0% to +1029% for the 0.6 μmol/g soil four-phenolic acid mixture (primary phenolic acids in wheat), but was not modified by the 0.6 μmol/g soil chlorogenic acid (primary phenolic acid in sunflower).

In nonautoclaved soil, the incorporation of wheat or sunflower tissue consistently inhibited cucumber seedling AGRSL, but had no effect on log CFU per gram root of phenolic acid–utilizing bacteria. Potential reasons for these results could be that phenolic acid–utilizers were not competitive in nonautoclaved soils and that these populations were already at carrying capacity. Another potential reason could be that cucumber inhibition was not due solely to phenolic acids, but to a complex mixture of molecules such as organic acids, volatiles, hydroxamic acids, etc., so selection pressure of phenolic acids was negated. In autoclaved soil (−25% reduction in initial populations), the log CFU per gram root of phenolic acid–utilizing rhizosphere bacteria consistently increased while cucumber seedling AGRSL declined only when sunflower tissue was incorporated into the soil. A possible reason for these results could be that phenolic acids released by wheat tissue stimulated rhizosphere bacteria that could utilize phenolic acids as a sole carbon source; the bacteria utilized the phenolic acids and prevented them from reaching the root surface. Conversely, levels of phenolic acids in sunflower tissue were much higher than in wheat, so the bacteria may not have utilized them all, thus cucumber seedling AGRSL was inhibited.

Blum et al. (2000) used the range of response by individual seedlings and their rhizosphere phenolic acid-utilizing bacteria to the 0.6 μmol/g soil equimolar four-phenolic acid mixture from several experiments to determine how changes in phenolic acid-utilizing rhizosphere bacteria might influence seedling inhibition and found that an increase of 500% in rhizosphere phenolic acid-utilizing bacteria would lead to a 5% decrease (e.g., 20–25%) in inhibition of absolute rates of leaf expansion. Attempts to model these relationships were not successful from our data due to low replicate numbers. The soils used by Blum et al. (2000) and for these experiments were collected at the same location but several years apart. For our soils, the log CFU of bacteria that could utilize phenolic acids as a sole carbon source at harvest were much higher. The log CFU per gram root for Blum et al. (2000) experiments were all below 11, whereas for our experiments they were all above 10 and ranged up to 13 log CFU per gram root. The inconsistent relationships between inhibition of AGRSL and the stimulation of rhizosphere phenolic acid-utilizing bacteria may thus have been a result of these high bacterial populations, since no response would be expected if bulk-soil and rhizosphere bacteria were phenolic acid limited and/or the resulting level of phenolic acids reaching the root surfaces were too low for inhibition. This observation is supported by the fact that consistent relationships were only observed in autoclaved soil (i.e., soils with lower microbial populations).

Since a simultaneous expression of inhibition for cucumber seedling AGRSL and stimulation of the community of rhizosphere bacteria that could utilize phenolic

acids as a sole carbon source occurred only for phenolic acid treatments and incorporated sunflower tissue in autoclaved soil, we are left to conclude for the present set of experiments that the strong selection pressure observed for individual phenolic acids or phenolic acid mixtures by Blum et al. (2000) was not consistently evident for either the phenolic acid mixture treatments or the incorporated wheat shoot and sunflower leaf tissue treatments in these nonautoclaved soils.

Inhibition of AGRSL, however, was observed for nonautoclaved soils in the absence of a stimulation of rhizosphere phenolic acid-utilizing bacteria for both incorporated wheat and sunflower tissue. This suggests that the inhibition observed for both autoclaved (only to reduce initial microbial populations) and nonautoclaved soil was due to phytotoxin mixtures, which may have included phenolic acids (see beginning of Discussion for potential list of phytotoxins). At the same time the effects of phenolic acids were clearly evident (i.e., stimulation of phenolic acid–utilizing bacteria) when phenolic acid-utilizing bacteria were free (e.g., could be due to the absence of more aggressive microbial species in autoclaved soil or that reduced microbial populations prevented phenolic acid carbon from being limiting) to respond to the phenolic acid carbon source.

In summary, our data suggest that given the right conditions (e.g., right levels of phenolic acid concentrations and bulk-soil and rhizosphere bacterial populations), it is possible to observe a simultaneous inhibition of seedling growth and stimulation of phenolic acid–utilizing rhizosphere bacteria as was hypothesized, and, thus, provide indirect evidence for phenolic acid transfer from sources within the soil (e.g., plant debris) to root surfaces. We suspect that such simultaneous behavior will probably be even more difficult to detect under field conditions and thus is unlikely to be a useful tool for characterizing phenolic acid transfer from the bulk soil to the rhizosphere of sensitive species. However, it is possible that this screening procedure may be useful for phytotoxins that are more unique than phenolic acids.

REFERENCES

ANDERSON, R. A., and TODD, J. R. 1968. Estimation of total tobacco plant phenols by their bonding to polyvinylpyrrolidone. *Tobacco Sci*. 12:107–111.

ARMSTRONG, G. M., ROHRBAUGH, L. M., RICE, E. L., and WENDER, S. H. 1970. The effect of nitrogen deficiency on concentration of caffeoylquinic acids and scopolin in tobacco. *Phytochemistry* 9:945–948.

ARMSTRONG, G. M., ROHRBAUGH, L. M., RICE, E. L., and WENDER, S. H. 1971. Preliminary studies on the effect of deficiency in potassium or magnesium on concentration of chlorogenic acid and scopolin in tobacco. *Proc. Okla. Acad. Sci.* 51:41–43.

BARNES, J. P., PUTNAM, A. R., and BURKE, A. 1986. Allelopathic activity of rye (*Secale cereale* L.), pp. 271–286, *in* A. R. Putnam and C. S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

BLUM, U., and SHAFER, S. R. 1988. Microbial populations and phenolic acids in soil. *Soil Biol. Biochem.* 20:793–800.

BLUM, U., DALTON, B. R., and SHANN, J. R. 1985. Effects of ferulic and *p*-coumaric acids in nutrient culture on cucumber leaf expansion as influenced by pH. *J. Chem. Ecol.* 11:1567–1582.

BLUM, U., GERIG, T. M., and WEED, S. B. 1989. Effects of mixtures of phenolic acids on leaf area expansion of cucumber seedlings grown in different pH Portsmouth A_1 soil materials. *J. Chem. Ecol.* 15:2413–2423.

BLUM, U., WENTWORTH, T. R., KLEIN, K., WORSHAM, A. D., KING, L. D., GERIG, T. M., and LYU, S. W. 1991. Phenolic acid content of soils from wheat–no till, wheat–conventional till, and fallow–conventional till soybean cropping systems. *J. Chem. Ecol.* 17:1045–1068.

BLUM, U., GERIG, T. M., WORSHAM, A. D., and KING, L. D. 1993. Modification of allelopathic effects of *p*-coumaric acid on morning-glory seedling biomass by glucose, methionine, and nitrate. *J. Chem. Ecol.* 19:2791–2811.

BLUM, U., KING, L. D., GERIG, T. M., LEHMAN, M. E., and WORSHAM, A. D. 1997. Effects of clover and small grain cover crops and tillage techniques on seedling emergence of some dicotyledonous weed species. *Am. J. Alt. Agric.* 12:146–161.

BLUM. U., AUSTIN, M. F., and SHAFER, S. R. 1999. The fates and effects of phenolic acids in a plant–microbial–soil model system, pp. 159–166, *in* F. A. Macias, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds.). Recent Advances in Allelopathy, Vol. I, A Science for the Future. Cadiz University Press, Cadiz, Spain.

BLUM, U., STAMAN, K. L., FLINT, L. J., and SHAFER, S. R. 2000. Induction and/or selection of phenolic acid-utilizing bulk-soil and rhizosphere bacteria and their influence on phenolic acid phytotoxicity. *J. Chem. Ecol.* 26:2059–2078.

BRADOW, J. M. 1991. Relationships between chemical structure and inhibitory activity of C_6 through C_9 volatiles emitted by plant residues. *J. Chem. Ecol.* 17:2193–2212.

BUTTERY, R. G., XU, C. J., and LING, L. C. 1985. Volatile components of wheat leaves (and stems): Possible insect attractants. *J. Agric. Food Chem.* 33:115–117.

CHOU, C. H., and PATRICK, Z. A. 1976. Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. *J. Chem. Ecol.* 2:369–387.

DEL MORAL, R. 1972. On the variability of chlorogenic acid concentration. *Oecologia* 9:289–300.

GERIG, T. M., and BLUM, U. 1991. Effects of mixtures of four phenolic acids on leaf area expansion of cucumber seedlings grown in Portsmouth B_1 soil materials. *J. Chem. Ecol.* 17:29–40.

HALL, A. B., BLUM, U., and FITES, R. C. 1982. Stress modification of allelopathy of *Helianthus annuus* L. debris on seed germination. *Am. J. Bot.* 69:776–783.

HALL, A. B., BLUM, U., and FITES, R. C. 1983. Stress modification of *Helianthus annuus* L. debris on seedling biomass production of *Amaranthus retroflexus* L. *J. Chem. Ecol.* 9:1213–1222.

HARRIS, R. F., and SOMMERS, L. E. 1968. Plate-dilution frequency technique for assay of microbial ecology. *Appl. Microb.* 6:330–334.

KOEPPE, D. E., ROHRBAUGH, L. M., RICE, E. L., and WEBDER, S. H. 1970. The effects of age and chilling temperatures on the concentration of scopolin and caffeoylquinic acids in tobacco. *Physiol. Plant.* 23:258–266.

LEHMAN, M., and BLUM, U. 1997. Cover crop effects on weed emergence as modified by environmental factors. *Allelopathy J.* 4:69–88.

LIEBL, R. A., and WORSHAM, A. D. 1983. Inhibition of pitted morningglory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J. Chem. Ecol.* 9:1027–1043.

LOPER, J. E., SUSLOW, T. V., and SCHROTH, N. 1984. Lognormal distribution of bacterial populations in the rhizosphere. *Phytopathology* 74:1454–1460.

LYNCH, J. M. 1977. Phytotoxicity of acetic acid produced in the anaerobic decomposition of wheat straw. *J. Appl. Bacterial* 42:81–87.

MACIAS, F. A., VARELA, R. M., TORRES, A., and MOLINILLO, J. M. G. 1993. Potential allelopathic guaianolides from cultivar sunflower leaves, var. SH-222. *Phytochemistry* 34:669–674.

MACIAS, F. A., VARELA, R. M., TORRES, A., OLIVA, R. M., and MOLINILLO, J. M. G. 1998. Bioactive norsesquiterpenes from *Helianthus annuus* with potential allelopathic activity. *Phytochemistry* 48:631–636.

MACIAS, F. A., MOLINILLO, J. M. G., VARELA, R. M., TORRES, A., and GALINDO, C. G. 1999. Bioactive compounds from the genus *Helianthus*, pp. 121–148, *in* F. A. Macias, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds). Recent Advances in Allelopathy, Vol. I, A Science for the Future. Cadiz University Press, Cadiz, Spain.

NIEMEYER, H. M., PESEL, E., CAPAJA, S. V., BRAVO, H. R., FRANKE, S., and FRANCKE, W. 1989. Changes in hydroxamic acid levels of wheat plants induced by aphid feeding. *Phytochemistry* 28:447–779.

PATRICK, Z. A. 1971. Phytotoxic substances associated with the decomposition in the soil of plant residue. *Soil Sci.* 111:13–18.

PUE, K. J., BLUM, U. GERIG, T. M., and SHAFER, S. R. 1995. Mechanisms by which noninhibitory concentrations of glucose increase inhibitory activity of p-coumaric acid on morning-glory seedling biomass accumulation. *J. Chem. Ecol.* 21:833–847.

SHAFER, S. R., and BLUM, U. 1991. Influence of phenolic acids on microbial populations in the rhizosphere of cucumber. *J. Chem. Ecol.* 17:369–389.

SHILLING, D. G., LIEBL R. A., and WORSHAM A. D., 1985. Rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) mulch: The suppression of certain broadleaved weeds and the isolation and identification of phytotoxins, pp. 243–271, *in* A. C. Thompson (ed.). The Chemistry of Allelopathy: Biochemical Interactions Among Plants. ACS Symposium Series 268. American Chemical Society, Washington, DC.

TANG, C. S., and WAISS, A. C. 1978. Short chain fatty acids as growth inhibitors in decomposing wheat straw. *J. Chem. Ecol.* 4:225–232.

WILLARD, J. I., and PENNER, D. 1976. Benzoxazinones: Cyclic hydroxamic acids found in plants. *Residue Rev.* 64:67–76.

WILSON, R. E., and RICE, E. L. 1968. Allelopathy as expressed by *Helinathus annuus* and its role in old-field succession. *Bull. Torrey Bot. Club* 95:432–448.

WILLIS R. J. 1985. The historical basis of the concept of allelopathy. *J. Hist. Biol.* 18:71–102.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

BUTYL ACETATE AND YEASTS INTERACT IN ADHESION AND GERMINATION OF *Botrytis cinerea* CONIDIA *IN VITRO* AND IN FUNGAL DECAY OF GOLDEN DELICIOUS APPLE

A. B. FILONOW

Department of Entomology & Plant Pathology
Oklahoma State University
Stillwater, Oklahoma 74078

(Received June 24, 2000; accepted December 13, 2000)

Abstract—Butyl acetate is a volatile aroma and flavor compound in apple. Conidia of three strains of *Botrytis cinerea*, a fungus that causes decay of apple fruit in postharvest storage, had greater adhesion to and greater germination on polycarbonate membrane filters on water inside sealed 500 cc glass jars that were injected with 4 μl butyl acetate than conidia not so exposed. Conidial germination was highly correlated with conidial adhesion. The yeasts *Sporobolomyces roseus* and *Cryptococcus laurentii*, but not *Saccharomyces cerevisiae*, reduced the adhesion and germination promoting effect of butyl acetate. Conidia did not readily utilize butyl acetate as a food source, as shown by lack of tetrazolium violet reduction, whereas *S. roseus* and *C. laurentii*, but not *S. cerevisiae* did. Butyl acetate added to suspensions of conidia increased the electrical conductivity of the suspensions and increased the loss of ^{14}C from ^{14}C-labeled conidia compared to conidia unexposed to butyl acetate. Uptake of [^{14}C]glucose by conidia was not increased by butyl acetate. Wounds of Golden Delicious apples inoculated with conidia (strain F-J-4) in a dilute solution of butyl acetate had greater decay than unexposed wounds. *S. roseus* and *C. laurentii*, but not *S. cerevisiae*, added with the conidia decreased the incidence or size of decay. Results indicated that butyl acetate increased conidial adhesion, stimulating conidial germination, and some yeasts can reduce this effect.

Key Words—Fungal spores, yeasts, germination, adhesion, butyl acetate, *Botrytis cinerea*, biological control, volatiles from apple, fungal ecology.

INTRODUCTION

Apple fruit produce acetate esters that influence aroma and flavor of the fruit (Mattheis et al., 1991; Knee, 1993; Fellman and Mattheis, 1995). In addition,

0098-0331/01/0400-0831$19.50/0

acetate esters may affect microbial growth and interactions on the surface of apple fruit. Filonow (1999) found that ethyl, butyl, and hexyl acetate stimulated *in vitro* germination of conidia of *Botrytis cinerea* Pers.:Fr, a fungus that causes a decay of apples and other fruits. These results support earlier findings (Brown, 1922) that ethyl acetate from apples stimulated germination of *B. cinerea* conidia. In addition, Filonow (1999) found that germination of conidia was stimulated by acetate esters on Golden Delicious apples, and apples in jars exposed to butyl acetate had more fungal decay than apples unexposed to butyl acetate. This work was done with one strain (F-J-4) of *B. cinerea*, and it is not known whether other strains will respond to acetate esters in a similar way. There appears to be no other study linking acetate ester exposure to enhanced fungal decay in apple. Furthermore, how acetate esters stimulate the germination of *B. cinerea* conidia is not known. Since adhesion of conidia of *B. cinerea* to substrates appears to be a necessary event preceding conidial germination (Doss et al., 1993, 1995), the present study assessed the link between acetate ester exposure and conidial adhesion.

Acetate esters from apple also may serve as a food source for some yeasts (Vishniac et al., 1997). Yeasts are common inhabitants of apple fruit, and several yeasts are known antagonists of filamentous fungi that cause fruit decay in postharvest storage (Janisiewicz et al., 1994; Filonow et al., 1996; Filonow, 1998). Filonow (1998) found that *Sporobolomyces roseus* FS-43-238 and *Cryptococcus laurentii* BSR-Y22 were highly effective in protecting apple wounds from decay caused by *B. cinerea*, but *Saccharomyces cerevisiae* BY-1 was not. In this study, nutrient competition for sugars played a role, but some other factor was most likely involved. In this regard, Filonow (1999) found that *C. laurentii* BSR-Y22, and *S. roseus* FS-43-238 were more effective than *S. cerevisiae* BY-1 in reducing the stimulatory effect that acetate esters have on *in vitro* germination of *B. cinerea* conidia. The mechanism by which some yeasts reduce this stimulatory effect is unknown.

The objectives of this research were: (1) to determine if conidia of other strains of *B. cinerea* are stimulated to germinate by butyl acetate; (2) to find the cause for the stimulatory effect, in particular to assess the role of conidial adhesion; and (3) to determine why some yeasts are more capable than others in reducing the stimulatory effect.

METHODS AND MATERIALS

Fungi and Culture. The sources for *Botrytis cinerea* F-J-4, *Sporobolomyces roseus* FS-43-238, *Cryptococcus laurentii* BSR-Y22, and *Saccharomyces cerevisiae* BY-1 were previously reported (Filonow, 1999). Strains WMA1 and WMG2 of *B. cinerea* were isolated by the author from infected Golden Delicious apple and geranium, respectively, obtained from a local supermarket. Maintenance of *B. cinerea* strains and the production of conidia on potato-dextrose agar

(PDA; Difco, Inc. Detroit, Michigan) have been reported elsewhere (Filonow et al., 1996). A PDA dish was inverted and tapped on a table to dislodge conidia on the inside of the dish lid. Conidia were suspended in sterile double-distilled (sdd) water with the aid of a rubber policeman, transferred to a 50-ml sterile centrifuge tube, and conidial density determined in a Brightline hemacytometer (Hausser Sci., Horsham, Pennsylvania). Suspensions were adjusted with water and conidial densities varied depending on the experiment. Maintenance of yeasts and their culture have been described (Filonow et al., 1996). Yeast densities in suspensions were 1×10^7 CFU/ml unless otherwise noted.

Effect of Butyl Acetate and Yeasts on Adhesion and Germination of Conidia In Vitro. Two hundred microliters of water (sdd) or a suspension of *S. roseus*, *C. laurentii*, or *S. cerevisiae* were aseptically pipetted into each of two autoclaved stainless steel wells (4 mm $\times$ 250 mm diam.) affixed to double-stick tape on a glass microscope slide. Membrane filters bearing 4×10^4 conidia of each strain of *B. cinerea* were prepared (Filonow, 1999). Membrane filters used in experiments were quarter sections cut from 47-mm-diam. polycarbonate membrane filters (1.0-μm-diam. pore; Osmonics, Inc, Livermore, California). Membranes were placed on the water or yeast suspensions, and two wells were placed inside an autoclaved 500-cc glass Mason jar (Kerr, Inc., Los Angeles, California) resting on its side. The jar was sealed with a lid fitted with a rubber septum. For each strain of *B. cinerea*, there were 24 jars, representing one experiment. Twelve jars were injected with 4 μl of butyl acetate (99.9% pure) (Fluka Chemika), whereas the others were not. There were three jars per treatment or control that were exposed or not-exposed to butyl acetate. Jars were randomized on a bench. After 24 hr at 22°C, one filter from a jar was assessed for germination of conidia (Filonow, 1999), and the other filter was pressed to the inside of a glass funnel (3 cm diam. $\times$ 6 cm stem). The funnel was put into a 15-ml glass centrifuge tube and positioned under a 10-ml glass disposable pipet containing 10 ml of water. The column of water in the pipet was held in place by pinch clamping a rubber tube attached to the top of the pipet, thereby stopping air pressure on the water column. The distance from the tip of the pipet to the top of the filter was 13 cm. Opening the pinch clamp caused the water to stream down on the filter, dislodging conidia. Residual water in the stem of the funnel was blown into the centrifuge tube and the tube capped. The filter in the funnel was discarded, the funnel washed with a squirt of water, and the process repeated for the next sample. Centrifuge tubes were centrifuged at 2800 rpm for 20 min. The supernatant was reduced to 2 ml by gentle suction into a vacuum flask. Then 2 ml were vortex mixed and the number of conidia per milliliter estimated in a hemacytometer from two counts per tube. The total number of conidia in nine fields was considered one count. Percentage adhesion was calculated from the mean number of conidia dislodged from a filter unexposed to butyl acetate minus the mean number dislodged from an exposed filter divided by the mean number dislodged from an unexposed filter $\times 100$.

Utilization of Butyl Acetate by Yeasts or Conidia of B. cinerea *Strains As Indicated by Tetrazolium Violet Reduction In Vitro.* Microorganisms capable of catabolizing certain organic substrates in a colorless tetrazolium violet (TV; Sigma Chemical, Inc., St. Louis, Missouri) solution reduce the TV to a violet-colored formazan (Bochner and Savageau, 1977). This method was used to assess the capability of yeasts or *B. cinerea* conidia to use butyl acetate as food. The TV preparation (TVP) consisted of 2.1 g K_2HPO_4, 0.9 g KH_2PO_4, 0.4 g proteose peptone (Difco, Inc.), 40 mg $MgSO_4 \cdot 7H_2O$, 100 μl 1 M NH_4NO_3, 20 μl 1 mM thiamine HCl, 20 μl 1 mM biotin, and 20 mg TV in 100 ml of water. Brief sonication at different steps helped solubilize some of the ingredients. The very pale yellow solution was filter-sterilized (0.2-μm pore) and stored in sterile centrifuge tubes at 4°C until use. One hundred microliters each of TVP, a suspension of test organisms, and water were aseptically pipetted into autoclaved 22-ml glass scintillation vials. Test organisms were suspensions (3×10^8 CFU/ml) of each yeast or conidia (3×10^7 conidia/ml) of each *B. cinerea* strain in sterile saline (0.9% NaCl). Vials were placed in upright glass jars, and the lids were sealed. Each test organism was in two vials per jar. Half of the jars had 4 μl of butyl acetate injected into them, and the other half did not. Some jars contained vials with TVP, a test organism, and 100 μl of 20 mM glucose or vials with TVP, water instead of a test organism, and 100 μl of 20 mM glucose. These jars were not injected with butyl acetate. There were three jars per treatment. After incubation at 22°C for 24 hr, the color of solutions in the vials was rated: 1 = no purple color, 2 = light purple, 3 = intermediate purple, and 4 = intense purple.

Effect of Butyl Acetate on Electrical Conductivity of Conidia in Water. Five milliliters of water (sdd) containing conidia ($5–7 \times 10^6$ conidia/ml) of each *B. cinerea* strain in 15 ml polypropylene centrifuge tubes were treated with 1 μl or 5 μl of butyl acetate. The tubes were capped, shaken horizontally at 22°C for 1–24 hr at 100 rpm, and electrical conductivity of suspensions measured at 1, 4, and 24 hr with a conductivity probe (Amber Science, Inc., San Diego, California) on the lab bench. Controls were water without conidia, but treated with 5 μl butyl acetate, and water with conidia but no butyl acetate. There were three replicates per treatment or control per experiment.

Effect of Butyl Acetate on Loss of ^{14}C from ^{14}C-Labeled Conidia. Conidia of each strain of *B. cinerea* were produced on 10-cm-diam. PDA dishes that were supplemented with 370 kBq of [^{14}C]glucose per dish. Solutions of [^{14}C]glucose (11.1 Gbq/mM; ICN Biomedicals) were filter-sterilized (0.2-μm-diam. pore) before use. Four hundred microliters of ^{14}C-labeled conidia ($2.4–5.0 \times 10^6$ conidia/ml) of each *B. cinerea* isolate or water (sdd) were aseptically pipetted into a sterile well affixed to double-stick tape on the inside of a 6.5-cm-diam. glass Petri dish cover. Also stuck to the tape was a 22-ml glass scintillation vial containing 200 μl of 1 M NaOH as a $^{14}CO_2$ trap. The cover dish was placed inside an upright 500 cc glass jar that was then sealed. Three jars containing each of the fungi and three

with water were injected with 4 μl of butyl acetate, and three jars each with fungi or water were not. After 24 hr at 22°C, 14 ml of Econolume scintillation cocktail (ICN Biomedicals) were added to the vial with NaOH, and the contents of a well was transferred by pipet to a membrane filter on a sintered-glass suction head and vacuum deposited on the filter. Two 200-μl water washings of the well were also added to the membrane. Liquid was collected in a scintillation vial inside the suction flask. The filter was dissolved in 200 μl chloroform, and the conidia suspended in 10 ml of 4% Cab-O-Sil cocktail (Filonow and Lockwood, 1983). Ten milliliters of Econolume cocktail were added to the liquid from inside the suction flask. Radioactivity was measured as disintegrations per minute (dpm). Total ^{14}C available in a jar prior to an experiment was equal to ^{14}C (dpm) in liquid + in conidia on a filter $+^{14}CO_2$. Percentage ^{14}C lost as leakage from conidia was calculated as (^{14}C in the liquid/total ^{14}C) $\times$ 100. Percentage $^{14}CO_2$ evolved from conidial respiration was calculated from ($^{14}CO_2$/total ^{14}C) $\times$ 100. Total percentage ^{14}C lost from conidia was the sum of ^{14}C leakage $+^{14}CO_2$ evolved.

Effect of Butyl Acetate on Uptake of ^{14}C-Labeled Sugars by Conidia. Two hundred microliters of ^{14}C-labeled glucose (370 kBq) in water were pipetted into a sterile well inside a small glass Petri dish cover. The cover also held a 22-ml scintillation vial containing 200 μl of 1 M NaOH. One membrane filter without conidia as a control or one bearing 1×10^6 conidia of each *B. cinerea* isolate was placed on the ^{14}C-labeled glucose and the dish cover placed inside an upright 500-cc glass jar. There were 24 jars per experiment: six jars with conidia of each isolate and six of a control. The jars were sealed and three jars with conidia of each isolate and a control were injected with 4 μl of butyl acetate. Jars without butyl acetate were the controls. Jars were completely randomized on a bench. After 9 hr at 22°C, 14 ml of Econolume cocktail were added to the scintilliation vial, and membrane filters were transferred to a sintered-glass suction flask and washed (200 μl) twice with cold water. Radioactivity in conidia was measured, as described above. One experiment was done.

Effect of Butyl Acetate and Yeasts on B. cinerea *Infection of Apples*. Golden Delicious apples were purchased from a local supermarket. Apples used in experiments weighed 110–135 g, had a mean firmness of 40–44 N and soluble solids of 11.5–12.7%, and were free of obvious bruises. Apples were used on the day of purchase or stored (4°C) for one week or less before use. Apples from cold storage were equilibrated at 22°C for 16–24 hr before use. Apples were washed with tap water and dried with paper towels. Two wounds (3 mm diam. $\times$ 3 mm deep) were made on opposite sides of an apple at the equator by puncturing the tissue with a nail. The nail was passed through a flame and briefly allowed to cool before the next puncture. Apples were incubated for 24 hr prior to inoculating wounds. Treatments used for inoculation were prepared in 12-ml glass vials by mixing 5 ml suspensions of isolate F-J-4 conidia with 5 ml of water or 5 ml of yeast suspension to give: (1) conidia + *S. roseus,* (2) conidia + *C. laurentii,*

(3) conidia + *S. cerevisiae,* or (4) conidia + water. Density of conidia was 2–4 $\times$ 10^4 conidia/ml, whereas yeast density was 1 $\times$ 10^7 CFU/ml. The control was 10 ml of water in a vial, and one vial per fungal suspension or water was prepared. Water and fungal mixtures (10 ml each) were spiked with 10 μl of butyl acetate or not. Vials were capped, the contents mixed well, and 20 μl of a treatment pipetted into both wounds per apple using a fresh pipet tip for each wound. Fifteen apples per treatment or control on a fruit tray were placed inside a 40-cm $\times$ 58-cm $\times$ 14-cm plastic storage box containing 500 ml of water, and sealed inside a plastic bag. Each box contained a different treatment and was randomized on a bench. After 10 days at 22°C, the number of infected wounds and lesion diameters (Filonow et al., 1996) were recorded.

Data Analysis. All experiments were repeated one or two times, unless otherwise noted. Results from repeated experiments were reproducible. Percentage adhesion of conidia exposed to butyl acetate and yeasts (Figure 2 below) is presented as an increase over the percentage adhesion of unexposed conidia. Percentage germination and percentage infection data were subjected to an arcsine square-root transformation prior to analysis of variance (ANOVA). A two-way ANOVA was performed on data from the apple infection experiments. Means were separated using the Student-Newman-Keul's test or Dunnett's test ($P \leq 0.05$). The relationship between conidial germination and conidial adhesion was analyzed by regression ($P \leq 0.05$).

RESULTS

Effect of Butyl Acetate and Yeasts on Adhesion and Germination of Conidia In Vitro. Preliminary experiments showed that centrifugation of a 10-ml suspension of conidia followed by suctioning off the supernatant consistently gave a 92% recovery of conidia unexposed to butyl acetate. The water-stream method for dislodging conidia yielded 85–88% of unexposed conidia within 3 min of conidia deposition on membranes, indicating a rapid adhesion of some conidia. Since the 85–88% recovery of conidia was consistent for several trials (data not shown), and the method was easy to use, it was considered suitable for assessing conidial adhesion.

Adhesion of unexposed conidia to membrane filters (experimental controls) increased over time (Figure 1), ranging from 11–26% after 4 hr to 22–42% after 48 hr of incubation. Germination of these conidia was typically 0% after 12 hr and only 2–10% after 48 hr (Figure 1). When conidia were exposed to butyl acetate (Figure 1), their adhesion increased 0.3- to 2.3-fold by 4 hr and 1.6- to 3.0-fold by 48 hr over the controls. Conidia exposed to butyl acetate also germinated more (Figure 1), with significant stimulation of germination beginning after 8–12 hr and increasing to 3- to 5-fold greater than controls after 48 hr. Data for germination and adhesion of exposed conidia (Figure 1) were subjected to regression

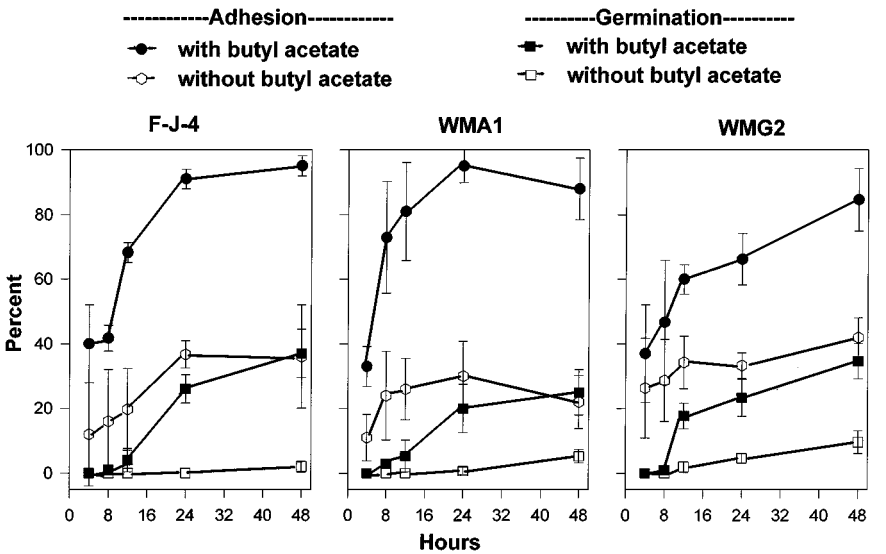


FIG. 1. Adhesion and germination of conidia of isolates F-J-4, WMA1, and WMG2 of *Botrytis cinerea* after 24 hr at 22°C on polycarbonate membrane filters on water inside 500-cc jars injected with 4 μl of butyl acetate. Controls were conidia not exposed to butyl acetate. Data points are the means of three replicates $\pm$ 1 SD.

analysis. Regression of percentage germination (G) to percentage adhesion (A) data best fit second order polynomial models for isolates F-J-4 ($G = 49.96 - 2.00A + 0.019A^2$; $r^2 = 0.99$), and WMA1 ($G = 30.44 - 1.38A + 0.014A^2; r^2 = 0.77$), and a linear model for WMG2 ($G = -31.46 + 0.79A; r^2 = 0.96$).

Sporobolomyces roseus and *C. laurentii* generally reduced the adhesion-promoting effect of butyl acetate on conidia to membrane filters (Figure 2), whereas *S. cerevisiae* did not. *Sporobolomyces roseus* inhibited adhesion of conidia more than *C. laurentii* for isolates F-J-4 and WMA1, but not WMG2. Generally the lower the level of conidial adhesion affected by the yeasts the lower the germination of conidia (Figure 2).

Utilization of Butyl Acetate by Yeasts and Conidia of B. cinerea Isolates As Indicated by Tetrazolium Violet Reduction In Vitro. All yeasts and conidia utilized glucose as a food as indicated by the production of a violet-colored precipitate (Table 1); however, only *S. roseus* and *C. laurentii* utilized butyl acetate. Controls consisting of an organism plus TVP without a food source were colored pale yellow, except for the *S. roseus* control, which often was light violet, but easily distinguishable from the medium-intense violet when *S. roesus* utilized butyl acetate or glucose. By 24 hr most of the formazan production had occurred, but *C. laurentii* often needed another 24 hr to improve color intensity.

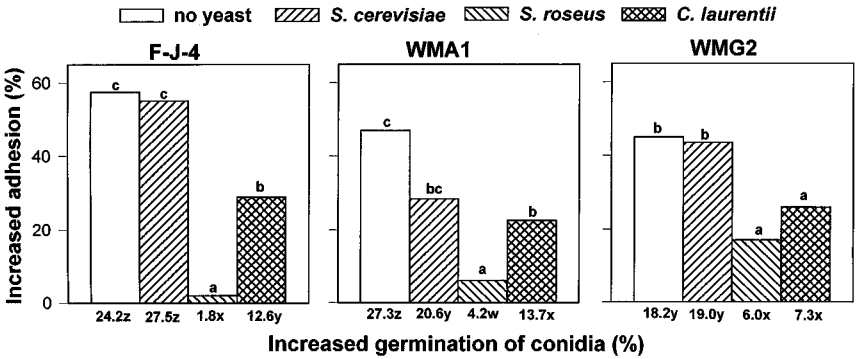


FIG. 2. Adhesion and germination of conidia of isolates F-J-4, WMA1, and WMG2 of *Botrytis cinerea* after 24 hr at 22°C on polycarbonate membrane filters on water or water suspensions of yeasts inside 500-cc jars injected with 4 μl of butyl acetate. Controls were conidia not exposed to butyl acetate. Bars are the means of three replicates and represent the increase in percentage adhesion and germination above controls. Bars with the same letter are not significantly ($P \leq 0.05$) different according to the Student-Newman-Keul's test.

Effect of Butyl Acetate on Electrical Conductivity of Conidia in Water. Addition of small amounts of butyl acetate to suspensions of conidia in water significantly increased the electrical conductivity of the suspensions beyond that of the controls. The conductivity of the suspensions dosed with 5 μl butyl acetate generally increased linearly over time (Figure 3), whereas the 1-μl dose increased conductivity 150–255% after 1 hr, but thereafter conductivity did not increase

TABLE 1. UTILIZATION OF GLUCOSE AND BUTYL ACETATE BY CONIDIA OF *Botrytis cinerea* AND YEASTS USING TETRAZOLIUM VIOLET REDUCTION AS INDICATOR

	Violet color rating with added substrate[a]		
Organism	Water	Glucose	Butyl acetate
F-J-4 conidia	1.0a	2.2b	1.0a
WMA1 conidia	1.0a	2.3b	1.0a
WMG2 conidia	1.0a	2.3b	1.0a
S. cervisiae	1.0a	2.7b	1.0a
C. laurentii	1.2a	2.7b	2.7b
S. roseus	2.0a	4.5c	3.8b

[a] Color was rated using a scale of 1–5, where 1 = no violet color and 5 = intense violet color. Values are the means of six replicates. Means in a horizontal row followed by the same letter are not significantly different using Dunnett's test ($P \leq 0.05$).

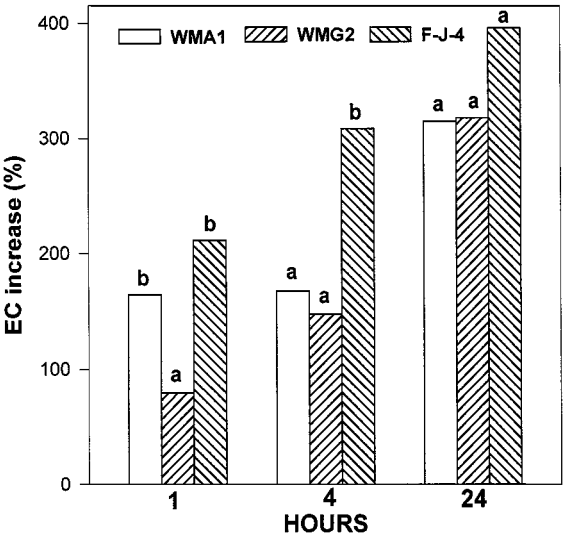


FIG. 3. Electrical conductivity (EC) at 22°C of suspensions of conidia of isolates F-J-4, WMA1, and WMG2 of *Botrytis cinerea* in water dosed with 5 μl of butyl acetate. Controls were conidia not exposed to butyl acetate. Bars are the means of three replicates and represent the EC increase in percentage above controls. Bars with the same letter are not significantly ($P \leq 0.05$) different according to the Student-Newman-Keul's test.

(data not shown). No isolate of *B. cinerea* consistently leaked more than others after butyl acetate exposure.

Effect of Butyl Acetate on Loss of ^{14}C from ^{14}C-Labeled Conidia. ^{14}C-Labeled conidia incubated on water and not exposed to butyl acetate lost 3.4–7.9% of their ^{14}C. Exposure to butyl acetate increased ^{14}C lost to 16.9–25.1% (Figure 4). Generally, respiration was the major route of ^{14}C lost from conidia of isolates WMA1 and WMG2, but not F-J-4,which also leaked considerable ^{14}C. Leakage of ^{14}C from conidia exposed to butyl acetate was 2.2–2.8 times greater than that from unexposed conidia.

Effect of Butyl Acetate on Uptake and Utilization of ^{14}C-Labeled Glucose by Conidia. Exposure of conidia to butyl acetate did not make them more permeable to glucose (Figure 5). Compared to unexposed conidia, neither [^{14}C]glucose uptake by conidia, respiration, nor total ^{14}C recovery from labeled conidia was increased by butyl acetate.

Effect of Butyl Acetate and Yeasts on B. cinerea Infection of Apples. Butyl acetate in water added to wounds of apples increased both the infection frequency and the lesion diameter caused by *B. cinerea* F-J-4 compared to the control (Table 2). Butyl acetate caused little additional disease in wounds treated with

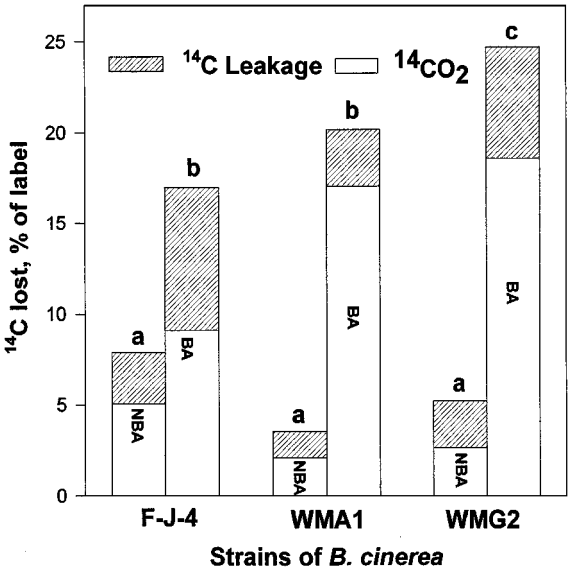


FIG. 4. ^{14}C lost as leakage or evolved CO_2 from ^{14}C-labeled conidia of isolates F-J-4, WMA1, and WMG2 of *Botrytis cinerea* after 24 hr at 22°C on water inside 500-cc jars injected with 4 μl of butyl acetate (BA) or not (NBA). Bars with the same letter are not significantly ($P \leq 0.05$) different according to the Student-Newman-Keul's test.

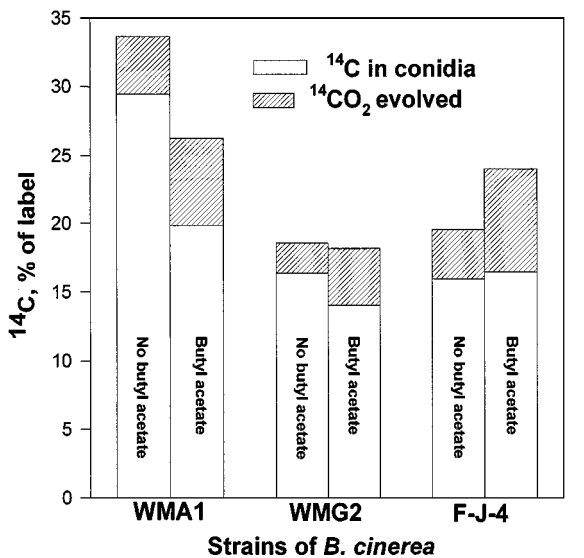


FIG. 5. Uptake and utilization of [^{14}C]glucose by conidia of isolates F-J-4, WMA1, and WMG2 of *Botrytis cinerea* after 24 hr at 22°C on water inside 500-cc jars injected with 4 μl of butyl acetate or not. No significant ($P \leq 0.05$) differences were found between conidia exposed or unexposed to butyl acetate.

TABLE 2. EFFECT OF YEASTS ON INFECTION BY CONIDIA OF *Botrytis cinerea* (F-J-4) IN WOUNDS OF GOLDEN DELICIOUS APPLES TREATED WITH BUTYL ACETATE[a]

Yeast	Infection frequency (%)		Lesion diameter (mm)	
	No butyl acetate	Butyl acetate	No butyl acetate	Butyl acetate
None	70.0aZ	90.0bZ	20.9aZ	31.0bZ
S. cerevisiae	60.0aZ	70.0aY	20.1aZ	23.0aZ
C. laurentii	46.7aY	43.3aX	10.4aY	11.9aY
S. roseus	6.7aX	26.7bW	5.5aY	9.1aY

[a] Values are the means of 15 replicates. Means in a horizontal row followed by a lowercase letter and means in a column followed by uppercase letters are not significantly different using the Student-Newman-Keul's test ($P \leq 0.05$).

yeasts. In wounds treated with butyl acetate, all yeasts reduced the infection frequency compared to the control; however, lesion diameter was only reduced by *S. roseus* and *C. laurentii.* Compared to *C. laurentii, S. roseus* reduced infection frequency but not lesion diameter.

DISCUSSION

Butyl acetate stimulated germination of conidia of *B. cinerea* on polycarbonate filter membranes, a finding that supports early work with butyl acetate and other acetate esters (Filonow, 1999). This phenomenon was found for two other isolates of *B. cinerea* in addition to F-J-4 (Filonow, 1999). My findings plus earlier work (Brown, 1922) suggest a general germination response of *B. cinerea* to acetate esters.

Butyl acetate increased the adhesion of *B. cinerea* conidia to membrane filters, which is a new finding. The percentage germination of conidia was strongly correlated with increasing adhesion. Other workers have reported that fungal spore adhesion plays a role in spore germination (Young and Kauss, 1984; Hamer et al., 1988; Doss et al., 1993, 1995). In the absence of butyl acetate, my results support the findings of Doss et al. (1993) that adhesion of freshly hydrated conidia of *B. cinerea* is a two-stage process with the first stage occurring very rapidly. A second stage, involving germ tube formation, occurs hours later, with adhesion increasing as germlings increase (Doss et al., 1995). Most likely, a minimum adhesive threshold may have to be surpassed by some *B. cinerea* conidia prior to germination.

Other mechanisms were investigated that could account for increased germination of conidia when exposed to butyl acetate. Conidia did not use butyl acetate as a food source, as indicated by the lack of tetrazolium violet reduction, although

this metabolic assay showed that conidia had an active metabolism capable of using glucose. Although tetrazolium dyes are widely used in microbial ecology as colorimetric indicators of organic substrate utilization, their use is sensitive to several factors (Bochner and Savageau, 1977; Konopka et al., 1998), and care must be used in assay interpretation. Nevertheless, under the conditions given in this paper, the TV assay was simple to use and gave unequivocal results.

Butyl acetate increased the permeability of conidia, as indicated by the increase in electrical conductivity (EC) of water suspensions of conidia treated with butyl acetate. Part of the EC rise was due to leakage of organic compounds. An increased loss of ^{14}C occurred from ^{14}C-labeled conidia of all isolates. Altered permeability appeared to favor efflux of material rather than influx, as suggested by the insignificant uptake and use of [^{14}C]glucose by conidia exposed to butyl acetate compared to unexposed conidia. The effect of butyl acetate on permeability of *B. cinerea* conidia is a new finding and raises the question of whether other fungal spores are likewise affected by acetate esters or other volatiles.

The present work also confirmed the finding (Filonow, 1999) that *Sporobolomyces roseus* and *C. laurentii*, but not *S. cerevisiae* reduced the stimulatory effect of butyl acetate on the *in vitro* germination of *B. cinerea* conidia. Reported here for the first time is the capability of some yeasts to reduce the adhesion-promoting effect of butyl acetate on fungal spores. *Saccharomyces cerevisiae* showed much less capability, a result consistent with the poor capability of *S. cerevisiae* to reduce butyl acetate stimulation of germination. The TV assay showed that *S. roseus* and *C. laurentii*, but not *S. cerevisiae* used butyl acetate as a food source, a finding supported by Vishniac et al. (1997) that some yeasts but not others can use acetate esters as food sources. Therefore, depletion of butyl acetate by *S. roseus* or *C. laurentii* from water films surrounding conidia, thereby reducing the adhesion-promoting effect of butyl acetate, is a plausible mechanism explaining the action of these yeasts. However, this hypothesis would be better tested by relating adhesion and germination to butyl acetate depletion rates induced by yeasts. This could be done *in vitro* but is now technically impossible in apple wounds.

Since butyl acetate caused increased leakage of organic compounds from conidia, it is also plausible that the released material or some component of the material may be an adhesive (Stahman et al., 1992; Doss et al., 1995). Depletion of butyl acetate by *S. roseus* and *C. laurentii* may reduce alterations in fungal permeability with less leakage of adhesive. Alternatively or concomitantly, these yeasts may feed on some component of the extracellular material altering adhesive capacity.

Filonow (1999) showed that exposing apples to a butyl acetate headspace in glass jars produced more infection by *B. cinerea.* The work herein expands on this finding to show that *S. roseus* and *C. laurentii*, but not *S. cerevisiae* reduced the infection-promoting effect of butyl acetate. Although this finding is consistent with *in vitro* germination and adhesion results, conclusive evidence awaits investigations of microbial interactions within apple wounds. In this study, wounds of apples were

directly treated with a dilute solution of butyl acetate and the apples incubate in a manner frequently used by workers in the biological control of postharvest diseases (Janisiewicz et al., 1994; Filonow, 1998). The wounds were also allowed to dry overnight to facilitate a quicker absorption of butyl acetate by wounds from the introduced solution.

Butyl acetate and other acetate esters are volatile aroma- and flavor-producing compounds readily emitted by apples (Knee, 1993; Fellman and Mattheis, 1995), but whose concentrations vary according to the physiological state of the apple (Mattheis et al., 1991; Fellman and Mattheis, 1995). Since butyl acetate is one of the most volatile of acetate esters, it has typical concentrations of 200–1000 nppb and 1–3 μppm in the flesh and peel, respectively. During storage, conidia of *B. cinerea* in apple wounds will be exposed to indigenous acetate esters and to volatile acetate esters that partition into and out of water films on the surfaces of apples. In the present study, conidia on filter membranes in glass jars were exposed to 4 μl of butyl acetate or about 7 μppm. Since butyl acetate is slightly soluble in water, it is reasonable to assume that concentrations of butyl acetate used in the *in vitro* studies were most likely similar to the ppb to ppm range typically found in apples.

The relevance of the present findings to the use of yeasts as biological control agents needs additional study, although these findings and those of Filonow (1998, 1999) indicate that other chemicals besides the standard nutrients, e.g., sugars, may influence interactions of fungal pathogen and biocontrol yeasts in apple wounds. The chemical ecology of fungal pathogen interactions with fruit appears to be exceedingly complex (Hamilton-Kemp et al., 1992; Vaughan et al., 1993; Eckert and Ratnayake, 1994; Archbold et al., 1997; Filonow, 1999) and may make the search for a single causal biocontrol mechanism difficult. If so many interacting chemicals affect microbial interactions in an apple wound, is it worthwhile to focus on single mechanisms of antagonism such as antibiosis or nutrient competition for the purposes of antagonist improvement?

Apart from this question, the present findings support the acquisition of further knowledge regarding the physiological and biochemical effects that volatile chemicals from apples or other fruits have on the ecology of fungal plant pathogens.

Acknowledgments—The author thanks W. J. Janisiewicz and H. S. Vishniac for donation of fungal culture. This work was supported by funds from the Division of Agricultural Science and Natural Resources, Oklahoma State University.

REFERENCES

ARCHBOLD, D. D., HAMILTON-KEMP, T. R., BARTH, M. M., and LANGLOIS, B. E. 1997. Identifying natural volatile compounds that control gray mold (*Botrytis cinerea*) during postharvest storage of strawberry, blackberry, and grape. *J. Agric. Food Chem.* 45:4032–4037.

BOCHNER, B. R., and SAVAGEAU, M. A. 1977. Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. *Appl. Environ. Microbiol*. 33:434–444.

BROWN, W. 1922. Studies in the physiology of parasitism. IX. The effect on the germination of fungal spores of volatile substances arising from plant tissues. *Ann. Bot.* 36:285–300.

DOSS, R. P., POTTER, S. W., CHASTAGNER, G. A., and CHRISTIAN, S. K. 1993. Adhesion of nongerminated *Botrytis cinerea* conidia to several substrate. *Appl. Environ. Microbiol.* 59:1786–1791.

DOSS, R. P., POTTER, S. W., SOELDNER, A. H., CHRISTIAN, J. K., and FUKUNAGA, L. E. 1995. Adhesion of germlings of *Botrytis cinerea. Appl. Environ. Microbiol.* 61:260–265.

ECKERT, J. W., and RATNAYAKE, M. 1994. Role of volatile compounds from wounded oranges in induction of germination of *Penicillium digitatum* conidia. *Phytopathology* 84:746–750.

FELLMAN, J. K., and MATTHEIS, J. P. 1995. Ester biosynthesis in relation to harvest maturity and controlled-atmosphere storage of apples, pp. 149–162, *in* R. L. Rouseff and M. M. Leahy (eds.) Fruit Flavors: Biogenesis, Characterization, and Authentication. American Chemical Society, Washington, DC.

FILONOW, A. B. 1998. Role of competition for sugars by yeasts in the biocontrol of gray mold of apple. *Biocontrol Sci. Technol.* 8:243–256.

FILONOW, A. B. 1999. Yeasts reduce the stimulatory effect of acetate esters from apple on the germination of *Botrytis cinerea* conidia. *J. Chem. Ecol.* 25:1555–1565.

FILONOW, A. B., and LOCKWOOD, J. L. 1983. Mycostasis in relation to the microbial nutrient sinks of five soils. *Soil Biol. Biochem.* 15:557–565.

FILONOW, A. B., VISHNIAC, H. S., ANDERSON, J. A., and JANISIEWICZ, W. J. 1996. Biological control of Botrytis cinerea in apple by yeasts from various habitats and their putative mechanisms of antagonism. *Biol. Control* 7:212–220.

HAMER, J. E., HOWARD, R. J., CHUMLEY, F. G., and VALENT, B. 1988. A mechanism for surface attachment in spores of a plant pathogenic fungus. *Science* 239:288–290.

HAMILTON-KEMP, T. R., MCCRACKEN, C. T., JR., LOUGHRIN, J. H., ANDERSEN, R. A., and HILDEBRAND, D. F. 1992. Effects of some natural volatile compounds on the pathogenic fungi, *Alternaria alternata* and *Botrytis cinerea. J. Chem. Ecol*. 18:1083–1091.

JANISIEWICZ, W. J., PETERSON, D. L., and BORS, B. 1994. Control of storage decay of apples with *Sporobolomyces roseus. Plant Dis.* 78:446–470.

KNEE, M. 1993. Pome fruits, pp. 325–346, *in* G. B. Seymour, J. E. Taylor, and G. A. Tucker (eds.) Biochemistry of Fruit Ripening. Chapman and Hall, New York.

KONOPKA, A., OLIVER, L., and TURCO, R. F., JR. 1998. The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbiol. Microb. Ecol.* 35:103–115.

MATTHEIS, J. P., FELLMAN, J. F., CHEN, P. M., and PATTERSON, M. E. 1991. Changes in headspace volatiles during physiological development of Bisbee Delicious apple fruit. *J. Agric. Food Chem.* 39:1902–1906.

STAHMAN, K.-P., PIELKEN, P., SCHIMZ, K.-L., and SAHM, H. 1992. Degradation of extracellular β-(1,3)(1,6)-D-glucan by *Botrytis cinerea. Appl. Environ. Microbiol*. 58:3347–3354.

VAUGHAN, S. F., SPENCER, G. F., and SHASHA, B.S. 1993. Volatile compounds from raspberry and strawberry fruit inhibit postharvest decay fungi. *J. Food Sci.* 58:793–796.

VISHNIAC, H. S., ANDERSON, J. A., and FILONOW, A. B. 1997. Assimilation of volatiles from ripe apples by *Sporidiobolus salmonicolor* and *Tilletiopsis washingtonensis. Antonie van Leeuwenhoek* 72:201–207.

YOUNG, D. H., and KAUSS, H. 1984. Adhesion of *Colletotrichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and to polystyrene. *Appl. Environ. Microbiol.* 47:616–619.

Journal of Chemical Ecology, Vol. 27, No. 4, 2001

COMPARISON OF DETOXIFICATION ENZYME mRNAS IN WOODRATS (*Neotoma lepida*) AND LABORATORY RATS

JOHN G. LAMB,[1] JENNIFER S. SORENSEN,[2] and M. DENISE DEARING[2]

[1]*Department of Pharmacology and Toxicology*
[2]*Department of Biology*
University of Utah
Salt Lake City, Utah 84112

(Received September 12, 2000; accepted December 10, 2000)

Abstract—To understand how mammalian herbivores process plant secondary compounds, we examined differences in expression of biotransformation enzyme mRNAs among populations of wild woodrats (*Neotoma lepida*) and laboratory rats. We compared expression of mRNAs for 10 biotransforming enzymes in five families (CYP, mEH, QOR, GST, and UGT) by using Northern blot analysis. We found significant differences in eight of 10 mRNAs tested. We suggest that the differences in mRNA expression among populations of woodrats and laboratory rats may be due to differences in the secondary compound composition of their diets. Our results provide background for future studies of detoxification strategies in mammalian herbivores that combine pharmacological techniques with an ecological perspective.

Key Words—Detoxification, biotransforming enzymes, plant secondary compounds, *Neotoma*, woodrats, xenobiotics.

INTRODUCTION

Mammalian herbivores ingest and detoxify a wide variety of plant secondary compounds. Detoxification and elimination is carried out by a multitude of enzymes collectively referred to as "xenobiotic biotransforming enzymes" or less precisely, "drug-metabolizing enzymes." These enzymes transform hydrophobic molecules into hydrophilic compounds that can be excreted as metabolites in the urine or feces.

To understand how mammalian herbivores detoxify secondary compounds, ecologists have focused primarily on the end products of detoxification by examining metabolites in the urine. Most studies have focused on the excretion of a single compound, glucuronic acid, which is an endogenous compound that can be

0098-0331/01/0400-0845$19.50/0

conjugated to a diversity of secondary compounds (Dearing, 1997; Foley, 1992; Lindroth and Batzli, 1983). Focus on this pathway is due in part to the fact that relatively straightforward, colorimetric assays for glucuronic acid have been developed (Blumenkrantz and Asboe-Hansen, 1973). Quantification of metabolites from other enzymatic pathways often entail more involved chemical analyses, such as gas chromatography, high pressure liquid chromatography, or mass spectrometry (Mangione, 1999; McLean et al., 1993).

In contrast, contemporary pharmacological studies approach mechanisms of detoxification of foreign (xenobiotic) compounds differently. In addition to quantifying metabolites excreted in the urine, *in vitro* techniques are used to identify individual enzymes involved in drug metabolism (Batt et al., 1992; Kato, 1979). One such technique that uses Northern blot analysis (Sambrook et al., 1989) detects messenger RNAs (mRNA) that encode for numerous biotransformation enzymes. This analysis gives an indication of the level of expression of mRNA for a particular detoxification enzyme. Screens of mRNA for biotransformation enzymes typically are employed to determine the pathways of biotransformation that are induced when a known drug is administered.

We took a pharmacological approach to investigate the detoxification mechanisms of mammalian herbivores consuming their natural diet. As a first step, we used Northern blot analysis to quantify the expression of mRNAs for several biotransformation enzymes in the liver of desert woodrats, *Neotoma lepida*. This species occurs in a wide variety of habitats across the American Southwest. We selected two populations of *N. lepida* known to consume different diets. *N. lepida* from the Great Basin feeds primarily on Utah juniper (*Juniperus osteosperma*), whereas *N. lepida* from the Mojave Desert consumes desert almond (*Prunus fasciculata*) and creosote (*Larrea tridentata*) (Egoscue, 1957; Karasov, 1989; Mangione, 1999). These plant species contain high levels of various secondary compounds, particularly terpenes and phenolics (Dearing et al., 2000; Mangione, 1999; Mangione et al., 2000). We compared the results from *N. lepida* consuming natural diets to those from laboratory rats (*Rattus norvegicus*) consuming standard laboratory chow.

For each population, we examined levels of mRNA from five families of enzymes involved in xenobiotic biotransforming enzymes: cytochrome P450s (CYP), microsomal epoxide hydrolase (mEH), NAD(P)H quinone oxidoreductase (QOR), glutathione *S*-transferases (GST), and UDP-glucuronosyltransferases (UGT) (Figure 1). These five families encompass the majority of known detoxification enzymes and include both functionalization (phase I) and conjugation enzymes (phase II). In addition, we probed for the heavy subunit of γ-glutamylcysteine synthase (γ-GCS). CYP, mEH, and QOR encode for functionalization enzymes, whereas GST and UGT produce conjugation enzymes. For CYP and UGT families, we restricted our investigation to isoforms that are known to metabolize foreign compounds.

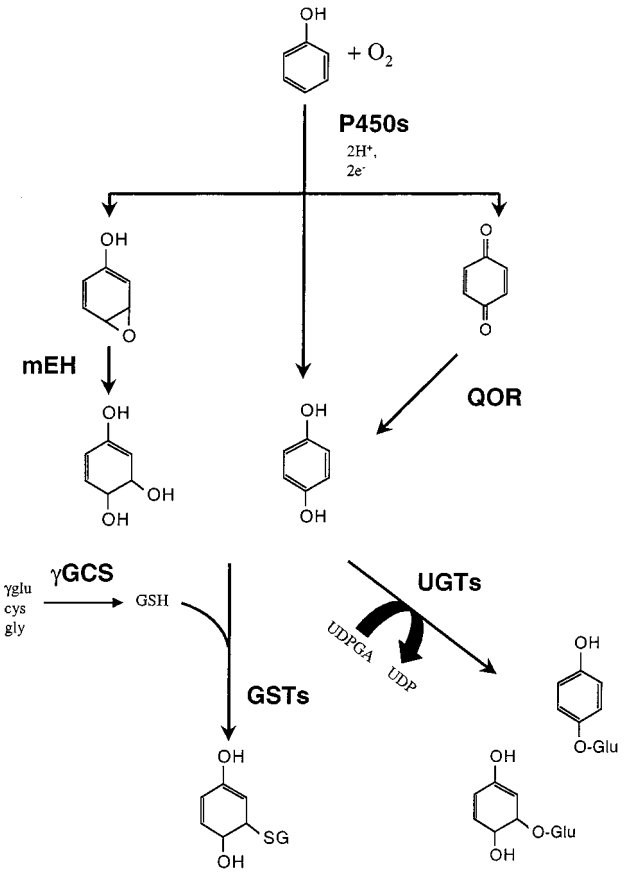


FIG. 1. General pathways of xenobiotic biotransformation. An example of a phenolic compound is shown. P450s: cytochrome P450 enzymes, mEH: microsomal epoxide hydrolase enzyme, QOR: NAD(P)H quinone oxidoreductase, UGTs: UDP-glucuronosyltransferase enzymes, γGCS: γ-glutamylcysteine synthase enzyme, GSH: glutathione, and GSTs: glutathione-S-transferase enzymes.

Enzymes from each of the five families modify xenobiotics in a specific way (Hardman and Limbird et al., 1996) (Figure 1). Cytochrome P450 enzymes catalyze a monooxygenation reaction by inserting one atom of molecular oxygen into the xenobiotic compound while the other oxygen atom is reduced to water. Microsomal epoxide hydrolase catalyzes the addition of water to alkane epoxides, which are commonly produced during cytochrome P450 reactions. QOR catalyzes the two-electron reduction of quinones by oxidation of NAD(P)H. GSTs catalyze conjugation of xenobiotic compounds with the tripeptide, glutathione. An

adequate supply of glutathione is critical for conjugation. The rate-limiting step in the production of glutathione is catalyzed by γ-GCS. UGTs conjugate xenobiotic compounds with a sugar acid moiety from the cosubstrate UDP-glucuronic acid (Dutton, 1980).

We had three objectives in this study: (1) to determine whether complementary DNA (cDNA) probes for biotransformation enzyme mRNAs in laboratory rats (*Rattus norvegicus*) were homologous to mRNAs in woodrats, (2) to determine detoxification pathways induced by woodrats consuming a natural diet, and (3) to compare levels of biotransformation enzyme mRNAs among populations of woodrats and laboratory rats. We hypothesized that *N. lepida* woodrats should express higher levels of mRNA for biotransformation enzymes compared to laboratory rats because woodrats consume plants with measurable quantities of secondary compounds, whereas laboratory rats consume formulated diets low in toxins.

METHODS AND MATERIALS

Laboratory Rats. Male laboratory rats (Muridae: Murinae: *Rattus norvegicus*: strain Sprague-Dawley; 160–180 g) were obtained from Simonson Laboratories and kept on a 12 hour light dark cycle and given free access to rat chow and water.

Collection of Neotoma. We collected *Neotoma lepida* (Muridae: Sigmodontinae) from two populations in Utah in October 1999. Four individuals were collected from a Mojave Desert habitat near Beaver Dam Wash, Utah (37°06'N, 113°58'W). The other four individuals were trapped in the Great Basin Desert near Jericho, Utah (39°57'N, 112°22'W). From here on, we refer to individuals trapped in the Mojave Desert as Mojave woodrats and those collected in the Great Basin Desert as Great Basin woodrats. For a description of each of these sites, see Dearing et al. (1998). Animals were live-trapped with Sherman traps and dispatched in the field with CO_2. Whole animal body mass was recorded. The liver was removed, weighed, and frozen quickly on dry ice. Samples of liver tissue were brought back to the laboratory for total RNA isolation.

Total RNA Isolation and Northern Blot Analysis. Total RNA was isolated from approximately 100 mg of frozen liver tissue by homogenization in 2 ml TRIzol (phenol–guanidine isothiocyante) reagent (Giboco/BRL) and isopropyl alcohol precipitation of the aqueous layer following chloroform treatment. Twenty micrograms of total RNA from each sample (concentration of total RNA was determined from 260 nm absorbance) was subjected to electrophoresis in 1% denaturing agarose gel containing formaldehyde. Sample integrity was determined by visualization of sharp 18S and 28S bands with ethidium bromide. Total RNA was transferred to a Nytran membrane (Schleicher and Schuell) by downward

TABLE 1. cDNAs USED FOR NORTHERN PROBES[a]

cDNA	Genbank	Region	Citation
CYP1A1	X00469	1511–2250	Yabusaki et al. (1984)
CYP2B	J00719	50–1567	Fujii-Kuriyama et al. (1981)
CYP3A	M10161	1079–2040	Gonzalez et al. (1985)
UGT1A1	U20551	2–870	Coffman et al. (1995)
UGT1A6	J02612	152–934	Iyanagi et al. (1986)
UGT2B1	M13506	8–710	Mackenzie (1986)
GSTYa	K00136	4–755	Pickett et al. (1984)
QOR	J02608	107–1495	Robertson et al. (1986)
mEH	M26125	107–1531	Porter et al. (1986)
γ-GCS	J05181	251–820	Yan and Meister (1990)

[a] The Genbank account numbers and approximate region of the cDNA are given.

alkaline transfer, cross-linked with UV light, and hybridized with cDNA probes labeled with [α-^{32}P]dCTP by random primed synthesis (Amersham Multiprime) according to the manufacturer's specifications. The rat cDNA probes used are given in Table 1.

Hybridized blots were washed twice for 30 min at 42°C in 2× SSC, 0.1% SDS, twice for 30 min at 42°C in 0.1× SSC, 0.1% SDS, and once for 45 min at 54°C in 0.1× SSC, 0.1% SDS. Autoradiographic film was exposed for 6–72 hr at −70°C with an intensifying screen, and the developed band intensity was determined by scanning densitometry with Molecular Analyst (Bio-Rad) software. To remove any effects of gel loading and transfer variations, all mRNA bands were normalized to a housekeeping gene, cyclophilin mRNA, in the same sample. Plasmids containing the cDNA probes were provided by Dr. J. Ritter, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia.

Statistics. Statistical analyses were performed by using ANOVA, followed by Fisher's multiple range test.

RESULTS

cDNA Probes. In general, cDNA probes generated from mRNAs for rat biotransforming enzymes gave good results when used on woodrat livers (Figure 2). All probes gave bands that were similar in size to mRNA bands from laboratory rats, with the exception of UGT2B1, where a larger transcript was detected in woodrat samples (Figure 2).

mRNAs for Detoxification Enzymes. Levels of mRNAs varied between woodrats and lab rats and among woodrats. In eight mRNAs tested, there were significant differences between woodrats and laboratory rats and among woodrats, but no consistent pattern in levels of mRNAs emerged. We found no difference in

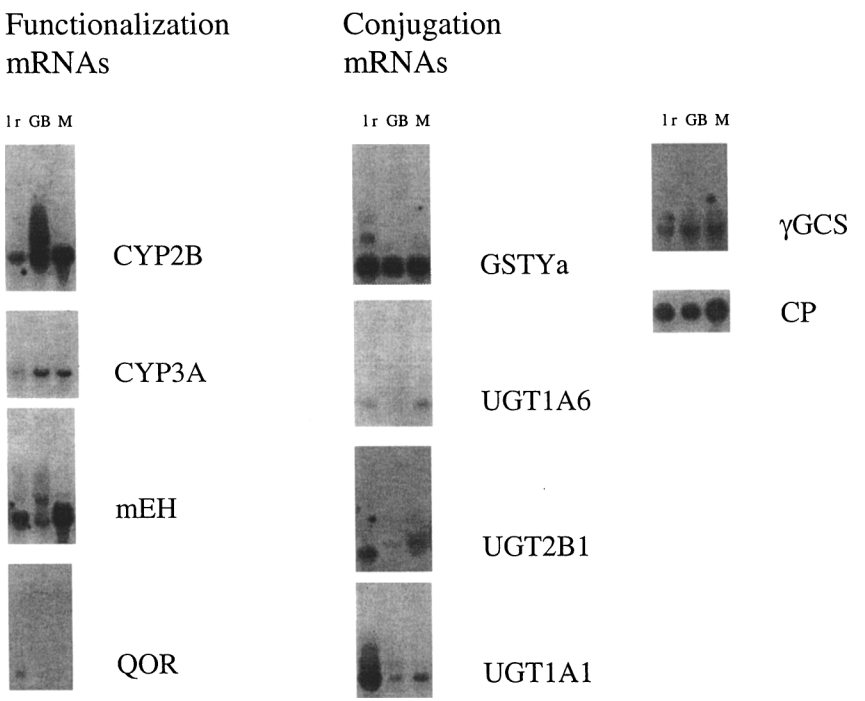


FIG. 2. Northern blots of liver mRNAs. Representative results are shown for each sample. Membranes were probed with the indicated ^{32}P-labeled cDNA probes (see Table 1) for each xenobiotic biotransformation enzyme. Membranes were also probed with ^{32}P-labeled cDNA for cyclophilin to compensate for gel loading and transfer variations. Band order is shown as laboratory rats (lr), Great Basin woodrats (GB), and Mojave woodrats (M).

mRNA levels for two of 10 mRNAs across the three animal groups tested. Details of the results for each mRNA are presented in the following sections.

mRNAs for Functionalization Enzymes. Among functionalization enzyme mRNAs, significant differences were present in mRNA levels for CYP2B and CYP3A. The level of mRNA for CYP2B was greater in Great Basin woodrats compared to Mojave woodrats and laboratory rats (Figure 3). Both populations of *N. lepida* had elevated levels of mRNA for CYP3A in comparison to laboratory rats. There was no detectable message for CYP1A1 in woodrat populations or laboratory rats (data not shown). The level of mEH mRNA was lower in Great Basin woodrats compared to laboratory rats and Mojave woodrats. The level of QOR mRNA was lower in Mojave woodrats than laboratory rats and Great Basin woodrats (Figure 3).

mRNA for γ-GCS and Conjugation Enzymes. The level of mRNA for γ-GCS differed among groups (Figure 4). Mojave *N. lepida* populations had higher

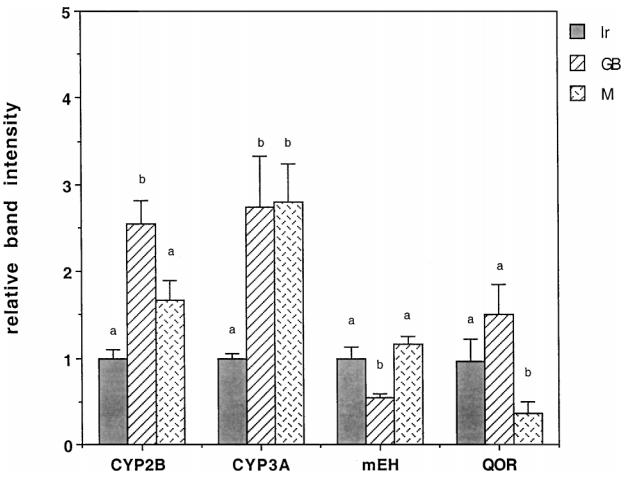


FIG. 3. Mean band intensities ± SE of northern blot analysis for functionalization enzyme mRNAs. Sample sizes were $N = 4$ for laboratory rats (lr), Great Basin woodrats (GB), and Mojave woodrats (M). Relative band intensities were normalized to laboratory rats. Different letters indicate significant differences among groups.

levels of γ-GCS compared to laboratory rats, but the difference between Great Basin woodrats and laboratory rats was not statistically significant. The Mojave population had γ-GCS levels five times that of laboratory rats and two times that of the Great Basin population. There was no difference in levels of mRNA for the GSTYa among woodrats and laboratory rats (Figure 4).

We found differences among animal groups with respect to mRNAs for glucuronic acid conjugation enzymes (Figure 4). Mojave woodrats had significantly higher levels of UGT1A6 mRNA compared to Great Basin woodrats and laboratory rats. Levels of mRNA for UGT1A6 were not significantly different between laboratory rats and Great Basin woodrats. Levels of mRNA for another glucuronidation enzyme, UGT2B1, showed the opposite pattern. Both populations of *N. lepida* exhibited significantly lower levels of mRNA for UGT2B1 compared to laboratory rats. Levels of UGT1A1 mRNA were also significantly lower in both *N. lepida* woodrat species compared to laboratory rats (Figure 4).

DISCUSSION

More than 25 years ago, Freeland and Janzen (1974) integrated the fields of pharmacology and ecology to generate numerous hypotheses on how herbivorous mammals cope with toxins in plants. Since then, diverse analytical techniques have been developed to understand the biotransformation pathways through which drugs

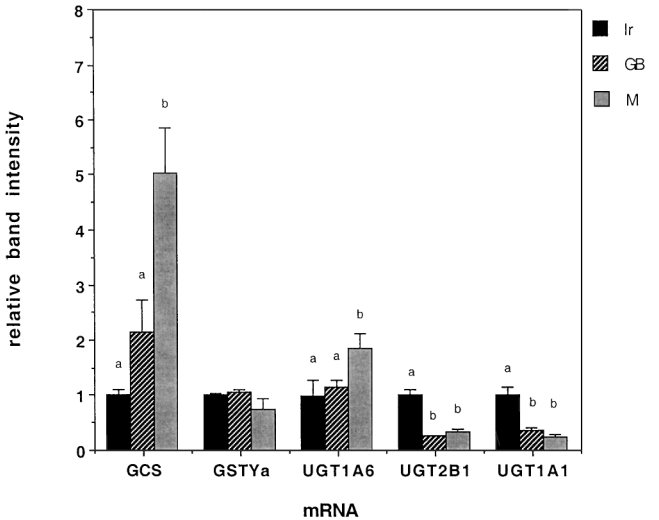


FIG. 4. Mean band intensities ± SE of northern blot analysis for γGCS and conjugation enzyme mRNAs. Sample sizes were $N = 4$ for laboratory rats (lr), Great Basin woodrats (GB), and Mojave woodrats (M). Relative band intensities were normalized to laboratory rats. Different letters indicate significant differences among groups.

and other xenobiotics are processed. Few of these techniques have been applied to studies of wild mammalian herbivores. A primary objective of our research was to join pharmacological knowledge and techniques to ecological questions of how wild herbivores detoxify secondary compounds in plants. Our results demonstrate that these approaches can be fruitful.

Use of cDNA Probes from Laboratory Rats in Woodrats. Overall, cDNA probes designed for detoxification mRNAs in laboratory rats gave good results in woodrats. Total RNA isolated from livers of woodrats and probed with cDNA for 10 biotransformation mRNAs of laboratory rats yielded bands of similar sizes to those found in laboratory rats (with the exception of UGT2B1). These results suggest that although laboratory rats and woodrats are different species, the sequences of their respective biotransforming enzyme mRNAs have been conserved. In the future, it would be valuable to have cDNA probes for the various biotransforming enzymes from *Neotoma* species. Knowledge of the exact DNA sequence of a specific biotransforming enzyme may yield valuable information on the evolution of detoxification systems (Lewis et al., 1998).

UGT2B1 did not yield results comparable to the other cDNA probes. The probe for UGT2B1 indicated a longer mRNA in woodrats than in laboratory rats. This result suggests that the mRNA for UGT2B1 in woodrats differs slightly from that of laboratory rats but is similar enough to hybridize to the probe.

Messenger RNA for CYP1A1 was not detected in either woodrat population. The CYP1A1 gene in woodrats may be significantly different from that of laboratory rats and, thus, was not detected by the probe for laboratory rats. This outcome, however, is unlikely given that species such as trout, which are more distantly related to laboratory rats than woodrats, exhibit a high degree of sequence similarity for CYP1A1 to laboratory rats (Heilman et al., 1988). It is more probable that levels of CYP1A1 mRNA in woodrats are undetectable, given that laboratory rats constitutively express CYP1A1 mRNA at low to undetectable levels.

mRNA for Functionalization Enzymes. The greater levels of mRNA for CYP2B in Great Basin woodrats compared to Mojave woodrats and laboratory rats may be attributable to high concentrations of α-pinene and/or other terpenes in the diet of Great Basin woodrats. α-Pinene is an abundant secondary compound in *Juniperus osteosperma*, the primary plant in the diet of Great Basin woodrats (Adams et al., 1981; Mangione, 1999; Mangione et al., 2000). This compound is not present in rat chow and occurs in low quantities in the diet of Mojave woodrats (Karasov, 1989; Mabry et al., 1977). In other studies, administration of α-pinene to laboratory rats increased quantities of the enzyme (P4502B) produced by CYP2B mRNA (Austin et al., 1988; Hiroi et al., 1995). The doses used in these experiments are comparable to those ingested by Great Basin woodrats in their natural diet (Dearing, personal observation). Thus, the elevated CYP2B mRNA in the Great Basin woodrats may be a response to α-pinene in its diet.

Both populations of woodrats had elevated levels of CYP3A mRNA compared to laboratory rats. In humans, the enzyme produced by CYP3A mRNA is responsible for the metabolism of over 60% of known pharmaceutical compounds (Gonzalez, 1989). Treatment of laboratory rats with essential wood oils, which are primarily terpenes, increased levels of the enzyme (P4503A) produced by CYP3A mRNA (Hiroi et al., 1995). It is plausible that the high levels of CYP3A mRNA in both populations of woodrats are induced by and required for detoxification of secondary compounds in their diets.

Neotoma lepida did not show higher levels of mEH and QOR mRNAs and in certain cases exhibited significantly lower levels than laboratory rats. The results imply that although both mEH and QOR mRNAs are subject to transcriptional induction by xenobiotic compounds (Hankinson, 1995; Daniel, 1993), the diets of *N. lepida* populations did not induce these two mRNAs.

Differences in Expression Levels of mRNA for Conjugation Enzymes. Conjugation with glutathione appears to be an important detoxification pathway for Mojave woodrats. Glutathione, a tripeptide thiol, is highly concentrated in liver cells (up to 10 mM) and is important in maintaining cellular redox status and metabolic and oxidative detoxification reactions. Synthesis of glutathione is rate-limited by the enzyme produced from γ-GCS mRNA (Packer, 1995). Mojave woodrats expressed significantly more γ-GCS mRNA than laboratory rats and Great Basin woodrats. However, there were no differences among woodrats and

laboratory rats in levels of mRNA GSTYa, which produces an enzyme that conjugates glutathione to xenobiotics. It is possible that the observed levels of GSTYa in woodrats produced sufficient quantities of enzyme for glutathione conjugation. In addition, there are several other forms of GST enzymes (GST μ and π) (Daniel, 1993) that we did not assay in this study. Thus, woodrats may be using other forms of GST enzymes for glutathione conjugation.

Conventional wisdom does not predict that mammalian herbivores would utilize glutathione (a tripeptide) as a primary detoxification pathway because nitrogen is typically a limiting resource to herbivores. Excretion of toxins conjugated to glutathione could result in marked losses of nitrogen. Utilization of the biochemically costly glutathione pathway by *N. lepida* suggests several intriguing possibilities. The amino acids in glutathione may not be limiting to *N. lepida*. If they are, the loss of amino acids can be minimized by recovering some of them in glutathione through transformation to mercapturic acids by kidney and intestinal γ-glutamyltransferase activity (Hardman and Limbird, 1996). Lastly, detoxification of the secondary compounds found in the diet of Mojave woodrats may be restricted to the glutathione pathway. Thus, elevated losses of glutathione may represent a biochemical cost of consuming plants containing toxins that can only be detoxified via the glutathione pathway. Further study of the use and costs of the glutathione pathway in Mojave woodrats is warranted.

It was interesting that both *N. lepida* populations expressed lower levels of mRNAs for two UGT mRNAs (UGT2B1 and UGT1A1) than laboratory rats. These two UGTs conjugate glucuronic acid to a number of plant secondary compounds common in the diets of *N. lepida*. UGT1A1 glucuronidates opiate alkaloids, while UGT2B1 conjugates "bulky" toxins, such as certain monoterpenes and phenolics (King et al., 1997). Several studies indicate that the glucuronic pathway is used extensively by woodrats (Mangione, 1999; Mangione et al., 2000). Mangione (1999) found that glucuronide conjugates comprised $\sim$70% of the conjugates examined in the urine of *N. lepida*. The *N. lepida* used by Mangione (1999) were obtained from the same site in the Mojave as this study and were fed creosote resin, a mixture of secondary compounds present in their natural diet. Our results in conjunction with those from the literature (Mangione, 1999; Mangione et al., 2000) suggest that *N. lepida* utilize UGTs other than UGT2B1 and UGT1A1 for glucuronidation. There are $\sim$19 different cDNA sequences for rat UGT enzymes (Mackenzie et al., 1997), and we have probed only for three.

Mojave woodrats did express slight but significantly higher levels of one UGT (UGT1A6) than either Great Basin woodrats or laboratory rats. The UGT1A6 enzyme metabolizes planar aromatic compounds (Jackson et al., 1988). The leaves of the creosote bush, a major component of the diet of Mojave woodrats, are coated with a resinous mixture of flavonoids and phenolics that can comprise up to 25% of the dry weight of the leaves (Mabry et al., 1977). It is possible that an increased

level of UGT1A6 expression is used to metabolize phenolic compounds in the diets of Mojave woodrats.

Detoxification in Woodrats and Laboratory Rats. The battery of detoxification enzymes used by woodrats differs from that in laboratory rats. Administration of phenobarbital to laboratory rats simultaneously induces CYP2B, mEH, and UGT2B1 mRNAs (Fujii-Kuriyama et al., 1981; Mackenzie, 1986; Porter et al., 1986). Great Basin woodrats exhibited high levels of CYP2B but had low expression of mEH and UGT2B1.

The pattern of mRNA expression in Mojave woodrats also differed from that of laboratory rats. In laboratory rats fed polycyclic aromatic hydrocarbons, transcription of UGT1A6 in the liver generally increases in concert with CYP1A1, GSTYa, and QOR (Hankinson, 1995). Mojave woodrats expressed slightly but significantly greater levels of UGT1A6 mRNA than Great Basin woodrats and laboratory rats. However, levels of GSTYa and QOR in Mojave woodrats were lower than or equal to those found in laboratory rats and Great Basin woodrats.

We propose two explanations for the differing patterns of functionalization and conjugation mRNAs in Great Basin and Mojave woodrats and laboratory rats. It is possible that the compounds woodrats consume in the wild are not processed through the same battery of enzymes as the drugs that have been given to laboratory rats. Alternatively, differences may be due to disparate constitutive expression of genes required to detoxify the various secondary compounds present in their typical diets. We plan to compare the detoxification series of laboratory rats and woodrats administered the same compound to distinguish between these two alternatives.

CONCLUSIONS

To our knowledge, this study is the first to examine biotransformation enzyme mRNAs of wild herbivores by using cDNA probes designed for laboratory rats. Overall, our results indicate that mRNA for biotransformation enzymes in woodrats and laboratory rats is similar enough that cDNA probes designed for laboratory rats work on woodrats. Our results also suggest that there are substantial differences in expression of biotransformation enzyme mRNAs between laboratory rats and woodrats and among populations of woodrats. Although more research is necessary to adequately address the ideas presented in this paper, the work provides a framework for future research.

Acknowledgments—We thank Drs. Michael Franklin, Gerald Yost, and Rebecca Boyle for editorial comments on this manuscript. We are grateful to Michael Bastiani for assistance in woodrat capture. Financial support was provided through the University of Utah's Seed Grant Program and the National Science Foundation (IBN 9809961) to M.D.D.

REFERENCES

ADAMS, R. P., ZANONI, T. A., RUDLOFF, E. V., and HOGGE, L. 1981. The south-western USA and northern Mexico one-seeded junipers: Their volatile oils and evolution. *Biochem. Syst. Ecol.* 9:93–96.

AUSTIN, C. A., SHEPHARD, E. A., PIKE, S. F., RABIN, B. R., and PHILLIPS, I. R. 1988. The effect of terpenoid compounds on cytochrome P-450 levels in rat liver. *Biochem. Pharmacol.* 37:2223–2229.

BATT, A. M., SIEST, G., MAGDALOU, J., and GALTEAU, M. M. 1992. Enzyme induction by drugs and toxins. *Clin. Chim. Acta* 209:109–121.

BLUMENKRANTZ, N., and ASBOE-HANSEN, G. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem.* 54:484–489.

COFFMAN, B. L., GREEN, M. D., KING, C. D., and TEPHLY, T. R. 1995. Cloning and stable expression of a cDNA encoding a rat liver UDP-glucuronosyltransferase (UDP-glucuronosyltransferase 1.1) that catalyzes the glucuronidation of opioids and bilirubin. *Mol. Pharmacol.* 47:1101–1105.

DANIEL, V. 1993. Glutathione *S*-transferases: Gene structure and regulation of expression. *Crit. Rev. Biochem. Mol. Biol.* 28:173–207.

DEARING, M. D. 1997. Effects of *Acomastylis rossii* tannins on a mammalian herbivore, the North American pika, *Ochotona princeps. Oecologia* 109:122–131.

DEARING, M. D., MANGIONE, A. M., and KARASOV, W. H. 2000. Diet breadth of mammalian herbivores: Nutrient versus detoxification constraints. *Oecologia* 123:397–405.

DEARING, M. D., MANGIONE, A. M., KARASOV, W. H., MORZUNOV, S., OTTESON, E., and ST. JEOR, S. 1998. Prevalence of hantavirus in four species of *Neotoma* from Arizona and Utah. *J. Mammal.* In press.

DUTTON, G. J. 1980. Glucuronidation of Drugs and Other Compounds. CRC Press, Boca Raton, Florida.

EGOSCUE, H. J. 1957. The desert woodrat: A laboratory colony. *J. Mammal.* 38:472–481.

FOLEY, W. J. 1992. Nitrogen and energy retention and acid–base status in the common ringtail possum. *Physiol. Zool.* 65:403–421.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory by mammals: the role of plant secondary compounds. *Am. Nat.* 108:269–289.

FUJII-KURIYAMA, Y., TANIGUCHI, T., MIZUKAMI, Y., SAKAI, M., TASHIRO, Y., and MURAMATSU, M. 1981. Construction and identification of a hybrid plasmid containing DNA sequence complementary to phenobarbital-inducible cytochrome P-450 messenger RNA from rat liver. *J. Biochem. (Tokyo)* 89:1869–1879.

GONZALEZ, F. 1989. The biology of cytochrome. *Pharmacol. Rev.* 40:243–288.

GONZALEZ, F. J., NEBERT, D. W., HARDWICK, J. P., and KASPER, C. B. 1985. Complete cDNA and protein sequence of a pregnenolone 16 alpha- carbonitrile-induced cytochrome P-450. A representative of a new gene family. *J. Biol. Chem.* 260:7435–7441.

HANKISON, O. 1995. The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* 35:307–340.

HARDMAN, J. G., and LIMBIRD, L. E. 1996. Goodman & Gilman's The Pharmacological Basis of Therapeutics. McGraw-Hill Health Professions Division, New York.

HEILMANN, L. J., SHEEN, Y.-Y., BIGELOW, S. W., and NEBERT, D.W. 1988. Trout P450IA1:cDNA and deduced protein sequence, expression in liver, and evolutionary significance. *DNA* 7:379–387.

HIROI, T., MIYAZAKI, Y., KOBAYASHI, Y., IMAOKA, S., and FUNAE, Y. 1995. Induction of hepatic P450s in rat by essential wood and leaf oils. *Xenobiotica* 25:457–467.

IYANAGI, T., HANUI, M., SOGAWA, K., FUJII-KURIYAMA, Y., WATANABE, S., SHIVELY, J. E., and ANAN, K. F. 1986. Cloning and characterization of cDNA encoding 3-methylcholanthrene inducible rat mRNA for UDP-glucuronosyltransferase. *J. Biol. Chem.* 261:15607–15614.

JACKSON, M. R., FOURNEL-GIGLEUX, S., HARDING, D., and BURCHELL, B. 1988. Examination of the substrate specificity of cloned rat kidney phenol UDP-glucuronyltransferase expressed in COS-7 cells. *Mole.* 34:638–642.

KARASOV, W. H. 1989. Nutritional bottleneck in an herbivore, the desert woodrat (*Neotoma lepida*). *Physiol. Zool.* 62:1351–1382.

KATO, R. 1979. Characteristics and differences in the hepatic mixed function oxidases of different species. *Pharmacol. Ther.* 6:41–98.

KING, C. D., RIOS, G. R., GREEN, M. D., MACKENZIE, P. I., and TEPHLY, T. R. 1997. Comparison of stably expressed rat UGT1.1 and UGT2B1 in the glucuronidation of opioid compounds. *Drug Metab.* and *Dispos.* 25:251–255.

LEWIS, D. F., IOANNIDES, C., and PARKE, D. V. 1998. Cytochromes P450 and species differences in xenobiotic metabolism and activation of carcinogen. *Environ. Health Perspect.* 106:633–641.

LINDROTH, R. L., and BATZLI, G. O. 1983. Detoxication of some naturally occuring phenolics by prairie voles: a rapid assay of glucuronination metabolism. *Biochem. Syst. Ecol.* 11:405–409.

MABRY, T. J., DIFEO, J., D. R., SAKAKIBARA, M., BOHNSTEDT, J., C. F., and SEIGLER, D. 1977. The natural products chemistry of *Larrea*, pp. 115–134, *in* T. J. Mabry, J. H. Hunziker and J. DiFeo, D. R. (eds.), Creosote Bush: Biology and Chemistry of *Larrea* in New World Deserts. Hutchinson and Ross, Stroudsberg, Pennsylvania.

MACKENZIE, P. I. 1986. Rat liver UDP-glucuronosyltransferase. Sequence and expression of a cDNA encoding a phenobarbital-inducible form. *J. Biol. Chem.* 261:6119–6125.

MACKENZIE, P. I., OWENS, I. S., BURCHELL, B., BOCK, K. W., BAIROCH, A., BELANGER, A., FOURNEL-GIGLEUX, S., GREEN, M., HUM, D. W., IYANAGI, T., LANCET, D., LOUISOT, P., MAGDALOU, J., CHOWDHURY, J. R., RITTER, J. K., SCHACHTER, H., TEPHLY, T. R., TIPTON, K. F., and NEBERT, D. W. 1997. The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* 7:255–269.

MANGIONE, A. 1999. Effects of creosote bush resin on the nutritional ecology of desert woodrats: Resin tolerance, detoxification, and impacts on water and energy balance. PhD thesis. University of Wisconsin, Madison, 128 pp.

MANGIONE, A., DEARING, M., and KARASOV, W. 2000. Interpopulation differences in tolerance to creoste bush resin in desert woodrats. *Ecology* 81:2067–2076.

MCLEAN, S., FOLEY, W. J., DAVIES, N. W., BRANDON, S., DUO, L., and BLACKMAN, A. J. 1993. Metabolic fate of dietary terpenes from *Eucalyptus radiata* in common ringtail possum (*Pseudocheirus peregrinus*). *J. Chem. Ecol.* 19:1625–1643.

PACKER, L. 1995. Bithiols in signal transduction and gene regulation. Methods *Enzymol.* 252:

PICKETT, C. B., TELAKOWSKI-HOPKINS, C. A., DING, G. J., ARGENBRIGHT, L., and LU, A. Y. 1984. Rat liver glutathione *S*-transferases. Complete nucleotide sequence of a glutathione *S*-transferase mRNA and the regulation of the Ya, Yb, and Yc mRNAs by 3-methylcholanthrene and phenobarbital. 259:5182–5188.

PORTER, T. D., BECK, T. W., and KASPER, C. B. 1986. Complementary DNA and amino acid sequence of rat liver microsomal, xenobiotic epoxide hydrolase. *Arch. Biochem. Biophys.* 248:121–129.

ROBERTSON, J. A., CHEN, H. C. and NEBERT, D. W. 1986. NAD(P)H:menadione oxidoreductase. Novel purification of enzyme cDNA and complete amino acid sequence, and gene regulation. *J. Biol. Chem.* 261:15794–15799.

SAMBROOK, J., FRITHSCH, E. F., and MANIATIS, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

YABUSAKI, Y., MURAKAMI, H., NAKAMURA, K., NOMURA, N., SHIMIZU, M., OEDA, K., and OHKAWA, H. 1984. Characterization of complementary DNA clones coding for two forms of 3-methylcholanthrene-inducible rat liver cytochrome P-450. *J. Biochem. (Tokyo)* 96:793–804.

YAN, N., and MEISTER, A. 1990. Amino acid sequence of rat kidney gamma-glutamylcysteine synthetase. *J. Biol. Chem.* 265:1588–1593.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

NEMATODE-ANTAGONISTIC TRICHOTHECENES FROM *Fusarium equiseti*[1]

JAMES K. NITAO,[2,*] SUSAN L. F. MEYER,[2] WALTER F. SCHMIDT,[3] JAMES C. FETTINGER,[4] and DAVID J. CHITWOOD[2]

[2]*Nematology Laboratory, USDA, ARS*
10300 Baltimore Avenue, Bldg. 011A, Rm. 165B
BARC-West, Beltsville, Maryland 20705-2350

[3]*Environmental Chemistry Laboratory, USDA, ARS*
BARC-West, Beltsville, Maryland

[4]*Department of Chemistry and Biochemistry*
University of Maryland
College Park, Maryland 20742

(Received July 31, 2000; accepted January 12, 2001)

Abstract—A strain of the fungus *Fusarium equiseti* isolated from soybean cyst nematode secretes nematode-antagonistic compounds. Bioassay-guided fractionation of an extract of the culture broth was undertaken to identify the compounds. Fractions were assayed for activity against a root-knot nematode (*Meloidogyne incognita*), a plant pathogen that attacks the roots of numerous plant species. Two trichothecene compounds were isolated that inhibited egg hatch and immobilized second-stage juveniles of this nematode: 4,15-diacetoxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one (4,15-diacetylnivalenol) and 4,15-diacetoxy-12,13-epoxy-trichothec-9-en-3-ol (diacetoxyscirpenol). This is the first published report of these compounds affecting plant-parasitic nematodes.

Key Words—*Fusarium equiseti*, fungus, *Meloidogyne incognita*, root-knot nematode, trichothecene, nematicide, 4,15-diacetoxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one, 4,15-diacetoxy-12,13-epoxytrichothec-9-en-3-ol, 4,15-diacetylnivalenol, diacetoxyscirpenol.

INTRODUCTION

Certain strains of *Fusarium* fungi, when grown in laboratory cultures, secrete compounds that are toxic to plant-feeding nematodes (Mani and Sethi, 1984; Meshram

[*]To whom correspondence should be addresssed.

[1]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

0098-0331/01/0500-0859$19.50/0

and Goswami, 1989; Hallman and Sikora, 1996; Nitao et al., 1999). The presence of these compounds is manifested by the inhibition of egg hatch or the immobilization of juvenile nematodes in culture broth filtrates; however, the precise identities of the nematode-antagonistic compounds generally are undetermined. In one of the few examples where structures were partially characterized, Mani et al. (1986) determined that a mixture of long-chain alkanes was responsible for the toxicity of *Fusarium solani* cultures to the root-knot nematode, *Meloidogyne incognita*, a major pathogen that attacks hundreds of crop and ornamental plant species. Although not isolated specifically from a nematode-antagonistic fungus strain, several commercially available mycotoxins commonly produced by *Fusarium* species were tested and found to be nematicidal to *Meloidogyne javanica* at low concentrations (Ciancio, 1995).

Identification of nematode-antagonistic compounds from *Fusarium* species is important for at least three reasons. First, nematode-antagonistic compounds potentially can be used to manage nematode pathogens (Jatala, 1986). Natural products from fungi and bacteria are being sought as alternatives to the use of fumigant and nonfumigant nematicides. Second, in studies searching for biological control agents of pathogenic nematodes, *Fusarium* strains are frequently isolated from nematodes or their egg masses (Qadri and Saleh, 1990; Hay and Skipp, 1993; Oduor-Owino and Waudo, 1996; Chen et al., 1996; Viaene and Abawi, 1998). Identifying the nematode-antagonistic compounds produced by these fungi would help determine if such compounds contribute to the detrimental effects of these antagonists. Third, although it is unknown if compounds produced by *Fusarium* in vitro play a role in natural interactions between fungi and plant-parasitic nematodes, identification of nematode-antagonistic compounds can be the first step toward examining such interactions in future studies.

As part of a study searching for biological control agents, the culture broths of over 250 fungi isolated from soybean cyst nematodes (*Heterodera glycines* Ichinohe) were screened for nematode-antagonistic activity (Meyer et al., 1998). *Fusarium* species were common among those isolates producing broth filtrates that inhibited in vitro egg hatch of soybean cyst nematode and root-knot nematode, *M. incognita*. One of these strains, an isolate of *Fusarium equiseti*, was especially active against *M. incognita* (Nitao et al., 1999), and bioassay-guided isolation of culture broth constituents that caused the nematode-antagonistic activity was undertaken. We report herein the isolation and structure of two *F. equiseti* metabolites responsible in part for inhibiting in vitro egg hatch and juvenile mobility of *M. incognita*.

METHODS AND MATERIALS

Root-knot Nematode Bioassay. Bioassays were conducted in vitro as described previously (Nitao et al., 1999). Eggs were obtained from *Meloidogyne*

incognita (Kofoid & White) Chitwood cultured on tomato plants and were surface-disinfested with 0.5% sodium hypochlorite (Nitao et al., 1999). Eggs were placed in 24-well tissue culture plates with sterile-filtered test solutions. Test compounds were solubilized in DMSO before mixing in water (final concentration: 0.5% DMSO). Control solution was 0.5% DMSO in water. After seven days, the number of hatched second-stage juveniles and the numbers of those juveniles that were mobile and immobile were recorded. The percentage of eggs that hatched and the percentage of juveniles that were mobile were calculated. Each bioassay trial consisted of five replicate wells per treatment (200–280 eggs/well).

Differences in egg hatch and juvenile mobility between test and control solutions were tested with one-way analysis of variance (ANOVA) followed by Dunnett's test to compare each treatment against the control. When two trials were conducted for each set of treatments, results from individual trials were observed to be normally distributed with equal variances. Pooling of data from trials resulted in nonnormality and/or unequal variances that could not be corrected by standard transformations. This indicated that combining data sets would be inappropriate. Therefore, data from trials were analyzed separately, but for brevity, means and standard errors are reported on pooled data since the pattern of means was similar among trials. Kruskal-Wallis test on ranks was used when results from one bioassay trial did not satisfy assumptions of normality and homogeneous variances.

Fungus Culture. *Fusarium equiseti* was isolated from soybean cyst nematode females (*Heterodera glycines*) collected in the People's Republic of China in cooperation with Dr. Xing-Zhong Liu (Chinese Academy of Agricultural Sciences) and was identified by Dr. Jean Juba (Fusarium Research Center, Pennsylvania State University) and Dr. Richard Humber (USDA, ARS, Plant Protection Unit, New York) (Meyer et al., 1998). The fungus was deposited and is maintained in the USDA, ARS, Nematology Laboratory, Beltsville, Maryland, as isolate L128. The fungus was grown on potato–dextrose agar for seven days at 25°C. One-liter Erlenmeyer flasks containing 250 ml potato–dextrose broth were inoculated by blending agar plate cultures into the broth. Broth cultures were incubated for three days on a shaker (240 rpm, 25°C).

Isolation of Nematode-Antagonistic Compounds. After incubation, the cultures were centrifuged, and the broth was filtered through a glass-filter funnel. A total of 18.75 liters of broth was processed in six batches. The broth filtrate was extracted by mixing for 2 hr with Amberlite XAD-16 resin equilibrated in H_2O (1 volume gel–6 volumes broth). The resin was removed from the broth, washed with 2.5 bed volumes H_2O, and eluted with 2.5 bed volumes methanol (MeOH). The MeOH eluate was evaporated under vacuum, and the residue (5.1 g) was partitioned in 7% MeOH in H_2O and ethyl acetate (EtOAc). Earlier studies demonstrated that the nematode-antagonistic activity resided in the EtOAc fraction after partitioning (Nitao et al., 1999).

After evaporating to dryness, the EtOAc fraction (0.68 g) was separated in batches of 70–430 mg by using vacuum liquid chromatography (VLC) (silica gel, Merck grade 9385) and eluted with 100% EtOAc followed by MeOH. Five 25-ml EtOAc fractions and one 50-ml MeOH fraction were collected. VLC fractions from one of these batches were bioassayed at 100 μg/ml in one trial (5 replicates).

The first two VLC EtOAc fractions and equivalent fractions from other VLC batches were combined after examining with thin-layer chromatography (TLC). Silica gel plates were developed in 100% EtOAc or 20 : 1 chloroform–methanol ($CHCl_3$–MeOH); C_{18} plates were developed in 70% acetonitrile in H_2O. Spots were visualized by 50% sulfuric acid spray and charring. The pooled VLC fraction (97 mg) was subjected to medium pressure liquid chromatography (MPLC) (silica gel, Merck, grade 9385). A step gradient (80 : 1, 40 : 1, 20 : 1, and 10 : 1 $CHCl_3$–MeOH, and 100% MeOH) was used to elute 21 fractions, and fractions were combined into nine fractions based on TLC. Of these pooled fractions, the five fractions eluted with 40 : 1 and 20 : 1 $CHCl_3$–MeOH had masses substantial enough for bioassay and further processing and were assayed at 50 μg/ml in two trials (five replicates per trial).

Nematode-antagonistic compounds were purified from MPLC fractions with high-performance liquid chromatography (HPLC) (10 $\times$ 250 mm Alltima C_{18} column, Alltech, Deerfield, Illinois; acetonitrile–H_2O gradient, 2 ml/min, 214 nm detection). Purified compounds were bioassayed at 25 μg/ml (two trials; five replicates per trial).

Identification of Compounds. Compounds were identified by mass spectrometry, NMR spectroscopy, and X-ray crystallography. ^{1}H NMR and ^{13}C NMR spectra were acquired in $CDCl_3$ on a Bruker QE 300-MHz spectrometer; chemical shifts are relative to $CDCl_3$. Mass spectra (EI, CI using NH_3) were obtained on a Finnegan INCOS 50 mass spectrometer and Finnegan MAT solid probe. X-ray crystallography was performed on a crystal obtained from aqueous CH_3CN. A colorless crystalline plate (0.43 $\times$ 0.06 $\times$ 0.03 mm) was optically centered on a Bruker SMART1000 single crystal CCD diffractometer. The SAINT+ data reduction program (Bruker Analytical X-ray Systems, Madison, Wisconsin) with 2θ max set to 55.0° was implemented to correct for Lorentz and polarization effects. An empirical absorption correction was applied to 11,861 reflections ($\pm h \pm k \pm l$) with 3328 being unique [R(int) = 0.0399] based upon equivalent reflection measurements using Blessing's method (Blessing, 1995) in the program SADABS (Sheldrick, 1996). The XPREP program (Sheldrick, 1994) was implemented to determine the space group and generate the initial files. The structure was determined by direct methods with the program XS (Sheldrick, 1990) and resulted in the successful location of many atoms comprising the molecule. Full-matrix least-squares refinement with the program XL (Sheldrick, 1993) coupled with difference-Fourier maps was used to locate, input, and complete the configuration of the nematode-antagonistic molecule and an accompanying water molecule.

Hydrogen atoms were placed initially in calculated positions and then allowed to refine freely; those hydrogen atoms comprising the water molecule were located directly from a difference-Fourier map and allowed to refine freely.

RESULTS

Isolation of Nematode-Antagonistic Compounds. Fractions from VLC had a significant effect on egg hatch when tested at 100 μg/ml (ANOVA, $P < 0.001$) (Figure 1). The proportion of eggs that hatched was lower in each fraction compared to the proportion in the control solution (Dunnett's test, $P < 0.05$). Egg hatch in VLC fractions 1 and 5 was 90% lower than that in the control. Of those juveniles that hatched, the percentage that was mobile also was affected by the *Fusarium* fractions (Kruskal-Wallis test, $P < 0.001$). The proportion of juveniles mobile in the fractions eluted with EtOAc (VLC fractions 1–4) was lower than in the control (Dunnett's test, $P < 0.05$). All or almost all of those juveniles that hatched in these fractions were immobile. However, mobility was not reduced by the MeOH fraction (VLC fraction 5) (Dunnett's test, $P > 0.05$).

Combined VLC fractions 1 and 2 were chosen for further separation on MPLC due to their relatively higher activity against both hatch and mobility and the larger

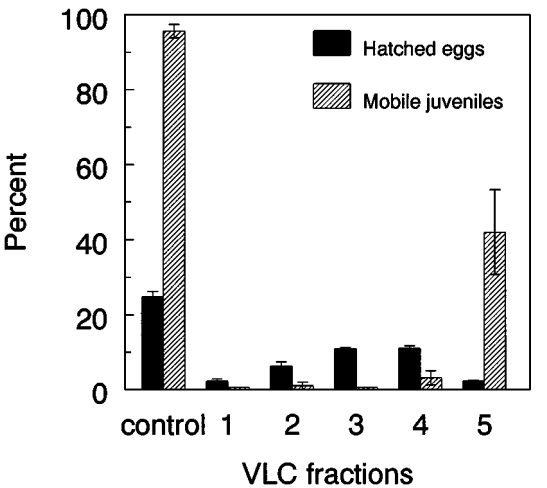


FIG. 1. Percentage of *Meloidogyne incognita* eggs hatched and percentage of hatched juveniles that were mobile after one week in vacuum liquid chromatography (VLC) fractions of *Fusarium equiseti* culture broth. VLC fractions 1–4 were eluted with ethyl acetate; fraction 5 was eluted with methanol. Fractions were tested at a concentration of 100 μg/ml. Data are mean $\pm$ SE of five replications in one trial (230 eggs/replicate).

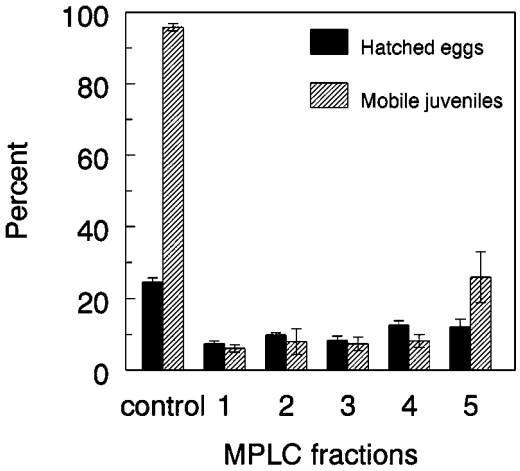


FIG. 2. Percentage of *Meloidogyne incognita* eggs hatched and percentage of hatched juveniles that were mobile after one week in medium-pressure liquid chromatography (MPLC) fractions of *Fusarium equiseti* culture broth. MPLC was performed on combined VLC fractions 1 and 2. Fractions were tested at a concentration of 50 μg/ml. Data are mean $\pm$ SE of 10 replications in two trials (250 and 280 eggs/replicate).

available quantity. In both bioassay trials of MPLC fractions, hatch and juvenile mobility were lower in each MPLC fraction than in the control when tested at 50 μg/ml (ANOVA, $P < 0.001$; Dunnett's test, $P < 0.05$) (Figure 2). Hatch was reduced 25–40% by the fungus fractions compared to hatch in the control solution, and the proportion of juveniles that were mobile was as much as 94% lower.

A major component contained in MPLC fractions 1–3 was purified by HPLC and crystallized (6 mg). X-ray crystallography revealed the crystal unit cell to consist of one molecule of 4,15-diacetoxy-3,7-dihydroxy-12,13-epoxytrichothec-9-en-8-one (4,15-diacetylnivalenol) and one water molecule in proximity to the epoxide group (Figure 3). Crystal data: monoclinic; $C_{19}H_{26}O_{10}$; $[C_{19}H_{24}O_9][H_2O]$; FW = 414.40; space group = $P2_1$; unit cell dimensions: $a = 10.4225(13)$ Å, $b = 7.7034(10)$ Å, $c = 11.9740(15)$ Å, $\beta = 100.072(2)^\circ$; $V = 946.6(2)$ Å^3; $Z = 2$; $D_x = 1.454$ g/cm^3; λ(MoKα) = 0.71073 Å; μ(MoKα) = 0.118 mm^1, F(000) = 40, $T = 173(2)$K. The absolute structure parameter (Flack, 1983), Flack(x), was refined to a value of $-1.0(8)$, indicating that while the structure is both correct and complete, its absolute configuration is inconclusive.

The identification of the molecule as 4,15-diacetylnivalenol (Figure 4) was consistent with NMR and MS analyses: ^{1}H NMR: δ 1.10 (3H, s, H-14), 1.90 (3H, br d, H-16), 1.91 (3H, s, H-20), 2.16 (3H, s, H-18), 3.08 (2H, m, H-13), 3.83 (1H, d, H-2), 4.22 (1H, dd, H-3), 4.27 (2H, dd, H-15), 4.73 (1H, d, H-11), 4.88 (1H, s, H-7), 5.23 (1H, d, H-4), 6.64 (1H, m, H-10). ^{13}C NMR APT: δ 7.5 (C-14, CH_3),

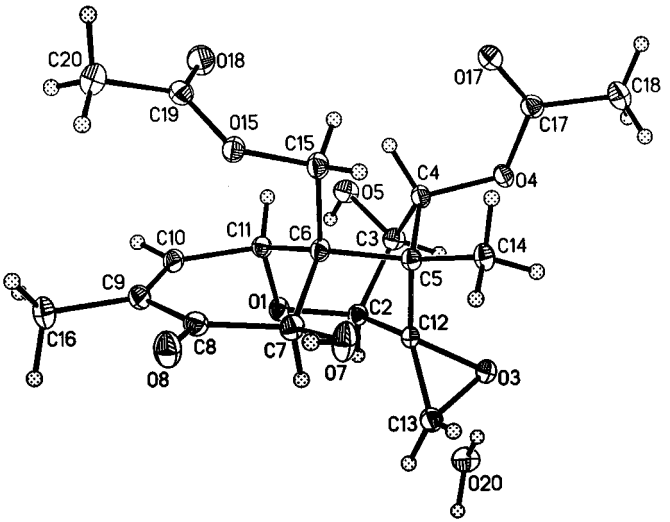


FIG. 3. Oak Ridge Thermal Ellipsoid Plot derived from X-ray crystallography showing the relative conformation of 4,15-diacetylnivalenol. The unit cell of the crystal included one water molecule (O20).

15.2 (C-16, CH_3), 20.6 (C-18, CH_3), 21.0 (C-20, CH_3), 46.1 (C-13, CH_2), 49.4 (C-5, C), 52.2 (C-6, C), 61.8 (C-15, CH_2), 64.3 (C-12, C), 69.3 (C-11, CH), 135.8 (C-9, C), 73.2 (C-7, CH), 78.4 (C-3, CH), 79.7 (C-2, CH), 83.5 (C4, CH), 138.3 (C-10, CH), 170.0 (C-17, C=O), 172.6 (C-19, C=O), 198.9 (C-8, C=O). EI-MS, *m*/*z* (rel. int.): 396 (1) $[M]^+$, 336 (6), 294 (9), 247 (10), 217 (10), 189 (30), 179 (100), 125 (37), 98 (53), 77 (57), 55 (48). CI-MS, *m*/*z*: 414 $[M + NH_4]$.

A second compound (3 mg) was purified from MPLC fraction 1. Based on NMR analysis, this compound was structurally similar to 4,15-diacetylnivalenol, but the hydroxyl group at C-7 and the carbonyl at C-8 were each replaced by

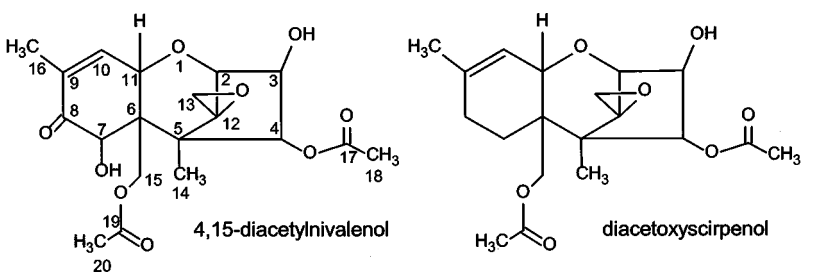


FIG. 4. Nematode-antagonistic trichothecene compounds isolated from *Fusarium equiseti*.

hydrogens. This compound was deduced to be 4,15-diacetoxy-12,13-epoxytrichothec-9-en-3-ol (diacetoxyscirpenol) (Figure 4): ^{1}H NMR: δ 0.82 (3H, s, H-14), 1.71 (3H, s, H-20), 1.72 (3H, br s, H-16), 1.90 (2H, m, H-13), 2.14 (3H, s, H-18), 2.77 (2H, d, H-9), 3.06 (2H, d, H-7), 3.69 (1H, d, H-2), 4.06 (2H, dd, H-15), 4.09 (1H, d, H-11), 4.16 (1H, dd, H-3), 5.12 (1H, d, H-4), 5.53 (1H, m, H-10). ^{13}C NMR APT: δ 6.9 (C-14, CH_3), 20.9 (C-18, CH_3), 21.0 (C-20, CH_3), 21.3 (C7, CH_2), 23.2 (C-16, CH_3), 27.9 (C-8, CH_2), 43.9 (C-13, CH_2), 47.8 (C-5, C), 48.7 (C-6, C), 63.6 (C-15, CH_2), 65.1 (C-12, C), 68.0 (C-11, CH), 84.9 (C-4, CH), 78.4 (C-3, CH), 78.9 (C-2, CH), 118.5 (C-10, CH). 140.5 (C-9, C), 171.7 (C-17, C=O), 173.8 (C-19, C=O). EI-MS, *m*/*z* (rel. int.): 366 (0.01), 306 (10), 278 (5), 233 (5), 187 (10), 175 (15), 159 (20), 124 (72), 105 (100), 91 (76), 79 (33), 55 (24). CI-MS, *m*/*z*: 384 [M + NH_4].

4,15-Diacetylnivalenol, diacetoxyscirpenol, and a third unidentified compound (2 mg) isolated from MPLC fraction 3 decreased egg hatch and juvenile mobility in both trials when tested at 25 μg/ml (ANOVA, $P < 0.001$; Dunnett's test, $P < 0.05$) (Figure 5). Egg hatch was reduced by approximately 40% compared to that in the control. The number of juveniles still mobile after hatching in

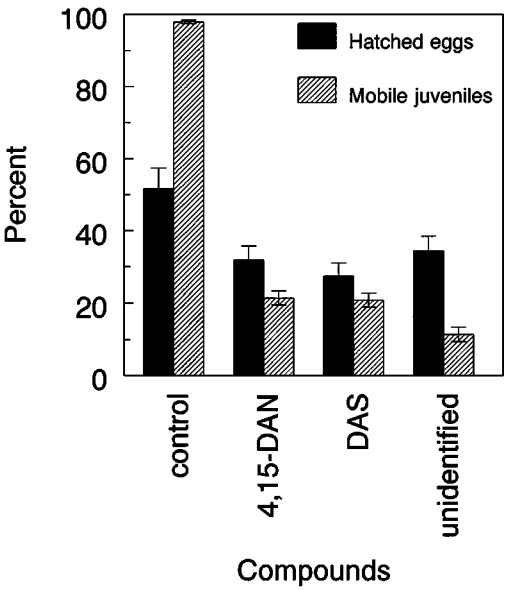


FIG. 5. Percentage of *Meloidogyne incognita* eggs hatched and percentage of hatched juveniles that were mobile after one week in compounds purified from *Fusarium equiseti* culture broth. Fractions were tested at a concentration of 25 μg/ml. 4,15-DAN = 4,15-diacetylnivalenol; DAN = diacetoxyscirpenol. Data are mean $\pm$ SE of 10 replications in two trials (200 and 250 eggs/replicate).

the test compounds was reduced by approximately 80% relative to the number in the control solution.

DISCUSSION

4,15-Diacetylnivalenol and diacetoxyscirpenol are trichothecenes, an important group of tricyclic sesquiterpene mycotoxins that inhibit protein synthesis and are commonly produced by *Fusarium* species (Sweeney and Dobson, 1998). These two compounds were first identified from *F. equiseti* as phytotoxins (Brian et al., 1961; Dawkins et al., 1965) but occur in a number of other *Fusarium* species (De Nijs et al., 1996). The broad-spectrum biological activity of diacetoxyscirpenol (DAS) has been especially well documented (Beasley, 1989); however, this is the first published report of activity against plant-parasitic nematodes for either compound.

The similarity in bioactivity between 4,15-diacetylnivalenol and diacetoxyscirpenol suggests that common structural elements are involved in their nematode-antagonistic activity. The C-7 hydroxyl group and the C-8 carbonyl group are not required for this activity, which suggests a hydrophilic binding site including the oxygens on C-3, C-4, and C-13 in a rigid conformation. The importance of these oxygens for biological activity is consistent with trichothecene structure–activity relationships known for protein synthesis inhibition and anticancer activity (Beasley, 1989). The X-ray data, which is the first such published analysis for 4,15-diacetylnivalenol, suggest a labile hydrophilic binding site could also occur that involves the two acetate groups. The acetate group attached to C-15 folds over the six-membered ring such that the C-16 and C-20 methyl groups are spatially close to each other, bringing the acetate carbonyl close to the C-9 double bond and the O-1 oxygen. The acetate group on C-15 along with that on C-4 is also involved in anticancer activity (Beasley, 1989). The hydrophobic portion of the molecule C-5, C-6, C-7, C-8 is structurally and/or conformationally different between the two compounds and appears not to be involved in nematode-antagonistic activity.

Nematode-antagonistic activity against *M. incognita* was detected in many different fractions during bioassay-guided isolation, indicating that compounds in addition to 4,15-diacetylnivalenol and diacetoxyscirpenol are contributing to the activity of the culture broth. Some of these other compounds, as well as the third nematode-antagonistic compound that was purified but unidentified, could also be trichothecenes, as *F. equiseti* is reported to produce at least four other trichothecenes (De Nijs et al., 1996). One of these, T-2 toxin, has been shown to be nematicidal to *Meloidogyne javanica* (Ciancio, 1995). Despite the predisposition of *Fusarium* species to produce mycotoxins, this should not discourage further investigation of *Fusarium* cultures to find useful natural products with a narrower activity range. Screening procedures to exclude broad-spectrum mycotoxins would increase efficiency of a search for nematode-antagonistic compounds.

It is not known if trichothecenes produced by *Fusarium* strains that parasitize plant nematodes play a role in interactions between fungi and nematodes under natural conditions. Enzymes secreted by parasitic fungi are known to help colonization of nematode eggs by penetrating the egg shell (Segers et al., 1991), but the contribution of nonenzymatic toxins to the success of parasitism has not been studied. If the biology of microbial colonization of insects is an appropriate analogy, then such a contribution would be probable. *Photorhabdus* bacteria that are released into insect hosts by entomophagous nematodes secrete toxins that kill the host and inhibit colonization of the carcass by competing microbes (Bowen et al., 1998). As *Fusarium* species are commonly found in soil environments, there should be ample opportunities for these fungi and their secondary metabolites to interact with root-feeding nematodes.

The broad-spectrum toxicity and nonspecific mode of action of trichothecenes indicate that they would be impractical by themselves as chemical control agents of plant nematode pathogens without significant modification. The production of trichothecenes in a strain isolated from a plant-parasitic nematode suggests that if *Fusarium* species are used for biological control, production of mycotoxins by these strains should be evaluated if incidental exposure to humans is a concern. The occurrence of mycotoxins with broad-spectrum toxicity in a potentially beneficial strain does not automatically preclude use of that fungus for biological control since artificial selection may eliminate or reduce mycotoxin production in that strain. On the other hand, should trichothecenes and other mycotoxins be found to enhance efficacy of parasitism and biological control, the benefits of their production will have to be balanced against their potential negative side effects.

Acknowledgments—We thank David DeVilbiss and Dr. Andre Raw for mass spectra; Dr. Muralee Nair for helpful comments and advice; and Paula Crowley, Steve Rogers, Rob Lee, and Christina Bennett for maintaining nematode cultures. Fungus collection was supported by funds from the Interagency Biological Control Coordinating Committee; the Agricultural Research Service negotiated this activity as part of the Sino-American Biological Control Laboratory Program.

REFERENCES

BEASLEY, V. R. (ed.). 1989. Trichothecene Mycotoxicosis: Pathophysiologic Effects, Volume I. CRC Press, Boca Raton, Florida.

BLESSING, R. H. 1995. An empirical correction for absorption anisotropy. *Acta Crystallogr*. A51:33–38.

BOWEN, D., ROCHELEAU, T. A., BLACKBURN, M., ANDREEV, O., GOLUBEVA, E., BHARTIA, R., and FFRENCH-CONSTANT, R. H. 1998. Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* 280:2129–2132.

BRIAN, P. W., DAWKINS, A. W., GROVE, J. F., HEMMING, H. G., LOWE, D., and NORRIS, G. L. F. 1961. Phytotoxic compounds produced by *Fusarium equiseti*. *J. Exp. Bot.* 12:1–12.

CHEN, S. Y., DICKSON, D. W., and MITCHELL, D. J. 1996. Pathogenicity of fungi to eggs of *Heterodera glycines*. *J. Nematol.* 28:148–158.

CIANCIO, A. 1995. Observations on the nematicidal properties of some mycotoxins. *Fundam. Appl. Nematol.* 18:451–454.

DAWKINS, A. W., GROVE, J. F., and TIDD, B. K. 1965. Diacetoxyscirpenol and some related compounds. *Chem. Commun.* 1965:27–28.

DE NIJS, M., ROMBOUTS, F., and NOTERMANS, S. 1996. *Fusarium* molds and their mycotoxins. *J. Food Safety* 16:15–58.

FLACK, H. D. 1983. On enantiomorph-polarity estimation. *Acta Crystallogr.* A39:876–881.

HALLMAN, J., and SIKORA, R. A. 1996. Toxicity of fungal endophyte secondary metabolites to plant parasitic nematodes and soil-borne plant pathogenic fungi. *Eur. J. Plant Pathol.* 102:155–162.

HAY, F. S., and SKIPP, R. A. 1993. Fungi and actinomycetes associated with cysts of *Heterodera trifolii* Goffart (Nematoda: Tylenchida) in pasture soils in New Zealand. *Nematologica* 39:376–384.

JATALA, P. 1986. Biological control of plant-parasitic nematodes. *Ann. Rev. Phytopathol.* 24:453–489.

MANI, A., and SETHI, C. L. 1984. Effect of culture filtrates of *Fusarium oxysporum* f. sp. *ciceri* and *Fusarium solani* on hatching and juvenile mobility of *Meloidogyne incognita*. *Nematropica* 14:139–144.

MANI, A., SETHI, C. L., and DEVKUMAR. 1986. Isolation and identification of nematoxins produced by *Fusarium solani* (Mart) Sacc. *Indian J. Nematol.* 16:247–251.

MEYER, S. L. F., HUETTEL, R. N., LIU, X.-Z., HUMBER, R. A., JUBA, J., and NITAO, J. K. 1998. Potential biocontrol fungi isolated from soybean cyst nematode eggs from China. *Int. Cong. Plant Pathol. Abstra.* 3:5.2.10.

MESHRAM, N. J., and GOSWAMI, B. K. 1989. Role of dominant rhizosphere fungi isolated from soil amended with mustard and karanj cakes on hatching and mortality of *Meloidogyne incognita* juveniles. *Indian J. Nematol.* 19:180–185.

NITAO, J. K., MEYER, S. L. F., and CHITWOOD, D. J. 1999. In-vitro assays of *Meloidogyne incognita* and *Heterodera glycines* for detection of nematode-antagonistic fungal compounds. *J. Nematol.* 31:172–183.

ODUOR-OWINO, P., and WAUDO, S. W. 1996. Effects of five fungal isolates on hatching and parasitism of root-knot nematode eggs, juveniles and females. *Nematol. Medit.* 24:189–194.

QUADRI, A. N., and SALEH, H. M. 1990. Fungi associated with *Heterodera schachtii* (Nematoda) in Jordan, II. Effect on *H. schachtii* and *Meloidogyne javanica*. *Nematologica* 36:104–113.

SEGERS, R., BUTT, T. M., KERRY, B. R., BECKETT, A., and PEBERDY, J. F. 1991. The role of the proteinase VCP1 produced by the nematophagous *Verticillium chlamydosporium* in the infection process of nematode eggs. *Mycol. Res.* 100:421–428.

SHELDRICK, G. M. 1990. Phase annealing in SHELX-90: Direct methods for larger structures. *Acta Crystallogr.* A46:467–473.

SHELDRICK, G. M. 1993. SHELXL93, Programme for the Refinement of Crystal Structures. Universtät Göttingen, Göttingen.

SHELDRICK, G. M. 1994. SHELXTL, Version 5.03. Siemens Analytical X-ray Instruments, Inc., Madison, Wisconsin.

SHELDRICK, G. M. 1996. SADABS "Siemens Area Detector Absorption Correction." Universtät Göttingen, Göttingen.

SWEENEY, M. J., and DOBSON, D. W. 1998. Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. *Int. J. Food Microbiol.* 43:141–158.

VIANE, N. M., and ABAWI, G. S. 1998. Fungi parasitic on juveniles and egg masses of *Meloidogyne hapla* in organic soils from New York. *Suppl. J. Nematol.* 30:632–638.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

OLFACTION IN THE QUEENSLAND FRUIT FLY, *Bactrocera tryoni*. I: IDENTIFICATION OF OLFACTORY RECEPTOR NEURON TYPES RESPONDING TO ENVIRONMENTAL ODORS

C. D. HULL* and B. W. CRIBB

Department of Zoology and Entomology and the Centre for Microscopy and Microanalysis
The University of Queensland, 4072
Queensland, Australia

(Received April 19, 2000; accepted January 1, 2001)

Abstract—The electroantennogram method was used to investigate the number of distinct olfactory receptor neuron types responding to a range of behaviorally active volatile chemicals in gravid Queensland fruit flies, *Bactrocera tryoni*. Three receptor neuron types were identified. One type responds to methyl butyrate, 2-butanone, farnesene, and carbon dioxide; a second to ethanol; and a third to *n*-butyric acid and ammonia. The receptor neuron type responding to methyl butyrate, 2-butanone, farnesene, and carbon dioxide consists of three subtypes. The presence of a limited number of receptor neuron types responding to a diverse set of chemicals and the reception of carbon dioxide by a receptor neuron type that responds to other odorants are novel aspects of the peripheral olfactory discrimination process.

Key Words—Olfaction, electroantennogram, *Bactrocera tryoni*, Diptera, Tephritidae, receptor type, environmental odorants, carbon dioxide.

INTRODUCTION

Bactrocera tryoni, a serious agricultural pest, has been shown to use environmental odorants in orientation behavior. Solvent extracts of the skin of fruits contain volatile chemicals that stimulate orientation to their source and elicit ovipositor probing and egg-laying (Pritchard, 1969; Eisemann, 1980). Olfactometer experiments have also been used to identify various chemicals that elicit positive orientation of female *B. tryoni* to the source (Eisemann and Rice, 1992). The flies are known to respond to stimulation with carbon dioxide (Rice, 1989; Stange, 1999),

* To whom correspondence should be addressed. e-mail: c.hull@ento.uq.edu.au

0098-0331/01/0500-0871$19.50/0

which may be part of the ovipositional orientation process. Female *B. tryoni* also respond positively to ammonia (Bateman and Morton, 1981), which may be related to their feeding behavior. From these experiments, we have selected chemicals that have a proven behavioral effect, and therefore must be detectable by the olfactory system, in order to undertake physiological investigations of the olfactory system.

The antennae are the principal organs for detecting olfactory signals in insects. The recording of electrical activity across the whole antenna in response to stimulation with odors is known as an electroantennogram (EAG). An EAG is thought to be the summed receptor potentials of the olfactory receptor neurons (Schneider, 1962), but the exact electrical processes are not known (Park and Hardie, 1998). The electrical potential may result from many "leakage" current loops around individual sensilla. Because an EAG indicates activity of the receptor potentials produced by olfactory receptor neurons, by using mixtures of chemicals the EAG technique can be used to show whether two chemicals are accepted on the same or different receptor neuron types. Such EAG methods have been used to indicate separate receptor neuron types for various chemicals in *Helicoverpa zea* (Lepidoptera) (Christensen et al., 1990), various species of *Glossina* (Diptera) (Den Otter, 1991), *Argyrotaenia velutinana* (Lepidoptera) (Baker and Roelofs, 1976), *Psila rosae* (Diptera) (Guerin et al., 1983a), and *Drosophila* (Diptera) (Borst, 1984). Knowing the number of olfactory receptor neuron types responding to a range of chemicals indicates how specific the receptors neurons are and provides information on how environmental information is processed by the nervous system.

Among the Tephritidae, EAG studies have been used to investigate the chemoreceptor response to plant volatiles for *Rhagoletis pomonella* (Fein et al., 1982; Averill et al., 1988), *Ceratitis capitata* (Light et al., 1988, Hernandez et al., 1996, Cossè et al., 1995), *B. dorsalis* (Light and Jang, 1987), *B. oleae*, and *R. cerasi* (Guerin et al., 1983b). However, none of these studies investigated the potential number of receptor neuron types. In this paper, EAGs are recorded from female *B. tryoni* in order to determine the number and specificity of olfactory receptor neuron types responding to a range of behaviorally significant, environmental odorants. These experiments also serve to increase our understanding of the peripheral processing of nonpheromonal semiochemicals and confirm the usefulness of the EAG technique for studying such processes.

METHODS AND MATERIALS

Insects. Bactrocera tryoni (Froggatt) were cultured at the Department of Entomology, University of Queensland, from flies originally obtained from the Department of Primary Industries, Brisbane, Australia. The larval flies were cultured as per Neale (1989). Adults were given unlimited access to sugar and water and regularly provided with protein hydrolysate. Only gravid flies were selected

for experimentation. Intact flies were wrapped in Blu-tack [Bostik (Australia) Pty Ltd] adhesive to constrain head movements and manipulated so that only the antennae were left protruding. We have found that Blu-tack has no discernible effect on the olfactory system of insects when used in electrophysiological experiments.

Electrophysiological Recording Technique. Ag–AgCl electrodes were used for both recording and indifferent electrodes. They were placed in disposable glass capillaries, about 1 cm from the tip. The glass capillaries, drawn to a point on a vertical pipet puller, were cut to a diameter of about 25 μm at the tip and filled with blowfly saline (Chen and Friedman, 1975).

The indifferent electrode was inserted at the base of the antenna, through the circumantennal sulcus. The recording electrode was inserted in the tip of the funiculus. The electrode output was first transmitted to a preamplifier (Grass P16D microelectrode DC amplifier), then to a differential amplifier (Tektronix 5A22N). Signals were displayed on a Tektronix 5103N storage oscilloscope and were also stored as a strip chart using the Delta program (Digital Solutions Pty Ltd). Some signals were collected by using a Syntech probe and data acquisition system and analyzed with the Syntech EAG 2.4 program on a personal computer. Electrical shielding was provided by a Faraday cage, with all metal surfaces within it being grounded.

Test Chemicals. Chemicals were selected because they elicit behavioral activity in *B. tryoni* [see Bateman and Morton (1981), Eisemann and Rice (1992) and Rice (1989)]: 2-butanone (Aldrich Chemical Company, claimed purity 99%), methyl butyrate (Aldrich Chemical Company, claimed purity 99%), *n*-butyric acid (British Drug Houses Ltd, claimed purity 99%), a mixture of isomers of farnesene (TCI Ltd, purity unknown), ethanol (Ajax Chemicals Pty Ltd, claimed purity 99.5%) and ammonia (Ajax Chemicals Pty Ltd 28.0–30.0% w/w), and carbon dioxide (taken from a cylinder of compressed food grade purity, provided by Commonwealth Industrial Gases, Brisbane, Australia).

Odorant Delivery. Humidified analytical grade compressed air was continuously blown over the fly at a rate of 500 ml/min, with the nozzle for the airstream placed 1 cm from, and directly in front of, the fly. The tube carrying the airflow was 3-mm-ID teflon tubing and was connected to a glass nozzle (same internal diameter). Test odorant samples were taken as saturated vapor, at room temperature, using gas-tight syringes. They were manually injected into the airstream through a rubber septum, 50 cm from the delivery point. Injection time was 0.5–1.0 secs. All samples were injected at the same time segment after the recording program had been activated, so that onset of stimulation could be easily identified. For carbon dioxide, a 100-ml flask was filled directly from a gas cylinder and then sealed with Parafilm (American National Can). A sample was then taken up by a gas-tight syringe inserted through the Parafilm. Preliminary experiments confirmed the method of odorant delivery for carbon dioxide and the other chemicals to be a reliable way of presenting specific quantities of chemicals to the

antenna. The relationship between the syringe volume and the actual concentration of the chemical at the preparation was not established because we only needed to know that the receptors were saturated. This was achieved by establishing dose–response curves (see below). For the mixture experiments, two chemicals were collected into one syringe and then injected into the continuous airstream. Stimulus delivery controls (see below) were undertaken to ensure volume artefacts were excluded.

Experimental Procedure. In all experiments the responses were compared to a standard of 50 μl methyl butyrate vapor. The standard was tested, followed by three injections of the test sample, then the standard again. At least 2 mins was left between each injection to allow disadaptation of the antenna. Responses from the three sample injections were averaged and expressed as a percentage of the standard. Standardizing each test vapor allowed comparison between individual flies and between different test chemicals and corrected for time dependent variability within preparations (Roelofs and Comeau, 1971; Light and Birch, 1979; Light, 1983). The EAG responses were initially measured in millivolts, recorded as the height of the initial depolarization.

Establishing Saturation Volumes of Test Chemicals. Saturation volumes are defined as the volume of vapor at which the standard error of the mean response overlapped the standard error of the mean response of the highest volume (Dickens, 1984). The saturating volumes had to be established, as it is a requirement of mixture experiments that all the receptor neuron types be fully activated. Differences in volatility, and therefore the number of molecules of each chemical in the test sample, will affect the size of the EAG (Brockerhoff and Grant, 1999). Applying enough of each chemical to maximally stimulate the receptor neurons will avoid this problem. To find the saturation points, the antennae were stimulated with volumes of vapor ranging between 5 μl and 400 μl.

Mixture Experiments. Chemicals were tested as binary mixtures to determine how many different receptor neuron types were present. First, the antennal response was determined for two individual chemicals at a volume in excess of the saturation volume (for all chemicals 400 μl was used). Second, a mixture containing 400 μl of each of the two chemicals was applied. Chemicals were always presented at a volume in excess of the saturation volume to ensure that the sensory dendrites for that chemical were maximally activated. The order of presentation of the mixture series and the chemicals within a mixture series were randomized.

Before statistical analysis, the mean result of stimulation with a control injection of clean air (see stimulus delivery controls below) was subtracted from the antennal response of each of the samples in the mixture experiments. The mixture experiments were then analyzed using paired sample *t* tests. Initially a one-way test was conducted to determine if the response to the mixture of chemicals was greater than the response of the largest of the two individual chemicals (i.e., a summating response). If the response to the mixture was summating, then a two-way test was

conducted to determine if the response to the mixture was equal to the calculated additive response of the two individual chemicals (i.e., fully summating).

Control Stimuli. Each test chemical used in the mixture experiments was applied using 400 μl and 800 μl of vapor to determine whether any of the responses to the binary mixtures were the result of artifacts of the stimulus delivery system. A larger EAG at 800 μl would indicate a stimulus delivery artifact, as the chemicals have been shown to saturate the antenna at volumes below 400 μl. To see if the delivery of a burst of odor produced a response in the nervous system, separate from the effect of the odor itself, 400 μl and 800 μl of clean air were tested. An EAG response to clean air was found and was therefore subtracted from all of the responses of the test chemicals and mixtures. This ensured that the calculated additive response for each mixture accurately reflected the EAG response to the individual chemicals. Consideration of extraneous elements in EAGs is particularly important when dealing with small EAG responses.

Local Potential Controls. Three of the mixtures were tested with the recording electrode in a different position to see if the results of the mixture experiments were influenced by local voltage potentials from specific sensillar fields at the tip of the antenna. The mixtures tested were methyl butyrate with ammonia, methyl butyrate with 2-butanone, and ethanol with 2-butanone. In these mixture experiments the recording electrode was inserted into the basal region of the funiculus, with all other parameters remaining as before.

Electrode Potential Controls. To determine whether any of the responses were electrode potentials (artifacts), methyl butyrate, 2-butanone, farnesene, ethanol, *n*-butyric acid, and ammonia were tested on recently killed flies. Flies were killed by freezing at -20°C, but were tested at room temperature. This technique shows how much of the EAG is the result of physiological activity within the antenna, and how much (if any) is the result of an interaction of the chemical with the electrodes. All other parameters were as previously mentioned.

RESULTS

Saturation Volumes of Test Chemicals. The largest responses were obtained with 2-butanone, followed by methyl butyrate, *n*-butyric acid, ethanol, carbon dioxide, ammonia and farnesene (Table 1). All the chemicals had antennal response values at 400 μl that were significantly larger than the response to stimulation with clean air, indicating a chemoreceptor response within the antenna, rather than an artifact. The actual EAG responses ranged from 0.2 to 4.0 mV.

Mixture Experiments. The results of the mixture experiments are displayed according to the outcome of the statistical tests, i.e., divided into fully summating, nonsummating, and partly summating groups (Figures 1 and 2). Ethanol was fully summating with methyl butyrate (two-way paired sample t test; $t = 0.836$)

TABLE 1. SATURATION VOLUMES AND EAG RESPONSES AT POINT OF SATURATION FOR CHEMICALS USED IN MIXTURE EXPERIMENTS

Chemical	Saturation volume (μl)	EAG response at saturation (mean $\pm$ 1 SE)
Methyl butyrate	100	119 ± 3
2-Butanone	250	151 ± 7
Farnesene	250	35 ± 9
n-Butyric acid	150	61 ± 10
Ammonia	250	40 ± 6
Ethanol	300	59 ± 3
Carbon dioxide	15	33 ± 11

(Figure 1A), 2-butanone (two-way paired sample t test; $t = 0.311$) (Figure 1D), farnesene (two-way paired sample t test; $t = 2.055$) (Figure 1G), carbon dioxide (two-way paired sample t test; $t = 0.574$) (Figure 1L), *n*-butyric acid (two-way paired sample t test; $t = 2.323$) (Figure 1J) and ammonia (two-way paired sample t test; $t = 1.946$) (Figure 1K). Ammonia also had fully summating responses when mixed with farnesene (two-way paired sample t test; $t = 1.650$) (Figure 1I), methyl butyrate (two-way paired sample t test; $t = 1.483$) (Figure 1C), 2-butanone (two-way paired sample t test; $t = 0.827$) (Figure 1F) and carbon dioxide (two-way paired sample t test; $t = 0.288$) (Figure 1N). Similarly, *n*-butyric acid was fully summating with farnesene (two-way paired sample t test; $t = 0.858$) (Figure 1H), methyl butyrate (two-way paired sample t test; $t = 0.055$) (Figure 1B), 2-butanone (two-way paired sample t test; $t = 0.714$) (Figure 1E) and carbon dioxide (two-way paired sample t test; $t = 0.942$) (Figure 1M).

The combination of ammonia and *n*-butyric acid gave a response that was not larger than the largest response of the individual chemicals (produced by the ammonia) (one-way paired sample t test; $t = -3.490$) (Figure 2F), that is nonsummating. Methyl butyrate was nonsummating when presented in combination with farnesene (one-way paired sample t test; $t = 0.612$) (Figure 2B), 2-butanone (one-way paired sample t test; $t = 1.133$) (Figure 2A) and carbon dioxide (one-way paired sample t test; $t = 0.263$) (Figure 2C). 2-Butanone was nonsummating when presented in combination with farnesene (one-way paired sample t test; $t = -2.498$) (Figure 2D) and carbon dioxide (one-way paired sample t test; $t = -3.259$) (Figure 2E).

The combination of farnesene with carbon dioxide resulted in a partly summating response. The mixture of these two chemicals gave a bigger response than the largest response of the two individual chemicals (produced by the carbon dioxide) (one-way paired sample t test; $t = 3.529$), but the mixture was not equivalent to the calculated additive response of the two individual chemicals (two-way paired sample t test; $t = 4.038$) (Figure 2G).

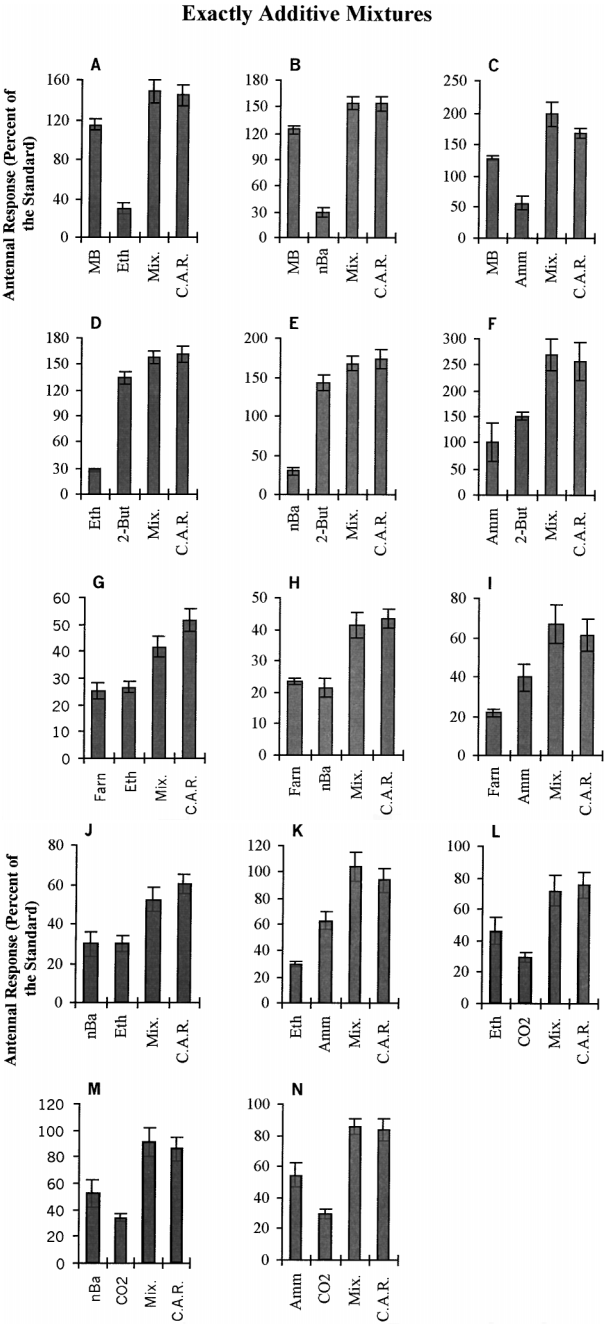


FIG. 1. Results of the mixture experiments showing those mixtures of chemicals that produced additive EAG responses. The EAG responses represent the mean response $\pm$ 1 SE ($N = 6$). MB = methyl butyrate, 2-But = 2-butanone, Farn = farnesene, Eth = ethanol, nBa = *n*-butyric acid, Amm = ammonia, CO2 = carbon dioxide, and C.A.R. = calculated additive response.

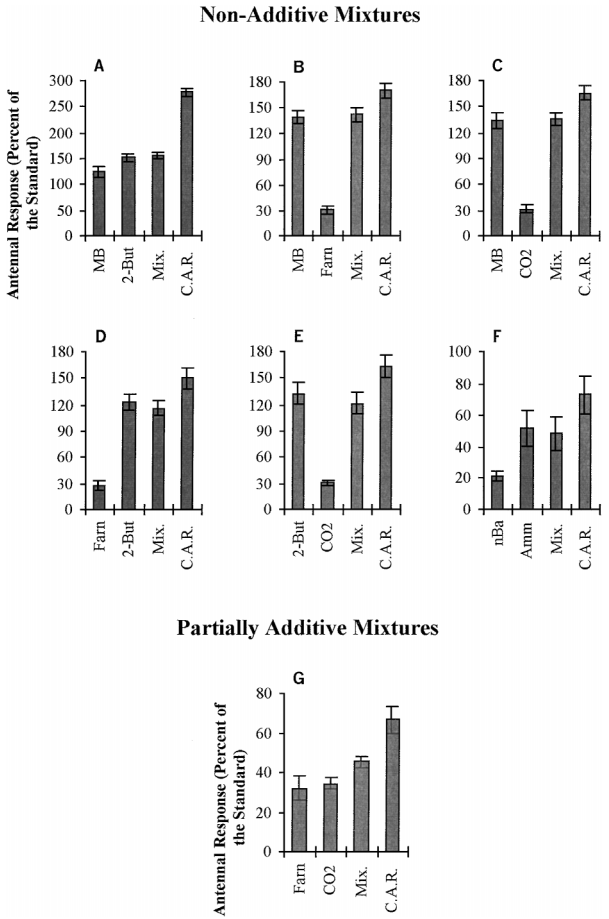


FIG. 2. Results of the mixture experiments showing those mixtures of chemicals that produced nonadditive or partially additive EAG responses. The EAG responses represent the mean response ± 1 SE ($N = 6$). MB = methyl butyrate, 2-But = 2-butanone, Farn = farnesene, Eth = ethanol, nBa = *n*-butyric acid, Amm = ammonia, CO2 = carbon dioxide and C.A.R. = calculated additive response.

Control Stimuli. We compared the mean responses from stimulating with 400 μ and 800 μl for each of the odors. The use of 800 μl of a single chemical simulates the presentation of a binary mixture, but with only one chemical present any increase in response compared to 400 μl represents a stimulus delivery artifact. The two means were compared by using two-way paired sample *t* tests. In all cases the null hypothesis (that the difference between the two means was equivalent to

zero) was accepted at the 95% significance level (ammonia, $t = 0.085$; 2-butanone, $t = 1.432$; carbon dioxide, $t = 0.553$; ethanol, $t = 0.775$; farnesene, $t = 0.990$; methyl butyrate, $t = 0.202$; *n*-butyric acid, $t = 0.119$). The means for the control stimulus experiments using 400 μl and 800 μl of analytical grade air were compared using a two-sample *t* test, and found to be equal ($t = 0.616$).

Local Potential Experiments. Three of the mixtures were tested with the recording electrode in different positions, to see whether the position of the electrode had any effect on the types of responses (i.e., nonsummating, partly summating, or fully summating). The mixture of ethanol with 2-butanone showed a fully summating response (two-way paired sample *t* test, $t = 0.562$). The mixture of methyl butyrate with 2-butanone showed a nonsummating response (one-way paired sample *t* test, $t = -0.227$). The mixture of methyl butyrate with ammonia showed a response that was fully summating (two-way paired sample *t* test, $t = 2.370$). In all three cases the types of summating and nonsummating responses are the same as for the initial mixture experiments, indicating that the position of the recording electrode in the funiculus was not a contributing factor.

Electrode Potential Experiments. In the experiments with recently killed flies, the EAGs were eliminated for most chemicals (Figure 3). In comparison, the responses from live flies showed large, negative potentials. Only ammonia showed any potential deflection. However, the potential generated for ammonia was small in comparison to those obtained from living flies and was a positive potential. The positive potential of approximately 0.1 mV compares to the average negative deflection obtained from living flies of -1.8 mV.

DISCUSSION

Number of Olfactory Receptor Neuron Types. The results of the mixture experiments indicate that at least three types of receptor neurons are being used by gravid *B. tryoni* in detecting the seven tested chemicals. One receptor neuron type detects ethanol, another type detects ammonia and *n*-butyric acid, and the third type detects farnesene, methyl butyrate, 2-butanone and carbon dioxide.

The division of the mixtures into fully, partly, or nonsummating categories was based solely on the results of the statistical tests. Ethanol, therefore, appears to be detected by a different receptor neuron type than the other six chemicals. The remaining chemicals can be divided into two groups based on the types of responses they elicited when presented in combinations. Ammonia is detected by the same receptor neuron type as *n*-butyric acid because a mixture of these two chemical vapors was not summating, not larger than the largest of the individual chemicals. However, these two chemicals showed fully summating responses with all other chemicals, indicating separate receptor neuron types. The nonsummating response to the mixture of ammonia and *n*-butyric acid is not likely to be due to an

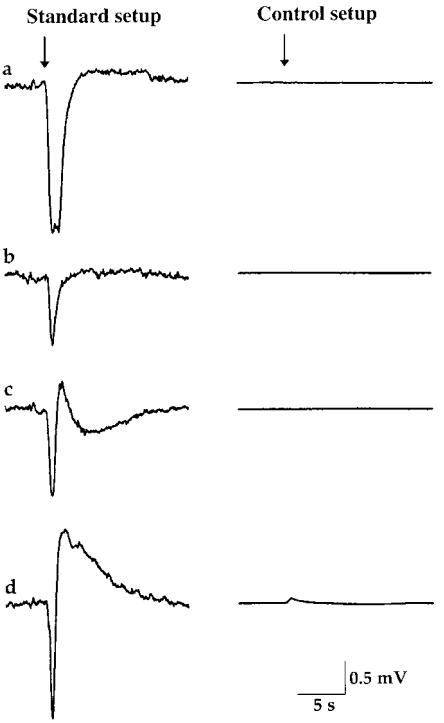


FIG. 3. Comparison of electroantennogram traces between standard and control preparations. The control preparation used a recently killed specimen; therefore any resultant potentials were artifacts. (a) 400 μl of methyl butyrate, (b) 400 μl of ethanol, (c) 400 μl of *n*-butyric acid, (d) 400 μl of ammonia. The arrow represents the point of stimulus injection.

interaction between the two chemicals in the syringe. We believe that significant association between the weak base ammonia and the weak acid butyric acid in the vapor phase can be ruled out on the grounds that there is no visible evidence of solid ammonium butyrate. This contrasts with the situation where an ammonium chloride "smoke" forms between hydrochloric acid vapor and ammonia.

Farnesene, methyl butyrate, 2-butanone, and carbon dioxide are likely to be detected by a separate, third type of receptor neuron, but the results are not as simple to interpret as the previous two groups. Methyl butyrate and 2-butanone are nonsummating when presented together, indicating common receptor neuron types. Farnesene is also nonsummating when tested in combination with either methyl butyrate or 2-butanone, showing that it is detected by the same receptor neuron types. Similarly, carbon dioxide is nonsummating when tested in combination with methyl butyrate or 2-butanone. If carbon dioxide and farnesene both

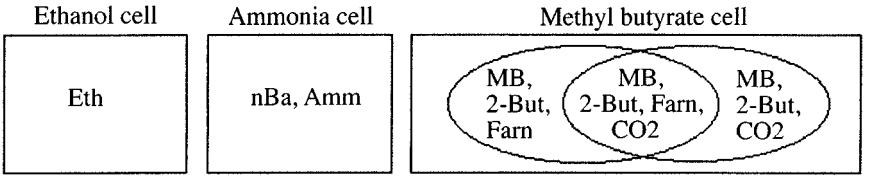


FIG. 4. Model of the olfactory receptor cell types. The receptors are divided into three broad types. The ethanol cell types only receives ethanol (Eth). The ammonia cell type receives *n*-butyric acid (nBa) and ammonia (Amm). The methyl butyrate receptor cell type is divided into three subgroups, comprising one that receives methyl butyrate (MB), 2-butanone (2-But), and farnesene (Farn); a second subgroup that receives MB, 2-But, Farn and carbon dioxide (CO2), and the third subgroup receives MB, 2-But, and CO2.

use the same receptor neuron types as methyl butyrate and 2-butanone, we would expect the mixture of farnesene and carbon dioxide to also produce a nonsummating response. Instead, a partly summating response was detected. This partly summating response for farnesene and carbon dioxide suggests that different receptor neuron types are used for these two chemicals but that there is some cross-reactivity between the two.

How, then, are farnesene and carbon dioxide both detected by the same receptor neuron types that detect methyl butyrate and 2-butanone? Rather than having one highly selective receptor mechanism, as was found for ethanol and for ammonia and *n*-butyric acid, we propose that farnesene, methyl butyrate, 2-butanone, and carbon dioxide are detected by a group of receptor neurons that show variation in their acceptance of odor ligands. The receptor neuron type for these four chemicals consists of three subgroups of the one basic receptor type. Figure 4 shows a model of these receptor subgroups that is consistent with the experimental results. One subgroup detects methyl butyrate, 2-butanone, and farnesene. A second subgroup detects methyl butyrate, 2-butanone, farnesene, and carbon dioxide; and the third subgroup detects methyl butyrate, 2-butanone, and carbon dioxide. In this model, when methyl butyrate or 2-butanone are tested, all three subgroups would be activated. There would therefore be nonsummating responses when farnesene or carbon dioxide were added to either methyl butyrate or 2-butanone, as all of the receptors would already be activated. When either farnesene or carbon dioxide was presented individually, only two of the three subgroups would be activated. When farnesene and carbon dioxide were presented together, however, all three subgroups would be activated, which would give a response that is larger than the response to farnesene or carbon dioxide on their own, but would not equal the calculated additive response for farnesene with carbon dioxide. The calculated additive response would be the equivalent of adding the two receptor groups for farnesene to the two receptor subgroups for carbon dioxide, assuming no cross-reactivity between the two. The larger EAG responses to methyl butyrate and

2-butanone compared to farnesene and carbon dioxide may also indicate that more receptor neurons respond to methyl butyrate and 2-butanone than for the other two chemicals, which supports our hypothesis.

Furthermore, if each of the subgroups of receptor neuron types for farnesene and carbon dioxide produced approximately the same amount of electrical potential when stimulated, then the model suggests that the response to the mixture of farnesene with carbon dioxide should be about 50% larger than the responses to individual treatments of farnesene or carbon dioxide. The experimental results come close to matching that prediction. The EAG response to farnesene was 32% $\pm$ 6, the response to carbon dioxide was 35% $\pm$ 3, while the response to the mixture of the two was 46% $\pm$ 3. These antennal responses to carbon dioxide are of great interest on their own (especially the nonsummating responses with methyl butyrate and 2-butanone) and will be investigated in more depth in a separate study.

The EAG results seem to support our model; however, they should not be seen to be conclusive. The EAG responses to some of the chemicals (*e.g.*, farnesene and carbon dioxide) were quite small, which makes interpretation of the results difficult. While our hypotheses were based on the results of statistical tests, single-unit electrophysiology will be needed to confirm the specificity of these receptor neuron types. Single-unit electrophysiology will also allow us to test whether the high stimulus dosages affect the responses of the receptor neurons. For instance, there may be inhibition of nerve impulses at high stimulus doses. High stimulus concentrations can also affect the selectivity of sensory cells, for example, the pheromone sensitive cells of *T. ni* lost some of their selectivity when high stimulus concentrations were applied (Mayer and Mankin, 1987). Finally, the receptor neuron types indicated in these experiments should not be seen as the only types present. Only a limited number of chemicals were tested, and it is likely that there are many more receptor neuron types on the antenna.

Comparison with Behavioral Experiments. Eisemann and Rice (1992) looked at behavioral responses of *B. tryoni* to some of the chemicals we tested and postulated that at least three olfactory receptor neuron types were responding. However, their predictions were based on assigning receptor neuron types according to gross differences in the chemical structure of the odors. They postulated that the long-chain hydrocarbons (such as farnesene) would be detected by separate receptor neuron types, compared to the short-chain aliphatic acids, alcohols, esters, and ketones, and that *n*-butyric acid would activate the same receptor neuron types as methyl butyrate and 2-butanone. However the electrophysiological results indicate that farnesene activates the same receptor neuron types as methyl butyrate and 2-butanone and that *n*-butyric acid activates separate receptor neuron types from the other short chain chemicals.

The chemicals for this EAG study were selected because they are known to have a behavioral effect on the flies. All the chemicals (except for ammonia) are volatile chemicals that have been found to be emitted from fruits (Nursten, 1970).

Ammonia is a common emission from bacterial activity and the attractancy of *B. tryoni* to it is most likely a feeding response (see Courtice and Drew, 1984; Drew et al., 1983; Lloyd, 1991). 2-Butanone is also produced by bacterial cultures (Drew, 1987). However, the concentrations of chemicals used may be much greater than normally experienced. As such they may negate or even reverse the behavioral action of the chemicals. Therefore, this study should not be taken out of context. Our experiments are looking at the physiological mechanisms associated with the initial reception and transduction of the odorants.

The EAG responses have provided us with valuable information on the initial reception of nonpheromonal chemicals in *B. tryoni*. By examining the antennal responses when stimulated with mixtures of behaviorally active chemicals, we have shown that three basic receptor neuron types are likely to be present and that there is little cross-reactivity between them. The use of these olfactory receptor neuron types represents an important part of the peripheral discrimination process and results in a selective neural input to the central nervous system. The results have also raised some important questions, particularly concerning the response of the olfactory receptor nurons that detect carbon dioxide as well as other environmental odorants. Carbon dioxide receptors are generally thought to be a specialist class of receptors (Stange, 1996). The responses of the carbon dioxide receptors are investigated in more detail in the accompanying paper.

Saturation Volumes of Test Chemicals. The saturation points for most of the chemicals were between 100 μl and 300 μl. Carbon dioxide saturated at only 15 μl; however, the figure is not directly comparable with the other chemicals, as the concentration of carbon dioxide in the syringe was close to 100%. The concentration of the other chemicals would have been lower, depending on the vapor pressures. Nevertheless, the EAG response to carbon dioxide was smaller than for methyl butyrate and 2-butanone, suggesting that there are fewer dendrites on the antenna sensitive to carbon dioxide compared to methyl butyrate or 2-butanone. Alternatively the receptor potentials produced by the dendrites receiving carbon dioxide may be small compared to the receptor potentials produced for the other chemicals.

Control Experiments. There was only one artifact identified from the control experiments: a small EAG response was found when clean air was injected, meaning that there is an antennal response in the absence of test odor stimulation. The response to the control stimulus air treatment was a stimulation technique artifact and was taken into account when interpreting the results. The antennal response to the air treatments may come from more than one source. First, mechanoreceptors, located solely on the scape and pedicel (Giannakakis and Fletcher, 1985; Hull and Cribb, 1997), may have been stimulated by the mechanical effect of the puff of air from the syringe. The response may also come from delivery-line contaminants, most likely Teflon plasticizers (Mayer et al., 1984). However, the continuous flushing of the stimulus delivery system would have negated the build-up of such

contaminants. The continuous air flow would also have reduced the chance of odorants from previous stimulations adhering to the tube walls and being released at a later time. The comparatively small response to the air treatments compared to the EAGs of the test chemicals shows that the larger part of the antennal responses resulted from an interaction of the chemicals with the olfactory receptor mechanisms of the antennae.

We found no local potential effect. Other researchers have found that local potentials (EAG responses that are more strongly influenced by sensilla in the immediate vicinity of the recording electrode) can be recorded from the antennae of tephritid flies (Crnjar et al., 1989; Bigiani et al., 1989). In the above cases the EAG response depended on the position of the recording electrode. Because sensilla are not equally distributed over the surface of the antenna, different responses occurred as a result of different recording sites. Our results showed that the types of EAG responses to the mixtures were consistent and did not depend on the position of the recording electrode. The difference between these findings is likely to be due to the recording method used: the authors referred to above gently pressed the tip of the recording electrode against the surface of the antenna. The recording electrode would only have been in contact with the external surface of the antenna. This may increase the resistance of the electrical connection, thus restricting the recording range of the electrode. Bigiani et al. (1989) estimated that the effect of local potentials was limited to a radius of about 30 μm around the recording electrode. Inserting of the electrode does have an effect on the dynamics of the potentials recorded. With a deep insertion, Kay (1971) found that some transient elements of the EAG response were eliminated. Nearly equal EAG responses were also found between different regions of the antenna of *Trichoplusia ni* when the electrodes were implanted through the cuticle (Payne et al., 1970). In our experiments, the recording electrode was inserted into the antenna. Therefore, we suggest that the different electrical contact achieved with our methods accounts for the absence of local potentials.

One potential source of error in EAG experiments is an interaction of the chemicals with the electrodes, resulting in artificial electrical potentials (so-called electrode potentials). Kafka (1970) recorded electrode potentials that were the result of interactions between chemicals and the electrodes, upon stimulation with both acid and alkaline substances. The aim of the control experiments with the dead fly was to see if significant electrode potentials were produced. The potentials from living flies had an initial negative deflection. With the dead fly the negative potentials were eliminated, showing that the chemicals were not significantly interacting with the electrodes. A small, positive electrode potential was produced with ammonia, but the effect of this potential with a live fly is minimal compared to the normal negative antennal response. The negative potentials recorded from the live flies in the dose–response and mixture experiments were therefore the result of physiological activity within the antenna.

Acknowledgments—The authors wish to thank Mr. Andrew Noskoff for technical assistance; Ms. Joan Hendrikz, the Faculty of Biological and Chemical Sciences, for assistance in the statistical analyses; Dr. Chris Moore, Department of Primary Industries, for help in interpreting some of the results and Dr. E. Rumbo for his constructive criticism and advice.

REFERENCES

AVERILL, A. L., REISSIG, W. H., and ROELOFS, W. L. 1988. Specificity of olfactory responses in the tephritid fruit fly *Rhagoletis pomonella*. *Entomol. Exp. Appl.* 47:211–222.

BAKER, T. C., and ROELOFS, W. L. 1976. Electroantennogram responses of the male moth, *Argyrotaenia velutinana* to mixtures of sex pheromone components of the females. *J. Insect Physiol.* 22:1357–1364.

BATEMAN, M. A., and MORTON, T. C. 1981. The importance of ammonia in proteinaceous attractants for fruit flies (Family: Tephritidae). *Aust. J. Agric. Res.* 32:883–903.

BIGIANI, A., SCALERA, G., CRNJAR, R., BARBAROSSA, I. T., MAGHERINI, P. C., and PIETRA, P. 1989. Distribution and function of the antennal olfactory sensilla in *Ceratitis capitata* Wied. (Diptera, Trypetidae). *Boll. Zool.* 56:305–311.

BORST, A. 1984. Identification of different chemoreceptors by electroantennogram-recording. *J. Insect Physiol.* 30:507–510.

BROCKERHOFF, E. G., and GRANT, G. G. 1999. Correction for differences in volatility among olfactory stimuli and effect on EAG responses of *Dioryctria abietivorella* to plant volatiles. *J. Chem. Ecol.* 25:1353–1367.

CHEN, A. C., and FRIEDMAN, S. 1975. An isotonic saline for the adult blowfly, *Phormia regina*, and its application to perfusion experiments. *J. Insect Physiol.* 21:529–536.

CHRISTENSEN, T. A., GEOFFRION, S. C., and HILDEBRAND, J. G. 1990. Physiology of interspecific chemical communication in *Heliothis* moths. *Physiol. Entomol.* 15:275–283.

COSSÈ, A. A., TODD, J. L., MILLAR, J. G., MARTÍNEZ L. A., and BAKER, T. C. 1995. Electroantennographic and coupled gas chromatographic–electroantennographic responses of the Mediterranean fruitfly, *Ceratitis capitata*, to male produced volatiles and mango odor. *J. Chem. Ecol.* 21:1823–1826.

COURTICE, A. C., and DREW, R. A. I. 1984. Bacterial regulation of abundance in tropical fruit flies (Diptera: Tephritidae). *Aust. Zool.* 21:251–268.

CRNJAR, R., SCALERA, G., LISCIA, A., ANGIOY, A. M., BIGIANI, A., PIETRA, P., and BARBAROSSA, I. T. 1989. Morphology and EAG mapping of the antennal olfactory receptors in *Dacus oleae*. *Entomol. Exp. Appl.* 51:77–85.

DEN OTTER, C. J. 1991. Olfactory responses of tsetse flies to phenols from buffalo urine. *Physiol. Entomol.* 16:401–410.

DICKENS, J. C. 1984. Olfaction in the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae): Electroantennogram studies. *J. Chem. Ecol.* 10:1759–1785.

DREW, R. A. I. 1987. Behavioural strategies of fruit flies of the genus *Dacus* (Diptera: Tephritidae) significant in mating and host-plant relationships. *Bull. Entomol. Res.* 77:73–81.

DREW, R. A. I., COURTICE, A. C., and TEAKLE, D. S. 1983. Bacteria as a natural source of food for adult fruit flies (Diptera: Tephritidae). *Oecologia* 60:279–284.

EISEMANN, C. H. 1980. An investigation of some stimuli influencing host finding and oviposition behaviours of the Queensland fruit fly, *Dacus (Bactrocera) tryoni* (Frogg.). MSc thesis. University of Queensland, Brisbane, Australia.

EISEMANN, C. H., and RICE, M. J. 1992. Attractants for the gravid Queensland fruit fly *Dacus tryoni*. *Entomol. Exp. Appl.* 62:125–130.

FEIN, B. L., REISSIG, W. H., and ROELOFS, W. L. 1982. Identification of apple volatiles attractive to the apple maggot, *Rhagoletis pomonella*. *J. Chem. Ecol.* 8:1473–1487.

GIANNAKAKIS, A., and FLETCHER, B. S. 1985. Morphology and distribution of antennal sensilla of *Dacus tryoni* (Froggatt) (Diptera: Tephritidae). *J. Aust. Entomol. Soc.* 24:31–35.

GUERIN, P. M., STÄDLER, E., and BUSER, H. R. 1983a. Identification of host plant attractants for the carrot fly, *Psila rosae*. *J. Chem. Ecol.* 9:843–861.

GUERIN, P. M., REMUND, U., BOLLER, E. F., KATSOYANNOS, B., and DELRIO, G. 1983b. Fruit fly electroantennogram and behavioural responses to some generally occurring fruit volatiles, pp. 248–251, *in* R. Cavalloro (ed.). Fruit Flies of Economic Importance. Balkema, Rotterdam.

HERNANDEZ, M. M., SANZ, I., ADELANTADO, M., BALLACH, S., and PRIMO, E. 1996. Electroantennogram activity from antennae of *Ceratitis capitata* (Wied.) to fresh orange airborne volatiles. *J. Chem. Ecol.* 22:1607–1619.

HULL, C. D., and CRIBB, B. W. 1997. Ultrastructure of the antennal sensilla of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). *Int. J. Insect Morphol. Embryol.* 26:27–34.

KAFKA, W. A. 1970. Molekulare wechselwirkungen bei der erregungeinzelner riechzellen. *Z. Vergl. Physiol.* 70:105–143.

KAY, R. E. 1971. Effect of odor composition and environment on olfactory receptor potential of the fly. *Am. J. Physiol.* 220:1473–1480.

LIGHT, D. M. 1983. Sensitivity of antennae of male and female *Ips paraconfusus* (Coleoptera: Scolytidae) to their natural aggregation pheromone and its enantiomeric components. *J. Chem. Ecol.* 9:561–583.

LIGHT, D. M., and BIRCH, M. C. 1979. Electrophysiological basis for the behavioral response of male and female *Trichoplusia ni* to synthetic female pheromone. *J. Insect Physiol.* 25:161–167.

LIGHT, D. M., and JANG, E. B. 1987. Electroantennogram responses of the Oriental fruit fly, *Dacus dorsalis*, to a spectrum of alcohol and aldehyde plant volatiles. *Entomol. Exp. Appl.* 45:55–64.

LIGHT, D. M., JANG, E. B., and DICKENS, J. C. 1988. Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata*, to a spectrum of plant volatiles. *J. Chem. Ecol.* 14:159–180.

LLOYD, A. C. 1991. Bacteria associated with *Bactrocera* species of fruit flies (Diptera: Tephritidae) and their host trees in Queensland. PhD thesis. University of Queensland, Brisbane, Australia.

MAYER, M. S., and MANKIN, R. W. 1987. A linkage between coding of quantity and quality of pheromone gland components by receptor cells of *Trichoplusia ni*. *Ann. N. Y. Acad. Sci.* 510:483–484.

MAYER, M. S., MANKIN, R. W., and LEMIRE, G. F. 1984. Quantitation of the insect electroantennogram: measurement of sensillar contributions, elimination of background potentials, and relationship to olfactory sensation. *J. Insect Physiol.* 30:757–763.

NEALE, C. F. 1989. Some aspects of the specific mate recognition systems of *Dacus tryoni* and *Dacus neohumeralis*. MSc thesis. University of Queensland, Brisbane, Australia.

NURSTEN, H. E. 1970. Volatile compounds: the aroma of fruits, pp. 239–268, *in* A. C. Hulme (ed.). The Biochemistry of Fruits and their Products. Academic Press, London.

PARK, K. C., and HARDIE, J. 1998. An improved aphid electroantennogram. *J. Insect Physiol.* 44:919–928.

PAYNE, T. L., SHOREY, H. H., and GASTON, L. K. 1970. Sex pheromones of noctuid moth: factors influencing antennal responsiveness in males of *Trichoplusia ni*. *J. Insect Physiol.* 16:1403–1055.

PRITCHARD, G. 1969. The ecology of a natural population of Queensland fruit fly *Dacus tryoni* II. The distribution of eggs and its relation to behaviour. *Aust. J. Zool.* 17:292–311.

RICE, M. J. 1989. The sensory physiology of pest fruit flies: conspectus and prospectus, pp. 249–272 *in* A. S. Robinson and G. H. S. Hooper (eds.). Fruit Flies—Their Biology, Natural Enemies and Control. World Crop Pests 3(A). Elsevier, Amsterdam.

ROELOFS, W. L., and COMEAU, A. 1971. Sex pheromone perception: electroantennogram responses of the red-banded leaf roller moth. *J. Insect Physiol.* 17:1969–1982.

SCHNEIDER, D. 1962. Electrophysiological investigation on the olfactory specificity of sexual attracting substances in different species of moth. *J. Insect Physiol.* 8:15–30.

STANGE, G. 1996. Sensory and behavioural responses of terrestrial invertebrates to biogenic carbon dioxide gradients, pp. 223–253, *in* G. Stanhill (ed.). Advances in Bioclimatology 4. Springer-Verlag, Berlin.

STANGE, G. 1999. Carbon dioxide is a close-range oviposition attractant in the Queensland fruit fly *Bactrocera tryoni*. *Naturwissenschaften* 86:190–192.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

OLFACTION IN THE QUEENSLAND FRUIT FLY, *Bactrocera tryoni*. II: RESPONSE SPECTRA AND TEMPORAL ENCODING CHARACTERISTICS OF THE CARBON DIOXIDE RECEPTORS

C. D. HULL* and B. W. CRIBB

Department of Zoology and Entomology and the Centre for Microscopy and Microanalysis
The University of Queensland
4072, Queensland, Australia

(Received April 19, 2000; accepted January 2, 2001)

Abstract—Single-unit electrophysiology was used to record the nerve impulses from the carbon dioxide receptors of female Queensland fruit flies, *Bactrocera tryoni*. The receptors responded to stimulation in a phasic-tonic manner and also had a period of inhibition of the nerve impulses after the end of stimulation, at high stimulus intensities. The cell responding to carbon dioxide was presented with a range of environmental odorants and found to respond to methyl butyrate and 2-butanone. The coding characteristics of the carbon dioxide cell and the ability to detect other odorants are discussed, with particular reference to the known behavior of the fly.

Key Words—Olfaction, electrophysiology, *Bactrocera tryoni*, Diptera, Tephritidae, sensilla, receptor, environmental odorant, carbon dioxide.

INTRODUCTION

Carbon dioxide receptors have been studied electrophysiologically for several hematophagous Diptera, including the mosquito *Aedes aegypti* (Kellogg, 1970), the sheep blowfly *Lucilia cuprina* (Stange, 1975), and the tsetse flies *Glossina palpalis* (Bogner, 1992) and *G. morsitans* (Den Otter and Van der Goes van Naters, 1992). In phytophagous insects these receptors have received less attention. The CO_2 concentration gradients around plants are far less than those encountered by hematophagous insects, so the receptors of phytophagous species must have high sensitivity (Stange, 1997). This has been found to be the case

* To whom correspondence should be addressed. e-mail: c.hull@ento.uq.edu.au

0098-0331/01/0500-0889$19.50/0

in the moths *Helicoverpa armigera* (Stange, 1992) and *Cactoblastis cactorum* (Stange, 1997; Stange et al., 1995). It is thought that CO_2 levels around plants alert the insect to potential feeding or oviposition sites (Rasch and Rembold, 1994; Stange, 1997). No electrophysiological studies are available for the CO_2 receptors of phytophagous Diptera, except for our electroanntenogram (EAG) study of the antennae of *Bactrocera tryoni* (Diptera: Tephritidae) (Hull and Cribb, 2001, the first in this series of papers), where we showed a response to CO_2 and developed a model of receptor types including one class containing three subsets: one that responds to methyl butyrate, 2-butanone, and farnesene; a second responding to methyl butyrate, 2-butanone, and CO_2; and a third responding to all four chemicals. Although EAGs do not indicate the significance of a response, Rice (1989) and Stange (1999) have shown that these flies respond behaviorally to CO_2. The putative CO_2 receptor cells of the Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae), with their characteristic lamellate dendrite in a multiporous peg, have been located using electron microscopy (Hull and Cribb, 1997). This makes them an ideal candidate for single unit electrophysiology.

Single-unit electrophysiology has been used previously to determine the number of receptors present on the dendrites of a sensory cell in adaptation experiments. Multiple olfactory receptor proteins were indicated on sensory cells of the beetle *Dendroctonus frontalis*, which correlated with EAG experiments from that species (Payne and Dickens, 1976). However, single-unit electrophysiological studies from Tephritidae are rare, because the small size and dense packing of the sensilla makes them difficult to record from. Dickens et al. (1988) have recorded from single trichoid sensilla of *C. capitata*. They found that the sensilla responded to trimedlure (a known behavioral attractant) by increasing the rate of firing of nerve impulses. In this paper single-unit electrophysiology is used to investigate the encoding characteristics of the carbon dioxide receptors of *B. tryoni* and test our model for the response spectrum developed from EAG data.

METHODS AND MATERIALS

Test Subjects. Bactrocera tryoni (Froggatt) were cultured as per Hull and Cribb (2001). Only gravid flies were selected for experimentation. Intact flies were wrapped in Blu-tack [Bostik (Australia) Pty Ltd] adhesive to constrain head movements and manipulated so that only the antennae were left protruding. The fly was then put onto a microscope slide, and a portion of Blu-tack was placed underneath the protruding antennae. Melted wax (melting point of approximately 52°C) was used to secure the antennae to the Blu-tack. Small drops of wax were placed at the tip of the funiculus and at the pedicel of the antenna.

Electrophysiological Recording Technique. Tungsten electrodes, mounted on micromanipulators, were used. Due to the small size and the dense packing of the

sensilla, the recording electrode had to be repositioned by chance until there was an adequate electrical connection. The recording electrode was always inserted into the same region of each antenna, which was between the sensory pit and the base of the funiculus. Preliminary experiments showed that this region contained numerous receptors responsive to CO_2. The indifferent electrode was inserted at the base of the antenna, through the circumantennal sulcus. The preparations remained viable for between 30 min to over 2 hr. The extracellularly recorded action potentials ranged in size from 0.2 to 1.5 mV. Signals were transmitted through a Grass high-impedance probe to a preamplifier (Grass P16D microelectrode DC amplifier). The signals were then transferred to a Syntech data acquisition system and analyzed with the Syntech AutoSpike V. 3.1 program on a personal computer. For all recordings, the nerve impulses were counted for the first 0.5 sec of stimulation. Electrical shielding was provided by a Faraday cage, with all metal surfaces within it being grounded.

Test Samples. The chemicals were carbon dioxide (Commonwealth Industrial Gases, food grade purity from a cylinder of compressed CO_2), 2-butanone (Aldrich Chemical Company, claimed purity 99%), methyl butyrate (Aldrich Chemical Company, claimed purity 99%), *n*-butyric acid (British Drug Houses Ltd, claimed purity 99%), a mixture of isomers of farnesene (TCI Ltd, purity unknown), ethanol (Ajax Chemicals Pty Ltd, claimed purity 99.5%), and ammonia (Ajax Chemicals Pty Ltd 28.0–30.0% w/w).

Dose–Response Relationship for Carbon Dioxide Receptor. The dose–response relationship for carbon dioxide was obtained using volumes of vapor between 5 and 800 μl. The saturation point was then determined from the curve and was taken to be the volume of gas at which the standard error of the mean response overlapped the standard error of the mean response of the highest volume of stimulation (Dickens, 1984).

Responses to Other Behavior-Modifying Chemicals. Once the response of the sensory cell to CO_2 had been characterized, the remaining test chemicals were applied. A volume of 400 μl was used in all cases, as it had been shown in previous EAG experiments that this volume was in excess of the saturation volume for each chemical. Using a volume of test chemical vapor in excess of the volume needed to maximally stimulate the cell meant that even chemicals having only a small effect on the receptor neurons would be detected. Those chemicals that produced an increase in the firing rate of the neurons were then tested in mixture experiments. One of the stimulating compounds (methyl butyrate) was also tested for a dose–response relationship to see whether the response curve was similar to that of carbon dioxide.

Mixture Experiments. Different cells within a sensillum may be difficult to identify if their nerve impulses are of similar size. If two chemicals are acting on the same sensory cell, then the binary mixture of the two should not produce more nerve impulses compared to the single most effective stimulant. A mixed injection

containing 400 μl of each chemical was applied. Chemicals were always presented at a volume in excess of the saturation volume to ensure the sensory dendrites were completely activated. All chemicals that produced an increase in the firing rate of the neurons were tested in combination with each other. The order of presentation of the mixture series and the chemicals within a mixture series were randomized. The responses to 400 μl of each chemical were analyzed with a random-blocks ANOVA, with a post-hoc Dunnett's test. They were compared to the response obtained with the average stimulation of clean, analytical grade air.

Odorant Delivery. Humidified analytical grade compressed air was continuously blown over the fly at a rate of 1 liter/min, with the nozzle for the airstream being placed 1 cm from the fly. The method of test odour injection was the same as for Hull and Cribb (2001). The relationship between the syringe volume and the actual concentration of the test samples at the preparation was not established.

Control Experiments. An experiment was conducted to see if the dilution of a chemical within a syringe would significantly affect the nerve impulse firing rate within the first 0.5 sec of the stimulation. 2-Butanone, 400 μl, was tested six times in a row and was then repeated a further six times using 400 μl of 2-butanone with an additional 400 μl of clean air. In each case the same number of odor molecules would have been present, but the concentration of the odorant within the stimulation burst would have differed. This experiment tested whether there were differences in spike numbers with variation in the volume of the stimulation burst, rather than as a result of the number of molecules of odor vapor. The experiment also tested if adding a small amount of carbon dioxide (present in 400 μl of analytical grade air) to a sample of odorant had a significant effect on the number of nerve impulses.

Injecting the test stimuli by hand involved a degree of variability in terms of the strength and time course of the injection (injections took between 0.5 and 1 sec). To gauge the reproducibility of the stimulation technique, a preparation was stimulated six times in a row with 400 μl of methyl butyrate, and then again with 400 μl of carbon dioxide (preliminary experiments indicated that the sensory cells did not become adapted with repeated stimulation). The six injections of 2-butanone from the previous experiment were also used to gauge the reproducibility of the stimulation technique. At least 2 min elapsed between each injection to allow disadaptation of the receptors after the stimulus-induced activity.

Identification of Sensillum Type. Scanning electron microscopy was used to identify the sensillum type we recorded from. After recording, the electrode was pushed further into the antenna to make a larger hole. The antenna was then removed from the insect and dehydrated through a graded series of alcohol. Following dehydration the ethanol was exchanged for hexamethyl disilazane (HMDS) and left for 2–4 hr. After the HMDS was removed, the antenna was air dried and mounted on an aluminum pin-type stub using double-sided adhesive tape and silver conductive paint. The preparations were then coated with gold in a sputter coater and viewed on a JEOl JSM6400F scanning electron microscope.

RESULTS

Response to Carbon Dioxide. The average basal firing rate for the carbon dioxide-sensitive receptor was 14 ± 2 nerve impulses per 0.5 sec (Figure 1). The saturation point was obtained with 50 μl of CO_2-saturated air and was 61 ± 8 nerve impulses for the first 0.5 sec of stimulation ($N = 9$).

The pattern of firing of the nerve impulses showed some variability according to the stimulus load. At low stimulus intensities, the responses were tonic in nature and showed an even level of firing throughout the stimulus burst (Figure 2B). At high stimulus intensities, the pattern changed to phasic-tonic, with an increase in the firing rate at the start of stimulation followed by a lower level of firing (Figure 2D). The increased level of nerve impulse firing continued for up to 2 sec, but did show some variability according to the stimulus strength (compare Figure 2C and 2D). This variability was associated with the method of stimulation, and so to avoid problems, the spikes were only counted for the first 0.5 sec of the stimulation burst. This initial period included the phasic portion for all of the responses. Although not ideal, the stimulation method is adequate for the large volumes tested. After stimulation with large quantities of CO_2-laden air (50–800 μl), the nerve impulses stopped for a period of up to 3 sec, although periods of 1–2 sec were more common. On some of the recordings the amplitude of the nerve impulses decreased when stimulated with large quantities of CO_2 (Figure 2D). However, this could usually be compensated for by the AutoSpike program, but in one recording (not shown) the response of the cell had become completely adapted after the initial phasic burst and the spikes eventually could

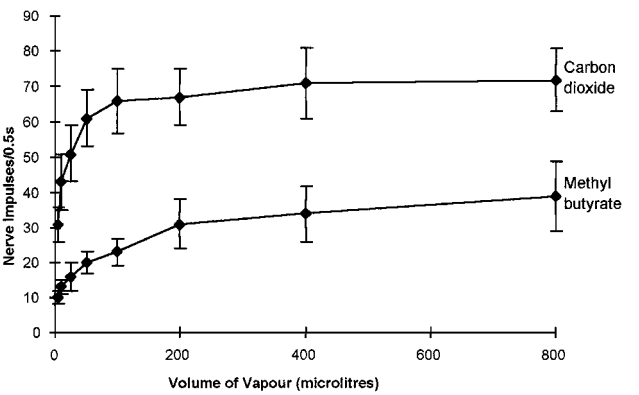


FIG. 1. Dose–response curves of receptor cells on the antenna of female *B. tryoni* in response to stimulation with carbon dioxide and methyl butyrate (each data point represents the mean number of nerve impulses/0.5 sec ± 1 SE; $N = 9$ for carbon dioxide, $N = 5$ for methyl butyrate).

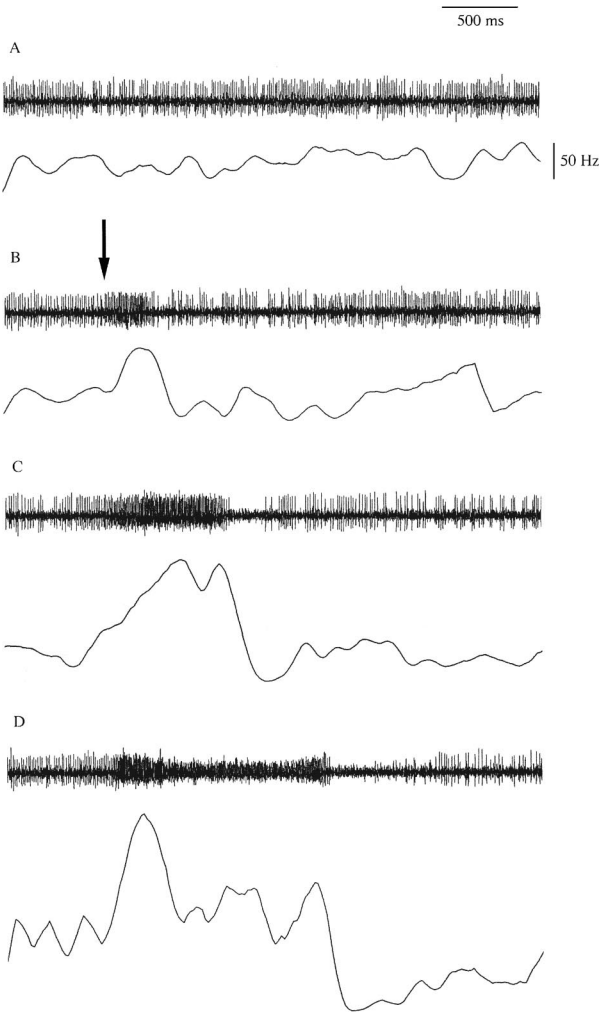


FIG. 2. Electrophysiological responses from a sensory cell on the antenna of female *B. tryoni* in response to stimulation with various concentrations of carbon dioxide. The line below each electrophysiological recording is a computer generated frequency curve, with the same time scale, showing the firing frequency of the nerve impulses. (A) Basal level of firing in the absence of stimulation (but with the background air supply on); (B) stimulation with 5 μl of carbon dioxide; (C) stimulation with 50 μl of carbon dioxide; (D) stimulation with 400 μl of carbon dioxide. Arrow indicates the point of stimulus injection.

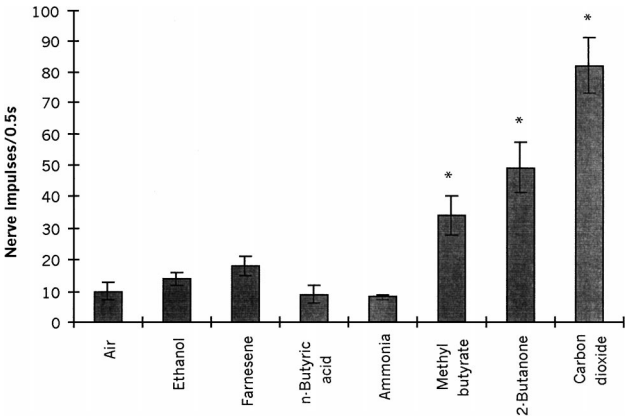


FIG. 3. Relative responses of carbon dioxide sensory receptors on the antenna of female *B. tryoni* to stimulation with 400 μl of each of the test chemicals. The asterisks indicate those chemicals that were significantly different from stimulation with clean air. Only methyl butyrate, 2-butanone, and carbon dioxide could be detected by the sensory cell. $N = 8$.

not be distinguished from the background noise, although they reappeared at the end of the stimulus burst and reached the original spike amplitude. The decrease in spike amplitude occurred after the first 0.5 sec of stimulation; therefore it did not affect the counting of nerve impulses during the initial phasic burst. Only one spike height could be reliably identified in the recordings. This was confirmed during stimulation with high concentrations of carbon dioxide: At the end of stimulation, when the spikes from the carbon dioxide receptor were inhibited, no other spikes were visible. Furthermore, if a second cell was firing, a more irregular pattern of nerve impulses would have been seen, and we should have seen some spikes of double the normal amplitude (produced when spikes from two cells coincide).

Responses to other Behavior-Modifying Chemicals. The relative responses of the sensilla containing the CO_2 receptors to other odorants are shown in Figure 3. The only chemicals that showed a significant increase in firing rate were methyl butyrate, 2-butanone, and CO_2. Farnesene, ethanol, *n*-butyric acid, and ammonia did not show a significant increase in the firing rate compared to stimulation with clean air. Ammonia often interacted with the electrodes to produce electrode potentials, which obscured the recordings for that chemical, although the onset of the electrode potentials was usually delayed long enough to determine the number of spikes within the first 0.5 sec of stimulation.

The responses to methyl butyrate and 2-butanone were not as strong as to carbon dioxide. The average number of spikes within the first 0.5 sec of stimulation with 400 μl of methyl butyrate-saturated air was 34 ± 6 spikes and for 2-butanone

it was 49 ± 8 spikes, compared to 82 ± 9 spikes for carbon dioxide. The pattern of spikes also showed some differences, with the responses to methyl butyrate and 2-butanone not showing a strong phasic response at the start of stimulation with large stimulus loads (Figure 4). The responses were tonic in nature. In most of the recordings (78%) a cessation of nerve impulses after the stimulus burst could be seen after stimulation with 400 μl of methyl butyrate and 2-butanone. The cessation of impulses after stimulation was similar to that for carbon dioxide, although the duration was smaller (0.8–1 sec; maximum of 2 sec). The duration of the increased level of firing after stimulation with methyl butyrate or 2-butanone

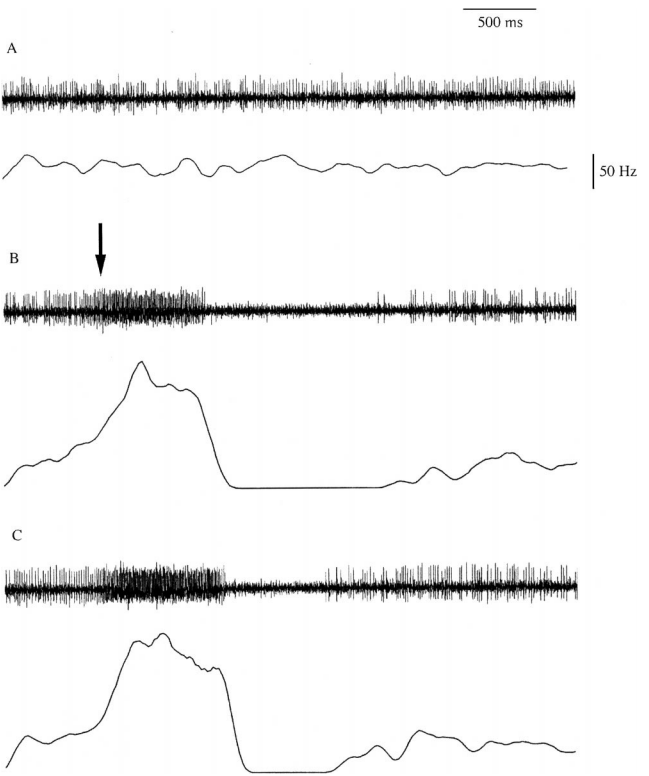


FIG. 4. Electrophysiological responses of a carbon dioxide sensory receptor on the antenna of female *B. tryoni* to other stimulating compounds. The line below each electrophysiological recording is a computer generated frequency curve, showing the firing frequency of the nerve impulses. Only methyl butyrate and 2-butanone produced significantly larger responses compared to an injection of clean air. (A) Control injection of 400 μl of clean air; (B) 400 μl of methyl butyrate saturated air; (C) 400 μl of 2-butanone saturated air. Arrow indicates the point of stimulus injection.

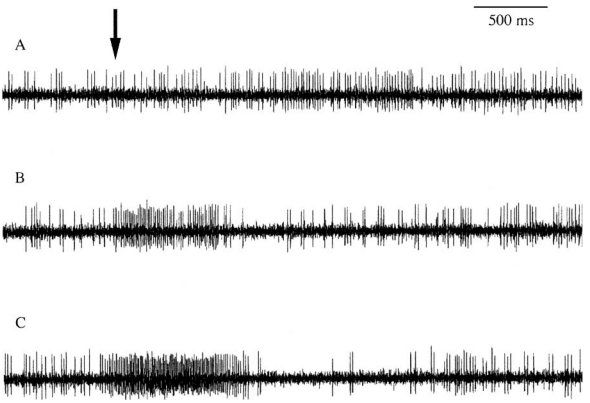


FIG. 5. Electrophysiological recordings from a carbon dioxide receptor cell on the antenna of female *B. tryoni* in response to stimulation with various concentrations of methyl butyrate. (A) Stimulation with 5 μl of methyl butyrate saturated air; (B) 100 μl of methyl butyrate saturated air; (C) 400 μl of methyl butyrate saturated air. Arrow indicates the point of stimulus injection.

was shorter compared to stimulation with carbon dioxide. At the higher stimulus intensities the duration of firing was between 1.0–1.5 sec.

The dose–response relationship for methyl butyrate is shown in Figure 1. The saturation volume for methyl butyrate is 200 μl, and resulted in 31 ± 7 nerve impulses for the first 0.5 sec of stimulation. The saturation volume is higher than that for carbon dioxide, and the number of nerve impulses obtained at all the stimulus volumes was lower for methyl butyrate compared to carbon dioxide. However, the shape of the dose–response curves are similar for both chemicals. A selection of the recordings of the methyl butyrate responses are shown in Figure 5.

Mixture Experiments. During the recordings, only one spike height could be reliably identified, regardless of the stimulating chemical. This result suggested that the nerve impulses of only one sensory cell were being recorded despite the range of stimulatory chemical types. To test this, mixture experiments involving methyl butyrate, 2-butanone, and CO_2 were conducted (Figure 6). The mixtures were analyzed by one-way paired sample *t* tests, to determine if the response to the mixture of chemicals was greater than the response of the largest of the two individual chemicals (i.e., an additive response). An additive response would indicate that the two chemicals are acting on different sensory cells within the sensillum. However, all three combinations of chemicals showed nonadditive responses (methyl butyrate with CO_2 $t = -2.205$; 2-butanone with CO_2 $t = -0.862$; and methyl butyrate with 2-butanone $t = -2.998$). The nonadditive responses confirm that only one receptor cell is responding to all three chemicals.

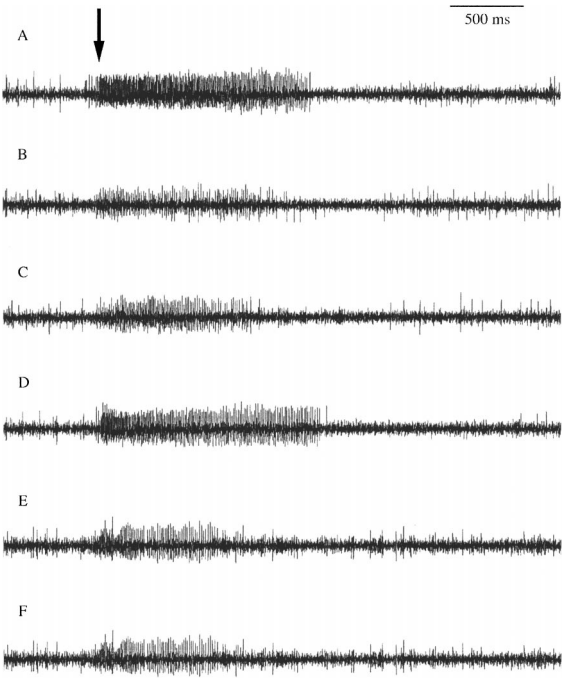


FIG. 6. Electrophysiological recordings from a sensory cell on the antenna of female *B. tryoni* in response to stimulation with mixtures of chemicals. (A) stimulation with 400 μl of carbon dioxide saturated air; (B) stimulation with 400 μl of methyl butyrate saturated air; (C) stimulation with 400 μl of 2-butanone saturated air; (D) stimulation with a mixture of carbon dioxide and methyl butyrate; (E) stimulation with a mixture of carbon dioxide and 2-butanone; (F) stimulation with a mixture of methyl butyrate and 2-butanone. Arrow indicates the point of stimulus injection.

Control Experiments. For the experiment on the effect of dilution of the chemicals, the mean number of spikes for the undiluted sample of 2-butanone was 32 ± 1.2 spikes for the first 0.5 sec. The mean for the sample of 2-butanone, diluted with clean air, was 30 ± 2.4 spikes for the first 0.5 sec. The two samples are not significantly different (two-sample t test, $t = 0.6903$), indicating that diluting the sample of a test chemical within the syringe does not effect the number of nerve impulses generated within the first 0.5 sec of stimulation. The addition of the small amount of carbon dioxide (present in the air used to dilute the sample) did not produce a significant change in the number of nerve impulses.

The percentage of the variation that is due to differences between the two runs (the R^2 value) of 2-butanone and 2-butanone plus air could be calculated. The R^2 value of 4.6% indicates that only a small amount of the variability between

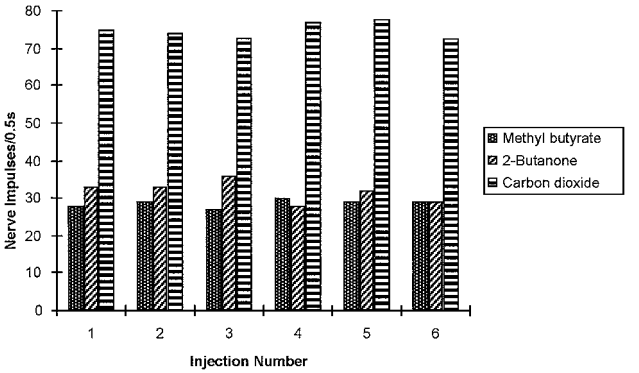


FIG. 7. Variability of the receptor cells and the stimulation technique. For each of the three test chemicals, six repeated stimulations (on the same sensillum) using 400 μl of test compound vapor produced little variability in the number of nerve impulses recorded. Methyl butyrate mean response: 29 $\pm$ 0.4 spikes, 2-butanone mean response: 32 $\pm$ 1.2 spikes, and carbon dioxide mean response: 75 $\pm$ 0.9 spikes.

the 12 values of the replicates can be accounted for by differences between the two treatments. The remaining variability is attributable to the pooled variation among the six replicates for each treatment. While most variability between the 12 values is attributable to the six replicates for each treatment, the actual level of variability for each treatment is low, as shown by the low standard errors for the treatments. The experiments testing the reproducibility of the stimulation technique showed a similarly low level of variation between the different repetitions (Figure 7). The standard error for the six repetitions of methyl butyrate was 0.4 impulses/0.5 sec, for carbon dioxide it was 0.9 impulses/0.5 sec, and for 2-butanone it was 1.2 impulses/0.5 sec. These results show that the method of injecting the odorants by hand did not contribute significant variability to the numbers of nerve impulses recorded. The use of large volumes of vapor may also have assisted in reducing variability. By having a large number of odor molecules presented to the antenna each time, any variability caused by the hand injections may have been compensated for, because both the diluted and undiluted stimuli are still at a level that saturates the receptors. However, for future experiments with low dosages, a stimulation method with even lower variability would be preferable.

Identification of Sensilla. The identification was made by locating the hole made by the insertion of the electrode. This was not possible for all of the recordings because some of the antennae required numerous attempts at inserting the electrode before a suitable recording site was found. In these antennae the large number of holes made identification of the correct one difficult. However, some recordings

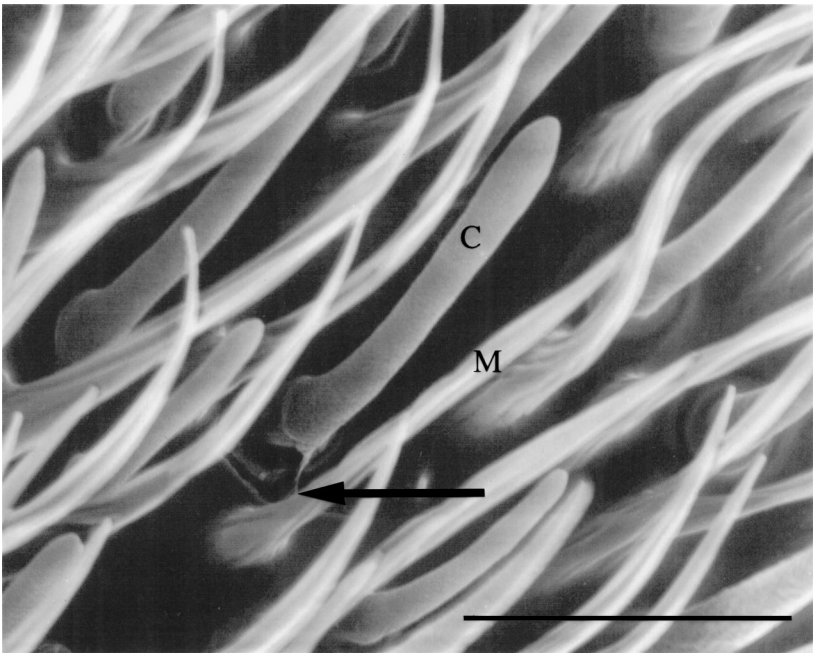


FIG. 8. Scanning electron micrograph identifying the type of sensillum recorded from in the single-unit electrophysiological experiments. The sensillum is a thin-walled multiporous type 3 (clavate) sensillum. The arrow shows the hole (enlarged for recognition) made by the insertion of the recording electrode at the base of the sensillum. C = clavate sensillum, M = uninnervated sensillum, bar = 10 μm.

were achieved with the first or second insertion attempt. Here, the position of the recording electrode was noted, and a larger hole made. Where identification was made (from four separate antennae), the sensilla were always subtype 3 Mp-sw thin-walled sensilla (clavate sensilla) (Figure 8).

DISCUSSION

Response of Cell to Carbon Dioxide. A high basal firing rate is common in general olfactory receptor cells even in the absence of stimuli, as well as in CO_2-receptive cells (Grant et al., 1995; Stange, 1996). In our study, the sensory cell was continuously exposed to a current of air (which contains CO_2), and was, therefore, constantly stimulated. The basal level of firing we recorded is then the plateau of maintained activity at that level of stimulation (the tonic response). Because CO_2 is present in the atmosphere, the tonic response we recorded can

be thought of as the normal level of firing. No tests were conducted to determine the rate of firing of the CO_2 receptor cells in *B. tryoni* to carbon dioxide–free air. The constant firing of the CO_2 sensory cells means that they must be adapted to signal changes in the concentration of CO_2 levels, which has been shown in other species (Stange, 1996). The signaling of changes in CO_2 concentration is achieved by either increasing or decreasing the level of firing of the action potentials. For *B. tryoni* the sensory cells showed a phasic-tonic response pattern to increased stimulation with CO_2. In other words, the firing of action potentials showed an initial increase, which leveled off to a lower, constant level of firing. Some of the sensory cells also showed a cessation of firing at the end of stimulation with high stimulus concentrations. The action potentials were inhibited, although the background airflow, containing CO_2, was still flowing over the antenna. Similar responses were found in the CO_2 receptors of the moth *C. cactorum* (Stange et al., 1995) and also in the pheromone receptors of the silkmoth *Bombyx mori* (Kaissling, 1986). The suppression of action potentials at the end of stimulation can be thought of as the equivalent of a phasic off-response. The ability to use both an increase and a decrease in action potential firing means that the insect can potentially encode a greater amount of information from this one stimulus and may correlate with the ability of CO_2 receptor cells to signal rapid changes in concentration (Rumbo and Kaissling, 1989).

Do CO_2 Receptors Detect Other Chemicals? During the periods of stimulation with CO_2 and when the antenna was exposed solely to the background airflow, only one action potential spike height could be reliably identified. This is despite the fact that the sensilla containing the CO_2-receptive cell also contains two other sensory cells (Hull and Cribb, 1997). When methyl butyrate and 2-butanone were presented to the antenna, the rate of firing of the nerve impulses increased. The results indicated that these compounds are being detected by the same sensory cell that detects CO_2. However, the possibility still existed that more than one sensory cell was responding during the recordings, but that all of them had identical spike heights. To test this, binary mixtures using methyl butyrate, 2-butanone, and CO_2 were conducted. If more than one sensory cell was responding, then a mixture of two of the chemicals should produce more action potentials than for either of the chemicals presented individually (Payne and Dickens, 1976). However, for all combinations of those chemicals, the mixtures produced responses that were no greater than for the larger of the two individual chemicals. These results confirmed that methyl butyrate, 2-butanone, and CO_2 are detected by the same sensory cells. This was a surprising result as CO_2-sensitive sensory neurons have generally been regarded as being specialized for that stimulus, and are, therefore, usually treated as a separate class of receptors from general odor and pheromone receptors (Stange, 1996). However, care should still be taken in interpreting these results as the response of the CO_2-sensitive sensory neurons to other volatiles may have been affected by the high dosage levels (see later discussion).

There are some cases where CO_2-sensitive cells respond to other chemicals. For instance, CO_2 receptors on the antennae of the termite *Schedorhinotermes lamanianus* can detect general odors, such as 1-pentanol, 1-hexanol, and 2-hexanol (Ziesmann, 1996). However, in this case the CO_2 had an inhibitory effect, whereas the general odors had an excitatory effect. Within the same sensillum was another sensory cell that also responded positively to general odors, but was not affected at all by CO_2. Ziesmann concluded that CO_2 was acting as a modulator of the olfactory signals and thereby allowed context specific signal interpretation. Modulation of sensory cell responses, similar to the termite CO_2 receptors, has been found in the moth *Trichoplusia ni*. In this case, dodecyl acetate was not detected by the sensory cells on its own, but when it was present in mixtures with other chemicals, it produced a modulation of the sensory cell response (O'Connell, 1986). However in *B. tryoni* no modulation of the receptors was evident. All of the detectable chemicals produced excitatory responses that were not inhibited when mixtures of chemicals were applied.

Responses of CO_2 receptors to other chemicals have also been found in *Rhodogastria* moths (Lepidoptera: Arctiidae). By using single-unit electrophysiology, the CO_2-sensitive cells in the labial palps were excited weakly by cyclopentane and inhibited by acetic acid (Bogner et al., 1986). The cells also responded positively to headspace odors of other insects and *Brassica* leaves, but this latter result is questionable. Bogner (1990) found the CO_2 receptors of *Pieris brassicae* also responded to headspace odors of insects and plants. Removing the carbon dioxide from the samples, however, greatly reduced the number of nerve impulses (from 100 impulses/sec to 0.2 impulses/sec). The receptors were, in this case, only responding to CO_2. This experiment shows the difficulty in interpreting results, particularly for CO_2 receptors, where the stimulant is present in other test samples. The CO_2 sensitive cells in the temporal organ on the head of the centipede *Thereuonema hilgendorfii* were also excited by amines, such as butylamine, amylamine, and hexylamine (Yamana et al., 1986). The same cells were inhibited by fatty acids, such as acetic acid and butyric acid, but alcohols had no effect. Volatile anesthetics have been shown to act on CO_2-sensitive cells, although the site of interaction in this case is not thought to be the primary transduction process, but a later stage of the signal cascade (Stange and Kaissling, 1995).

The response of the carbon dioxide sensory cell to methyl butyrate and 2-butanone may be due to the high stimulus concentrations used. In the gypsy moth, *Lymantria dispar*, the specialist receptor cell for disparlure (which contains only one type of receptor molecule) responds to analogs of disparlure when they are applied in high concentrations (Schneider et al., 1977). However, for *B. tryoni*, methyl butyrate and 2-butanone were the only two chemicals of the five other chemicals tested that produced a significant increase in firing of the carbon dioxide cell. The selectivity of the cell, therefore, suggests that there is some

functional significance in its ability to detect methyl butyrate and 2-butanone. Furthermore, both methyl butyrate and 2-butanone are behaviorally active chemicals (Eisemann and Rice, 1992), which adds strength to our assertion that the response of the carbon dioxide receptor to these other chemicals has a functional role. Whether the behavioral response to carbon dioxide, methyl butyrate, and 2-butanone is mediated by these receptor cells alone cannot be determined from this study. The physiological responses of only one cell type within a single sensillum type have been determined. Other cells in the antenna may also respond to some or all of these chemicals. Our EAG studies indicate another subtype of CO_2 receptor is present (Hull and Cribb, 2001). The full content of the information may only become apparent to the insect when it is taken in the context of all the responding cell types.

To determine if the cell could respond to low concentrations of an odor chemical, methyl butyrate was tested at a range of volumes. The dose–response relationship shows that the cell can detect methyl butyrate at lower volumes, although the sensitivity of the cell is lower for methyl butyrate compared to carbon dioxide. Even at the smallest volume tested (5 μl) the cell could detect carbon dioxide (i.e., the response is larger than the basal firing rate). For methyl butyrate, the responses did not increase above the basal firing rate until the stimulus volume was around 50 μl of odor-saturated air. The lower sensitivity of the cell to methyl butyrate was evident at all of the odor volumes tested. The difference in apparent sensitivity may be because the carbon dioxide samples were taken directly from a gas cylinder, and, therefore, would have been at a much greater concentration compared to the chemicals that had been taken as saturated air samples. Nevertheless, this result shows that the CO_2-sensitive sensory neurons can detect methyl butyrate at volumes well below the saturation level.

Temporal Firing Patterns and Variability. The generalist olfactory receptor cells of *Bombyx mori* show variability among cells of the same type (Heinbockel and Kaissling, 1996). Variability was also found in the firing characteristics of the carbon dioxide receptors of *H. armigera* (Stange, 1992). Therefore, it was not unexpected when we found some variability among the carbon dioxide receptors of *B. tryoni*. A range of response patterns for each receptor type may enable the insect to encode more information. However, confirmation of this hypothesis will require data from a larger number of receptor cells. Also, we recorded only from one region of the antenna. The clavate sensilla are distributed over the entire funiculus, and the patterns of nerve impulses of carbon dioxide receptors from other regions may show even larger differences.

The ability to detect changes in CO_2 concentration may be affected by changes in temperature (Stange and Wong, 1993). Small-scale temperature compensation may account for why only some of the recordings showed a cessation of firing after stimulation with high concentrations of CO_2. However, all of the recordings were

conducted in a laboratory maintained at a temperature of $24 \pm 2^{\circ}$C and with the same level of background air flow; therefore, any compensation of the responses would have been minimal.

How Do Responses of CO_2 Receptor Relate to Insect Behavior? At concentrations similar to that of vertebrate breath, CO_2 increases general flight activity (Rice, 1989). However, at low CO_2 concentrations (similar to concentrations produced by plants) gravid *B.tryoni* are attracted to the source (Stange, 1999). The difference in behavioral action may be encoded for at the peripheral level. Our experiments have shown differences in the firing characteristics of the cells that are dependent on the stimulus concentration. The behavioral activity at high concentrations may be encoded for in the initial phasic burst or in the longer period of nerve impulse firing or inhibition. Furthermore, the detection of other chemicals by the CO_2 sensitive sensory neurons may signify that the old demarcation of carbon dioxide as a separate olfactory class may not be appropriate in all cases; to *B. tryoni*, carbon dioxide is probably just another behavioral marker, which is processed along with the other olfactory stimulants.

All of the recordings were acquired from sensilla within a particular region of the funiculus, at the base near the olfactory pit. The sensilla were identified as subtype 3 Mp-sw thin-walled sensilla (clavate sensilla). These sensilla are distributed all over the funiculus, but are more abundant in the basal region. There are around 270 clavate sensilla on female *B. tryoni* antennae out of a total of about 2500 (Giannakakis and Fletcher, 1985). The relatively large number of carbon dioxide receptors confirm that this sensory modality is a significant one for the insect. The subtype 3 Mp-sw thin-walled sensilla house up to three sensory cells (Hull and Cribb, 1997). The lamellated dendrite of one of the sensory cells, extending up past the dendritic sheath, marks it as the carbon dioxide receptor (Zacharuk, 1985; Sutcliffe, 1994). The function of the other two sensory cells in the sensilla has not been determined. They did not produce an identifiable basal level of firing, and none of the other test chemicals elicited a response. Characterization of these other cells will require a greater range of test chemicals.

Our first paper in this series (Hull and Cribb, 2001). developed a hypothesis, from EAG data, that there was one class of receptor (the methyl butyrate receptor) within which three subtypes existed. Two of these subtypes responded to carbon dioxide as well as other general odorants; one responded to CO_2, methyl butyrate, and 2-butanone and the other responded to CO_2, methyl butyrate, 2-butanone, and farnesene. This present paper tests the hypothesis by looking at the response of individual sensilla and supports it in the occurrence of at least one of these two subtypes. The second subtype may be located on a separate region of the antenna and awaits further experimentation. In this previous paper, we also concluded, from EAG data on mixture experiments, that these receptors were broadly tuned rather than specific. This current study supports our EAG findings with electrophysiological data from individual dendrites.

Acknowledgments—The authors wish to thank Mr. Andrew Noskoff for technical assistance; Ms. Joan Hendrikz, the Faculty of Biological and Chemical Sciences, for assistance in the statistical analyses; and Dr. G. Stange for his helpful discussions.

REFERENCES

BOGNER, F. 1990. Sensory physiological investigation of carbon dioxide receptors in Lepidoptera. *J. Insect Physiol.* 36:951–957.

BOGNER, F. 1992. Response properties of CO_2-sensitive receptors in tsetse flies (Diptera: *Glossina palpalis*). *Physiol. Entomol.* 17:19–24.

BOGNER, F., BOPPRÉ, M., ERNST, K. D., and BOECKH, J. 1986. CO_2 sensitive receptors on labial palps of *Rhodogastria* moths (Lepidoptera: Arctiidae): Physiology, fine structure and central projection. *J. Comp. Physiol. A* 158:741–749.

DEN OTTER, C. J., and VAN DER GOES VAN NATERS, W. M. 1992. Single cell recordings from tsetse (*Glossina m. morsitans*) antennae reveal olfactory, mechano- and cold receptors. *Physiol. Entomol.* 17:33–42.

DICKENS, J. C. 1984. Olfaction in the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae): electroantennogram studies. *J. Chem. Ecol.* 10:1759–1785.

DICKENS, J. C., HART, W. G., LIGHT, D. M., and JANG, E. B. 1988. Tephritid olfaction: Morphology of the antennae of four tropical species of economic importance (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 81:325–331.

EISEMANN, C. H., and RICE, M. J. 1992. Attractants for the gravid Queensland fruit fly *Dacus tryoni*. *Entomol. Exp. Appl.* 62:125–130.

GIANNAKAKIS, A., and FLETCHER, B. S. 1985. Morphology and distribution of antennal sensilla of *Dacus tryoni* (Froggatt) (Diptera: Tephritidae). *J. Aust. Entomol. Soc.* 24:31–35.

GRANT, A. J., WIGTON, B. E., AGHAJANIAN, J. G., and O'CONNELL, R. J. 1995. Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. *J. Comp. Physiol. A* 177:389–396.

HEINBOCKEL, T., and KAISSLING, K.-E. 1996. Variability of olfactory receptor neuron responses of female silkmoths (*Bombyx mori* L.) to benzoic acid and (±)-linalool. *J. Insect Physiol.* 42:565–578.

HULL, C. D., and CRIBB, B. W. 1997. Ultrastructure of the antennal sensilla of Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). *Int. J. Insect Morphol. Embryol.* 26:27–34.

HULL, C. D., and CRIBB, B. W. 2001. Olfaction in the Queensland fruit fly, *Bactrocera tryoni* I. Identification of olfactory receptor neuron types responding to environmental odours. *J. Chem. Ecol.* 27:871–887.

KAISSLING, K.-E. 1986. Temporal characteristics of pheromone receptor cell responses in relation to orientation behaviour of moths, pp. 193–199, *in* T. L. Payne, M.C. Birch and C. E. J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

KELLOGG, F. E. 1970. Water vapour and carbon dioxide receptors in *Aedes aegypti* (L.). *J. Insect Physiol.* 16:99–108.

O'CONNELL, R. J. 1986. Electrophysiological responses to pheromone blends in single olfactory receptor neurones, pp. 217–224, *in* T. L. Payne, M. C. Birch, and C. E. J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

PAYNE, T. L., and DICKENS, J. C. 1976. Adaptation to determine receptor system specificity in insect olfactory communication. *J. Insect Physiol.* 22:1569–1572.

RASCH, C., and REMBOLD, H. 1994. Carbon-dioxide—highly attractive signal for larvae of *Helicoverpa armigera*. *Naturwissenshaften* 81:228–229.

RICE, M. J. 1989. The sensory physiology of pest fruit flies: conspectus and prospectus, pp. 249–272, *in* A. S. Robinson and G. H. S. Hooper (eds.). Fruit Flies—Their Biology, Natural Enemies and Control. World Crop Pests 3(A). Elsevier, Amsterdam.

RUMBO, E. R., and KAISSLING, K.-E. 1989. Temporal resolution of odour pulses by three types of pheromone receptor cells in *Antheraea polyphemus*. *J. Comp. Physiol. A* 165:281–291.

SCHNEIDER, D., KAFKA, W. A., BEROZA, M., and BIERL, B. A. 1977. Odor receptor responses of male gypsy and nun moth (Lepidoptera, Lymantriidae) to disparlure and its analogues. *J. Comp. Physiol. A* 113:1–15.

STANGE, G. 1975. Linear relation between stimulus concentration and primary transduction process in insect CO_2-receptors, pp. 207–211, *in* D. A. Denton and J. P. Coghlan (eds.). Olfaction and Taste V. Academic Press, New York.

STANGE, G. 1992. High resolution measurement of atmospheric carbon dioxide concentration changes by the labial palp organ of the moth *Heliothis armigera* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A* 171:317–324.

STANGE, G. 1996. Sensory and behavioural responses of terrestrial invertebrates to biogenic carbon dioxide gradients, pp. 223–253, *in* G. Stanhill (ed.). Advances in Bioclimatology 4. Springer-Verlag, Berlin.

STANGE, G. 1997. Effects of changes in atmospheric carbon dioxide on the location of hosts by the moth, *Cactoblastis cactorum*. *Oecologia* 110:533–539.

STANGE, G. 1999. Carbon dioxide is a close-range oviposition attractant in the Queensland fruit fly *Bactrocera tryoni*. *Naturwissenschaften* 86:190–192.

STANGE, G., and KAISSLING, K.-E. 1995. The site of action of general anaesthetics in insect olfactory receptor neurons. *Chem. Senses* 20:421–432.

STANGE, G., and WONG, C. 1993. Moth response to climate. *Nature* 365:699.

STANGE, G., MONRO, J., STOWE, S., and OSMOND, C. B. 1995. The CO_2 sense of the moth *Cactoblastis cactorum* and its probable role in the biological control of the CAM plant *Opuntia stricta*. *Oecologia* 102:341–352.

SUTCLIFFE, J. F. 1994. Sensory bases of attractancy: morphology of mosquito olfactory sensilla—a review. *J. Am. Mosq. Control Assoc.* 10:309–315.

YAMANA, K., TOH, Y., and TATEDA, H. 1986. Electrophysiological studies on the temporal organ of the Japanese house centipede, *Thereuonema hilgendorfii*. *J. Exp. Biol.* 126:297–314.

ZACHARUK, R. Y. 1985. Antennae and sensilla, pp. 1–69, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Volume 6. Pergamon Press, London.

ZIESMANN, J. 1996. The physiology of an olfactory sensillum of the termite *Schedorhinotermes lamanianus*: Carbon dioxide as a modulator of olfactory sensitivity. *J. Comp. Physiol. A* 179:123–133.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

EVALUATION OF THE SYNTHETIC MAJOR COMPONENT OF THE SEX PHEROMONE OF *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE)

FERNANDO A. A. FERRARA,[1] EVALDO F. VILELA,[2] GULAB N. JHAM,[3,*] ÁLVARO E. EIRAS,[4] MARCELO C. PICANÇO,[2] ATHULA B. ATTYGALLE,[5] ALES SVATOS,[5] ROSA T. S. FRIGHETTO,[6] and JERROLD MEINWALD[5]

[1]*Colégio Técnico Agrícola Ildefonso Bastos Borges*
Universidade Federal Fluminense
Bom Jesus do Itabapoana, RJ, Brazil

[2]*Dept. de Biologia Animal, and*

[3]*Dept. de Química*
Universidade Federal de Viçosa, MG, Brazil

[4]*Lab. de Proteção de Plantas*
Universidade Estadual Norte Fluminense
Campos dos Goytacazes, RJ, Brazil

[5]*Department of Chemistry and Chemical Biology*
Cornell University, Ithaca, New York

[6]*CNPMA/Embrapa*
Jaguariúna, SP, Brazil

(Received March 29, 2000; accepted January 4, 2001)

Abstract—In wind-tunnel bioassays, dispensers loaded with 1 μg of the synthetic major component (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrienyl acetate (TDTA) of the sex pheromone emitted by *Tuta absoluta* (Meyrick) females were found to be highly attractive to conspecific males. Field experiments were conducted to evaluate the efficacy of five trap designs. The best trap, baited with 100 μg of the synthetic sex pheromone caught on average 1200 males per trap per night, while those baited with virgin females caught only 201 males. The male response to this pheromone is restricted to the same early-morning time window during which females exhibit calling behavior. The high biological activity of the synthetic pheromone suggests that it could be useful for pest monitoring and in mating disruption.

Key Words—*Tuta absoluta*, sex pheromone, (3*E*,8*Z*,11*Z*)-3, 8,11-tetradecatrienyl acetate, field tests, monitoring.

* To whom correspondence should be addressed. e-mail: gulab@mail.ufv.br

0098-0331/01/0500-0907$19.50/0

INTRODUCTION

Tuta (=Srobipalpuloides) absoluta (Meyrick) (Lepidoptera:Gelechiidae) is a key pest of tomato *(Lycopersicon esculentum* Mill.) due to the damage it causes and the lack of ecologically acceptable methods for its control. It has been considered to be the major limiting factor for tomato production in South America (Bahamondes and Mallea, 1969; Quiroz, 1976).

IPM programs for *T. absoluta* may be achieved through the use of sex pheromone. Populations of other Gelechiidae, such as *Keiferia lycopersicella* (Walsingham) have been efficiently monitored with traps baited with sex pheromone (Jenkins et al., 1990). Trumble and Alvarado-Rodriguez (1993) reported a successful management strategy (sex pheromone for monitoring the population as well as for mating disruption) for the control of *K. lycopersicella* in industrial tomato crops. For *Phthorimae operculella* (Zeller), pheromone traps have been used as part of IPM strategies (Kennedy, 1975; Raman, 1984; Salas et al., 1985).

Using *T. absoluta* virgin females, Quiroz (1978) captured more than 100 males/trap/day, and Uchôa-Fernandes and Vilela (1994), using the same technique, compared different trap designs, heights, and displacement in tomato fields for capturing *T. absoluta* males. They reported high specificity and sensitivity of traps baited with natural pheromone, besides being more economical and convenient than the light traps.

In this work we report laboratory and field evaluations of the major synthetic component of the sex pheromone of *T. absoluta*, (3*E*, 8*Z*, 11*Z*)-3,8,11-tetradecatrienyl acetate (TDTA), which was identified and synthesized by our group (Attygalle et al., 1995, 1996). Its structure was confirmed by Griepink et al. (1996).

METHODS AND MATERIALS

Insect Rearing. Larvae of *T. absoluta* were collected on tomato in fields around Viçosa, MG, Brazil and reared on artificial diet (Pratissoli, 1995) at 23 ± 2°C, 75 ± 5% relative humidity and a 14L:10D photoregime. After separation of females and males (Coelho and França, 1987), pupae were maintained in separate rooms to avoid exposure of males to female sex pheromone and consequent development of habituation (Bartell, 1977).

Wind-Tunnel Bioassays. Bioassays were conducted in a wind tunnel (1.5 m long and 0.5 m height) at 24 ± 1.5°C, 83 ± 3%, relative humidity, and air flux of 30 mm/sec. The TDTA source was placed at a height of 0.3 m and 1.0 m from the point of insect release. After each bioassay, the tunnel and its accessories were thoroughly cleaned with neutral detergent and water. The synthetic pheromone was dissolved in doubly distilled hexane and appropriate volumes were impregnated on a rubber septum and allowed to air dry for 24 hr. For field trials, treated septa were sealed in aluminum foil and transported in an ice box. All septa used for

impregnating the pheromone were previously extracted for 24 hr with distilled CH_2Cl_2 in a Soxhlet extractor.

The attractiveness of the synthetic acetate (0.01, 0.1, 1 and 10 μg/septum) was compared to that of nine virgin females during their calling period (Hickel and Vilela, 1991; Hickel et al., 1991; Uchôa-Fernandes et al., 1995a). Hexane (100 μl) was placed on a rubber septum, air dried, and used as control. The experimental design was entirely randomized with 10 replications, each consisting of three males (1 to 3 days old), released from glass tubes open on both sides. Each observation lasted 5 min and the response of males was computed once, after which they were eliminated.

Field Experiments. Field experiments were conducted in two tomato crops (staked and industrial) to evaluate the efficiency of five types of traps as well as to determine the attractiveness of TDTA. In addition, the variation of insect capture during the day and during the tomato plant phases was studied.

Staked Tomatoes. These experiments were conducted in a 20-ha staked (commercial) tomato crop (Santa Clara variety), in Araguari, MG, Brazil during April 1–11, 1994. The following five trap designs were evaluated: (1) commercial Delta sticky trap (AgriSense Ltd.) with a vertical height of about 7 cm at the triangular opening and a base of 29 $\times$ 30 cm; (2) CICA-Q trap (home made), which consisted of a white square plastic tray (24 $\times$ 37 $\times$ 6 cm) covered with a thin plate (V shaped) and held together with four strong metal wires; (3) CICA-R trap (home made) consisted of two black round trays (commonly used for house gardening in Brazil), with the larger one (32 cm in diameter) serving as the base and the smaller one (20 cm in diameter) serving as the top. The two plates were held together by three strong metal wires equidistant from each other. Both traps contained water and detergent to trap the insects; (4) home made PVA 200 trap (Uchôa-Fernandes and Vilela, 1994) made up of a white PVC tube (200 mm diameter) and an adhesive cardboard inside, similar to the Delta trap, and (5) home made PVC-M 200 trap, which was a modification of PVA 200 trap with two lateral longitudinal openings.

Each trap was baited with 1 μg of TDTA and placed at 30-m intervals and at 1.2 m above ground (Uchôa-Fernandes and Vilela, 1994) during three days. The experimental design was a Latin square (Perry et al., 1980), and the number of insects caught in each trap, with five replicates (25 traps) was counted daily. The highest number of insects caught was with CICA-R traps and, hence, some of the subsequent tests were conducted with this type of trap.

The periodicity of male capture over a 24-hr period was evaluated by using Delta traps containing 1 μg of the synthetic TDTA impregnated on a rubber septa, with four replicates, for four 24-hr periods.

Industrial Tomatoes. These experiments were conducted in Petrolina, PE, Brazil during June 25–July 5, 1994 on a commercial 20-ha industrial tomato crop (cultivar IPA). The five traps described in the previous experiment were evaluated at 0.4 m above the ground over a five-day period. Each trap was baited with a

rubber septum containing 1 μg of the synthetic TDTA and its position remained unchanged during the experiment. The experiment was arranged in a randomized block with five replications.

In order to evaluate the variation of insect capture during the day, traps containing 1 μg of the TDTA (five replications) were spaced 30 m apart. To evaluate the effect of trap height, CICA-R traps were placed in three different crop phases. CICA-R traps containing 1 μg of the TDTA were placed at 0.2, 0.4, 0.6, 0.8, and 1.0 m above ground in the first crop phase, i.e., just before planting. CICA-R traps containing 1 μg of the TDTA were placed at 0.2, 0.4, 0.6, 0.8, and 1.0 m above ground in second crop phase (0.2-m high plant). CICA-R traps containing 1 μg of the TDTA were placed at 0.2, 0.4, 0.6, 0.8, and 1.0 m above ground in the third crop phase, which was crop with flowers. Five replications were used.

Statistical Analyses. The data obtained in wind-tunnel bioassays were transformed into arcsine (X). After that they started to present normal distribution and were statistically analyzed at 5% significance (Scott and Knott, 1974). The following statistical analyses were utilized for three parameters evaluated: in field experiments: The attraction data were transformed into log ($X + 1$). After that, they started to present normal distribution and they were statistically analyzed at 5% significance (Scott and Knott, 1974). The data of periodicity of male capture were transformed into $\sqrt{(X + 0.5)}$. After that, they started to present normal distribution and were statistically analyzed at 5% significance (Duncan). The trap design data with staked tomatoes presented normal distribution and were analyzed without transformation. The trap design data obtained in the field experiments with industrial tomatoes were transformed into $\sqrt{X}$. After that they started to present normal distribution and were statistically analyzed at 5% significance (Scott and Knott, 1974).

The data for height of traps for the first phase (before planting) as well as for the second phase, (20-cm-high plants), presented normal distribution and were analyzed without transformation, whereas the data for the third phase (plants with flowers) were transformed into $\sqrt{X}$. All data were subjected to ANOVA analysis and the averages were compared by Scott-Knott test (Scott and Knott, 1974) or the Duncan test at 5% significance.

RESULTS

Wind-Tunnel Bioassays. The response of *T. absoluta* males to the synthetic TDTA was immediate (within 1–5 min), with males landing on the pheromone source, indicating a possible application in insect trapping. The highest response was obtained with 0.1, 1.0 and 10.0 μg (Figure 1). Male wing fanning was significantly higher with 1 μg than that obtained with other doses of the synthetic pheromone (Figure 1).

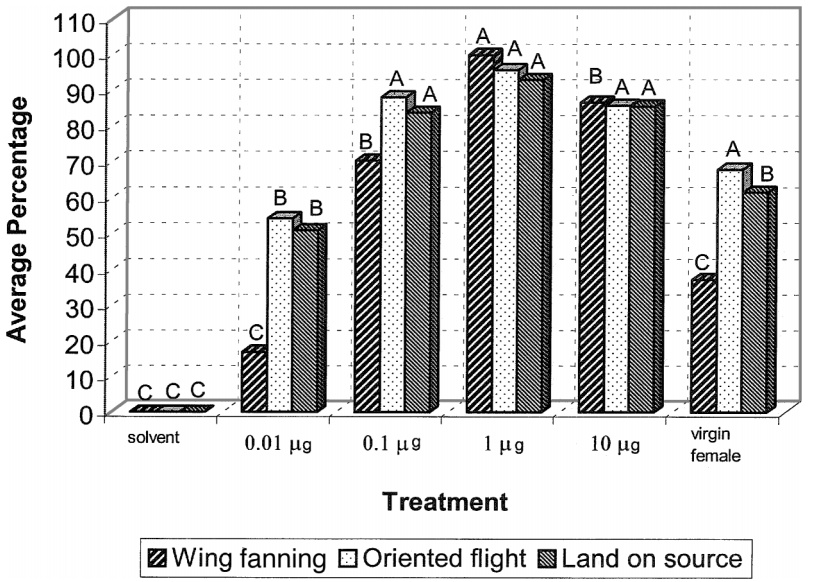


FIG. 1. Average percentage of *T. absoluta* males that respond to different amounts of TDTA, to virgin females, and to the solvent in a wind tunnel. Bars followed by the same letter are not significantly different ($\alpha = 0.05$, Scott-Knott test, $N = 10$).

Response to Trap Design in the Field. The results of the experiments with staked and industrial tomatoes are shown in Figures 2 and 3, respectively. In staked tomato plantations, both CICA-R and CICA-Q traps provided the best insect captures (Figure 2) while in the industrial tomato crops, at initial insect infestation (low), the CICA-R and PVC traps captured the highest number of males, but the captures were not significantly different from the CICA-Q and Delta traps (Figure 3). The adhesive cardboard of the PVC traps presented the disadvantage of not retaining newly attracted insects due to surface saturation. This was not observed with traps that used water and detergent to trap the insects in highly infested fields. Consequently, a greater number of insects was trapped with both CICA-Q and CICA-R traps. However, in a low infestation situation, CICA-Q traps did not present significant difference in the number of insect trapped. The CICA-R trap, which was black and totally open, was more efficient probably due to its format.

Concentrations of TDTA in the Field. The highest response of *T. absoluta* males was obtained at a concentration of 100 μg of the main component of the sex pheromone TDTA per trap (Figure 4).

Periodicity of Male Captures. In the staked and industrial tomato crops, the capture of the males occurred between 5 and 9 AM, with a peak around 7 AM

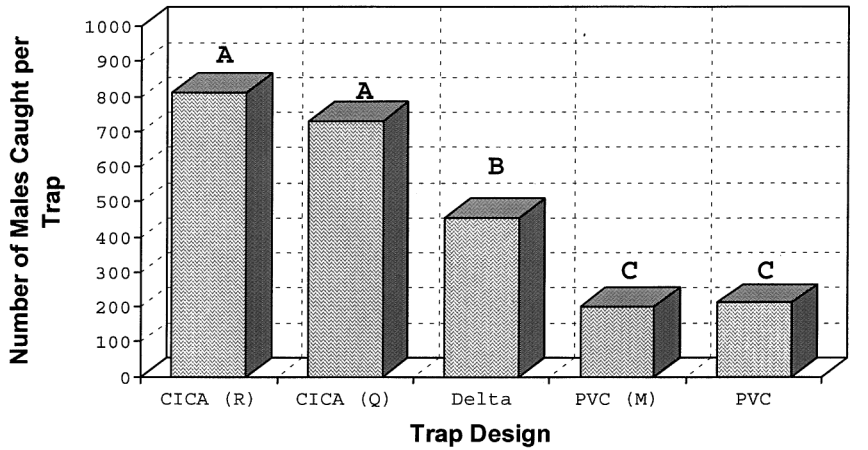


FIG. 2. Average number of males of *T. absoluta* caught per morning per trap in a staked tomato crop in Araguari, MG, Brazil, during 1994, with five different trap designs. Each trap was baited with 1 μg of synthetic TDTA. Histograms followed by the same letter are not significantly different ($\alpha = 0.05$, Scott-Knott test, $N = 5$).

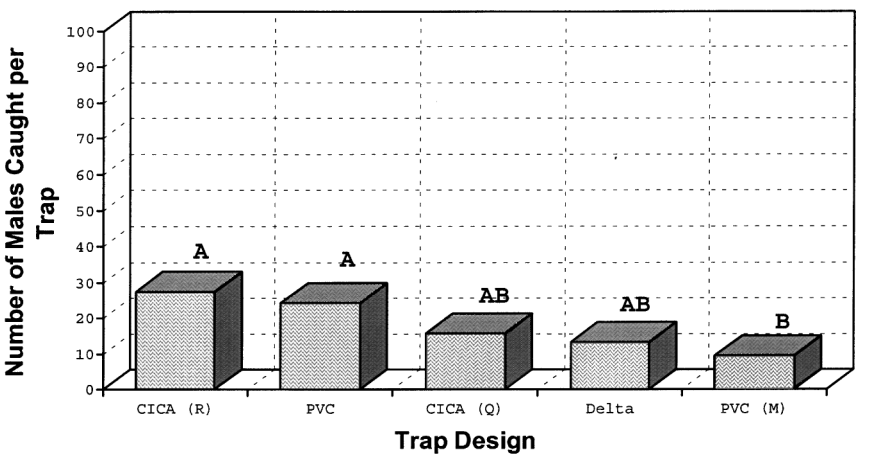


FIG. 3. Average number of males of *T. absoluta* caught per morning per trap in an industrial tomato crop in Petrolina, PE, Brazil, during 1994, with five different trap designs. Each trap was baited with 1 μg of synthetic TDTA. Histograms followed by the same letter are not significantly different ($\alpha = 0.05$, Duncan test, $N = 5$).

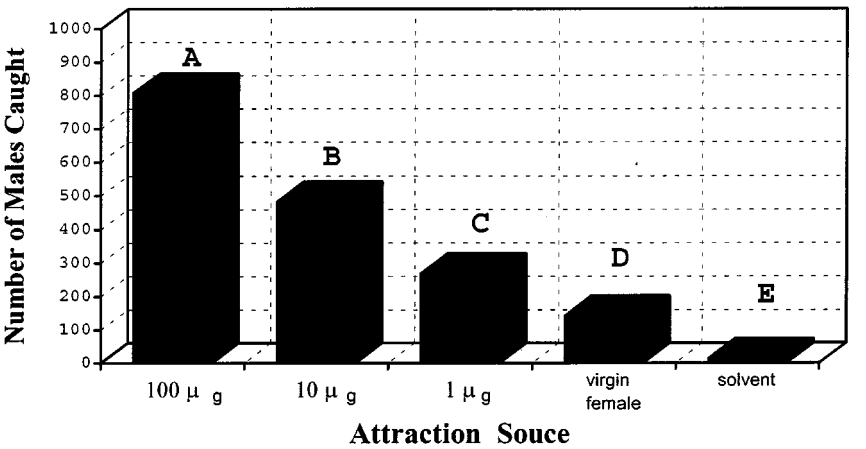


FIG. 4. Average number of males of *T. absoluta* caught per morning per trap in a staked tomato crop in Araguari, MG, Brazil, during 1994, with different concentrations of the synthetic TDTA. Histograms followed by the same letter are not significantly different ($\alpha = 0.05$, Scott-Knott test, $N = 5$).

(Table 1). Insect infestation was lower during the evaluation period in the industrial tomato crops compared to the staked tomato crops. Insect capture with synthetic pheromone baits was affected by temperature. Earlier capture generally took place when the temperature was lower ($r = -0.79$, $P < 0.01$). However, no effect of relative humidity was noted ($r = 0.79$, $P = 0.14$).

Height of Traps. In the industrial tomato crops, when the soil was prepared for tilling, traps placed at a height of 20 cm above ground presented the best results, capturing the highest number of *T. absoluta* males (Figure 5). When the tomato plants were about 20 cm high, traps placed at heights of 20, 40, and 60 cm captured

TABLE 1. AVERAGE NUMBER ($N = 4$) OF MALES OF *Tuta absoluta* CAUGHT PER MORNING PER TRAP, IN DELTA TRAPS BAITED WITH 1 μG OF SYNTHETIC TDTA IN A FIELD WITH STAKED TOMATO CROP (ARAGUARI, MG, BRAZIL, 1994)

Evaluation date	5-6 AM	6-7 AM	7-8 AM	8-9 AM	General average
April 8	13.98cAB	511.91aA	190.00Ba	3.48dA	111.02A
April 9	22.90cA	432.48aB	48.53bB	0.00dA	69.03B
April 10	2.30aC	6.69aC	5.68aC	2.00aA	3.93C
April 11	5.96aBC	9.98aC	0.46bC	0.00bA	2.97C
General average	9.83c	151.79a	36.27b	1.06c	

Averages followed by the same small letter in a row or capital letters in a column did not differ significantly (Duncan test, $P < 0.05$).

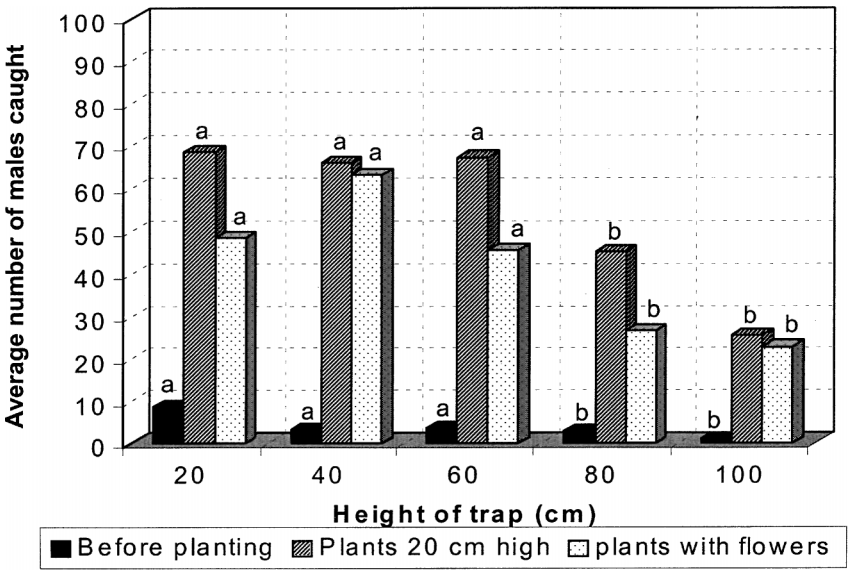


FIG. 5. Average number of males of *T. absoluta* caught per morning per trap in three different stages of an industrial tomato crop in Petrolina, PE, Brazil, during 1994. Each trap was baited with 1 μg of synthetic TDTA. In each stage, histograms followed by the same letter are not significantly different ($\alpha = 0.05$, Scott-Knott test, $N = 5$ for each stage).

a higher number of insects. However, the results obtained from different heights were not significantly different.

DISCUSSION

Wind-Tunnel Bioassays. Typical responses were recorded in wind-tunnel bioassays. Wing fanning represents a state of high sexual excitement in males (Cardé, 1984) and most of the time resulted in landing on the pheromone source. Males flew towards the source impregnated with 0.1, 1.0, and 10 μg of TDTA, as well towards virgin females, whereas there was no significant difference between 0.01 μg and solvent (Figure 1). Similar results have been reported by Hickel et al. (1991), working with virgin females.

Response to Trap Design in the Field. The best results were obtained with the CICA-R trap, which was probably due to its completely open shape. These results were in agreement with those of Wyman (1979), working on monitoring of *Keiferia lycopersicella* using synthetic pheromone. He concluded that a high trap efficiency was related to ease access of insects to traps. The cost of each unit of the CICA-R trap, Delta trap and its sticky cardboard, CICA-Q trap, and PVC

trap was of US$2.95, 5.00, 7.00, and 1.92 (not including the sticky cardboard), respectively.

Concentrations of TDTA in the Field. Each higher concentration of TDTA trapped more insects, possibly due to greater amounts of pheromone released. Tumlinson et al. (1994) have reported a higher release rate of the synthetic pheromone of *Manduca sexta* (L.) in the field from the rubber septa impregnated with higher amounts.

Periodicity of Male Captures. In the staked tomato crops, the results on periodicity of male captures were in agreement with those of Uchôa-Fernandes et al. (1995b), who used virgins female as baits. The difference in the number of insects captured in the two areas was most likely due to the lower insect infestation during the evaluation period in the industrial tomato crops compared to the staked tomato crops.

The influence of temperature on pheromone catch is in agreement with that reported by Cardé and Roelofs (1973), for *Holomelina immaculata* (Rearkirt), for which the temperature influenced both the initiation and periodicity of captures. Cardé et al. (1974) confirmed that temperature can modify the calling period or the male response. McNeil (1991) reported that several factors affect the emission and reception of pheromones, mainly temperature, which affected the calling period of the females and, consequently, the male response.

In summary, these results suggest that a partial application of low cost CICA-R traps loaded with 100 μg of the synthetic pheromone (TDTA) for monitoring *T. absoluta* populations. In soil prepared for sowing, the height of the trap should be 20 cm, and for plants that are around 20 cm high, the traps should be placed at a height of 20–60 cm and should remain in place until harvest. With growing plants, the height of the traps should be about 60 cm, since a higher degree of attack takes place in the medium and top parts of the canopy (Coelho and França, 1987; Pratissoli, 1995; Picanç et al., 1995).

Acknowledgments—We thank the Gessy Lever S/A-Brazil and the following Brazilian research agencies: FAPEMIG, CAPES, CNPq and FINEP for the financial support.

REFERENCES

ATTYGALLE, A. B., JHAM, G. N., SVATOS, A., FRIGHETTO, R. T. S., MEINWALD, J., VILELA, E. F., FERRARA, A., and UCHÔA-FERNANDES, M. A. 1995. Microscale, random reduction: Application to the characterization of (3*E*,8*Z*,11*Z*)-3,8,11-tetradecatrienyl acetate, a new lepidopteran sex pheromone. *Tetrahedron Lett*. 36:5471–5474.

ATTYGALLE, A. B., JHAM, G. N., SVATOS, A., FRIGHETTO, R. T. S., MEINWALD, J., VILLELA, E., and FERRARA, F. A. 1996. (3*E*,8*Z*,11*Z*)-3,8,11- Tetradecatrienyl acetate: A major component of sex pheromone of tomato pest *Scrobipalpuloides absoluta*. *Biol. Med. Chem.* 4:305–314.

BAHAMONDES, L. A., and MALLEA, A. R. 1969. Biologia en Mendoza de *Scrobipalpula absoluta* (Meyrick) Povolny (Lepidoptera: Gelechiidae), espécie nueva para la Republica Argentina. *Rev. Fac. Cienc. Agric*. 15:96–104.

BARTELL, R. J. 1977. Behavioral responses of Lepidoptera to pheromones, pp. 201–203, *in* H. H. Shorey and J. J. Mckevey Jr. (eds.). and Chemical Control of Insect Behavior, Theory and Application. John Wiley, New York.

CARDÉ, R. T. 1984. Chemo-orientation in flying insects, pp. 111–121, *in*: W. J. BELL, and R. T. CARDÉ (eds.). Chemical Ecology of Insects. Chapman and Hall, New york.

CARDÉ, R. T., and ROELOFS, W. L. 1973. Temperature modification of male sex pheromone response and factors affecting female calling in *Holomelina immaculata* (Lepidoptera: Arctiidae). *Can. Entomol*. 105:1505–1512.

CARDÉ, R. T., DOANE, C. C., and ROELOFS, W. L. 1974. Dual periodicity of male sex pheromone response and female attractiveness in the gypsy moth (Lepidoptera: Lymantriidae). *Can. Entomol*. 106:479–484.

COELHO, M. C. F., and FRANÇA, H. F. 1987. Biologia, quetotaxia da larva e descriçáo da pupa e do adulto da traça do tomateiro. *Pesq. Agropec. Bras*. 22:129–135.

GRIEPINK, F. C., VAN BEEK, T. A., POSTHUMUS, M. A, DE GROOT, A., VISSER, J.H., and VOERMAN, S. 1996. Identification of the sex pheromone of *Scrobipalpula absoluta*: Determination of double hand positions in triple unsaturated straight chain molecules by means of dimethyl disulfide derivatization. *Tetrahedron Lett*. 30:411–414.

HICKEL, E. R., and VILELA, E. F. 1991. Comportamento de chamamento e aspectos do comportamento de acasalamento de *Scrobipalpula absoluta* (Lepidoptera: Gelechiidae), sob condições de campo. *An. Soc. Entomol. Bras*. 20:173–182.

HICKEL, E. R., VILELA, E. F., LIMA, J. O. G., and DELLA LUCIA, T. M. C. 1991. Comportamento de acasalamento de *Scrobipalpula absoluta* (Lepidoptera: Gelechiidae). *Pesq. Agropec. Bras.* 26:827–835.

JENKINS, J. W., DOANE, C. C., SCHUSTER, D. J., MCLAUGHIN, J. R., and JIMENEZ, M. J. 1990. Development and commercial application of sex pheromone for control of the tomato pinworm, pp. 269, *in*: R. L. Ridgway, R. M. Silverstein, and M. N. Inscoe (eds.). Behavior-Modifying Chemical for Insect Management. Marcel Dekker, New York.

KENNEDY, G. G. 1975. Trap design and other factors influencing capture of male potato tuberworm moths by virgin female baited traps. *J. Econ. Entomol.* 68:305–308.

MCNEIL, J. N. 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. *Annu. Rev. Entomol.* 36:407–430.

PERRY, J. N., WALL, C., and NOWAY, A R. 1980. Square designs in field experiments involving insect sex attractants. *Ecol. Entomol.* 5:385–396.

PICANÇO, M. C., LEITE, G. L. D., and JHAM, G. N. 1995. Intensidade de ataque de *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera: Gelechiidae) ao dossel de três espécies de tomateiro. *Pesq. Agropec. Bras.* 30:429–433.

PRATISSOLI, D. 1995. Bioecologia do Trichogramma pretiosum Riley, 1879 nas traças *Scrobipalpuloides absoluta* (Meyrick,1917) e *Phthorimaea operculella* (Zeller, 1873) em tomateiro. Thesis, ESALQ/USP, Piracicaba, SP, Brazil, 135 pp. (in Portuguese).

QUIROZ, C. E. 1976. Nuevos antecedentes sobre la biología de la polilla del tomate, *Scrobipalpula absoluta* (Meyrick). *Agric. Tec.* 36:82–86.

QUIROZ, C. E. 1978. Utilización de trampas con hembras vírgenes de *Scrobipalpula absoluta* (Meyrick) (Lep., Gelechiidae) en estudios de dinámica de población. *Agric. Tec.* 38:94–97.

RAMAN, K. V. 1984. Evaluation of a synthetic sex pheromone funnel trap for potato tuberworm moths (Lepidoptera: Gelechiidae). *Environ. Entomol.* 13:61–64.

SALAS, J., PARRA, A., and ALVARES, C. 1985. Evaluation preliminar de la feromona sexual sintetica del minador grande de la hoja del tomate *Phthorimaea operculella* en la capture de machos. *Agron. Trop.* 35:139–144.

SCOTT, A. J., and KNOTT, M. A. 1974. A cluster analysis method for grouping means in the analysis of variance. *Biometrics* 30:507–512.

TRUMBLE, J. T., and ALVARADO-RODRIGUEZ, B. 1993. Development and economic evaluation of an IPM program for fresh market tomato production in Mexico. *Agric. Ecol. Environ.* 43:267–84.

TUMLINSON, J. H., MITCHELL, E. R., DOOLITTLE, R .E., and JAKSON, D. M. 1994. Field tests of synthetic *Manduca sexta* sex pheromone. *J. Chem. Ecol.* 20:579–591.

UCHÔA-FERNANDES, M. A., and VILELA, E. F. 1994. Field trapping of the tomato worm *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera:Gelechiidae) using virgin females. *An. Soc. Entomol. Bras.* 23:271–277.

UCHÔA-Fernandes, M. A., DELLA LUCIA, T. M. C., and VILELA, E. F. 1995a. Mating, oviposition and population of *Scrobipalpuloides absoluta* (Meyrick) (Lepidoptera: Gelechiidae). *An. Soc. Entomol. Bras.* 24:159–164.

UCHÔA-FERNANDES, M. A., VILELA, E. F., and DELLA LUCIA, T. M. C. 1995b. Ritmo diário de atração em *Scrobipalpuloides absoluta* (Lepidoptera: Gelechiidae). *Rev. Bras. Bio.* 55:67–73.

WYMAN, J. A. 1979. Effect of trap design and sex attractant release rates on tomato pinworm catches. *J. Econ. Entomol.* 72:865–868.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

TASK-RELATED CHEMICAL ANALYSIS OF LABIAL GLAND VOLATILE SECRETION IN WORKER HONEYBEES (*Apis mellifera ligustica*)

TAMAR KATZAV-GOZANSKY,[1,*] VICTORIA SOROKER,[1,3] ARMIN IONESCU,[1] GENE E. ROBINSON,[2] and ABRAHAM HEFETZ[1]

[1]*Department of Zoology, George S. Wise Faculty of Life Sciences*
Tel Aviv University
Ramat Aviv, Tel Aviv, Israel

[2]*Department of Entomology*
University of Illinois
Urbana, Illinois

(Received October 4, 1999; accepted January 10, 2001)

Abstract—Chemical analyses revealed that the labial gland complex of worker honeybees possesses a series of hydrocarbons dominated by odd-numbered carbon chain alkanes along with minor amounts of alkenes and branched alkanes. Foragers contained significantly more secretion than nurse bees. Experiments with bees from colonies induced to have a division of labor independent of age revealed that the differences in the amount of secretion were task, but not age dependent.

Key Words—Honeybees, labial glands, exocrine glands, secretion, hydrocarbons, forager bees, task specificity.

INTRODUCTION

The labial (salivary) gland complex comprises two pairs of glands, one in the head and one in the thorax, both of which connect through a common duct to the mouth (Cruze Landim, 1967). They are intermittently developed in bees, and little is known about the chemistry or function of their secretion. In the mason bee, *Chalicodoma siculum*, the head gland secretion is composed of hydrocarbons that are used to waterproof brood cells (Kronenberg and Hefetz, 1984). Male carpenter

* To whom correspondence should be addressed. e-mail: katzavt@post.tau.ac.il
[3] Present address: Department of Entomology, Institute of Plant Protection, Agricultural Research Organization, Volcani Center, Beit Dagan, 50250 Israel.

0098-0331/01/0500-0919$19.50/1

bees, *Xylocopa varipuncta*, presumably use the secretion from the thoracic gland as a long-range sex attractant, but the chemistry remains elusive (Minckley et al., 1991). The chemistry and function of the labial gland secretion of male bumble bees was extensively studied, revealing a plethora of compounds (Bergström et al., 1981; Genin et al., 1984). The secretion is species-specific, and in the species studied it is used for marking the flight path to which queens and other males are attracted (Kullenberg et al. 1970, 1973; Bergman and Bergström 1997; Hovorka et al., 1998; Kindl et al., 1999). The labial glands are also well developed in *Bombus terrestris* queens and contain mostly a series of dodecyl esters (Hefetz et al., 1996).

The posterior part of the head gland and the thoracic gland were reported to be involved in partial food digestion in the honeybee. The secretion of the thoracic gland contains watery saliva that dissolves sugars, whereas the head gland produces an oily secretion of unknown function (Simpson, 1960; Arnold and Delge-Derachen, 1978). We present here a comparative analysis of the volatile constituents of the head and thoracic gland secretions of worker honey bees as a function of age and task.

METHODS AND MATERIALS

Bee and Hive Manipulations. Workers of European honeybees (*Apis mellifera ligustica*) were obtained mostly from the Tzrifin apiary (Ministry of Agriculture), Israel, and from one colony from an apiary at the University of Illinois Bee Research Facility. Nurse bees were collected on brood combs and foragers returning with pollen were collected at the hive entrance. To dissociate possible effect of age and behavioral status on glandular composition, a single-cohort colony was established with about 1000 1-day-old adult bees from a field colony. These bees were obtained by taking frames containing old pupae and placing them in an incubator (34°C and 80% relative humidity). Newly emerging adults were marked with paint dots on the thorax. The colony was given a single frame of food and a mated queen. Under these conditions some of the workers become precocious foragers, whereas three weeks later some workers still remained as old nurses (Robinson et al., 1989). Four groups of bees were collected for comparative analyses: young (precocious) foragers and young nurses were collected after one week, and old foragers and old (over-aged) nurses were collected after three weeks. Bees for the GC-MS analyses were obtained from several commercial colonies at the Tzrifin apiary. Quantitative analyses were conducted using bees from one "typical colony" and one "single cohort colony."

Chemical Analyses and Compound Identification. Head and thoracic labial glands were dissected under a stereo microscope ($\times 20$) and extracted in 100 μl dichloromethane (to enable proper detection of the peaks by gas chromatography

each sample comprised a pool of glands from five bees). The head glands were separated from the hypopharyngeal glands and the thoracic glands were separated from the thoracic muscles. Extracts were analyzed by combined capillary gas chromatography–mass spectrometry (EI 70 eV, and CI using methane as a reagent gas) using a 30-m DB-5 fused silica column that was temperature programmed from 120°C to 300°C at a rate of 3°/min with initial hold of 3 min. Quantitative analyses were done by gas chromatography using a 30-m SE-54 or DB-1 capillary column that was temperature programmed from 60°C to 100°C at 20°/min and to 270°C at 5°/min (final hold: 30 min). Identity of the components was verified by comparing their retention time with standard compounds. Quantification of the glandular secretion was performed by peak integration (FID detector) using eicosane (1 μg/sample) as an internal standard.

RESULTS AND DISCUSSION

Table 1 presents a list of the volatile components found both in the head and thoracic labial gland secretion of honeybee, nurses, and foragers. The main components in both worker groups were straight-chain alkanes ranging from C_{21} to C_{35}, accompanied by minor amounts of alkenes and methyl-branched alkanes. In most cases the major components in all groups of workers were the same (e.g., odd-numbered carbon chain alkanes), but there was a degree of specificity in the minor components.

Quantification of the glandular extracts revealed that the total amount of secretion was normally distributed among samples (Kolmogorov-Smirnov $P > 0.2$, for both head and thoracic glands). We assessed the differences between treatments (age and task) by ANOVA followed by Fisher post-hoc test. There was a significant effect of task, but not age on the total amount of secretion for both the head and thoracic glands (Table 2, ANOVA). Foragers had higher amounts of secretion than nurses, irrespective of their age.

For the head glands, specific comparison between the group of bees showed that among the hive bees (of undetermined age) foragers had larger amounts of secretion than nurses in the head but not in the thoracic glands (Table 3, typical colony, ANOVA followed by Fisher's PLSD; $P < 0.0001$). Analyses of bees from a single-cohort colony indicated that the quantitative differences in the head gland secretion are mostly related to differences in behavior, and not to age (Table 3, single cohort colony bees). Although old foragers that originated from the single cohort colony tended to have less secretion in the head labial glands as compared to foragers from a typical colony, it was not significant ($P = 0.057$). The amount of secretion in precocious foragers was lower than that of foragers from a typical colony, but not than of old foragers from the single cohort colony ($P = 0.03$ and $P = 0.68$, respectively). Foragers from the single-cohort colonies had greater

TABLE 1. CHEMICAL COMPOSITION OF LABIAL GLAND SECRETIONS OF NURSE AND FORAGER HONEYBEES[a]

	Head gland		Thoracic gland	
Compound	Nurses	Foragers	Nurses	Foragers
Alkanes				
C_{17}	—	*	—	—
C_{19}	*	t	*	—
C_{21}	—	*	*	*
C_{22}	*	t	—	***
C_{23}	***	***	**	**
C_{24}	*	*	*	*
C_{25}	**	****	**	**
C_{26}	*	*	**	**
C_{27}	**	***	**	**
C_{28}	—	*	—	—
C_{29}	**	*	*	*
C_{30}	—	*	—	—
C_{31}	***	*	*	*
C_{33}	**	t	**	*
C_{35}	**	t	**	—
Alkenes				
$C_{23:1}$	—	t	—	*
$C_{25:1}$	*	t	—	—
$C_{31:1}$	—	t	—	—
$C_{33:1}$	—	*	—	*
Methylalkanes				
4-MeC_{22}	*	—	—	—
3-MeC_{24}	—	—	—	*
9-MeC_{25}	*	—	—	—
3-MeC_{25}	—	—	—	*

[a] The results are presented in relative proportions: —, not detected; t, trace; *1–5%; **5–15%; ***16–25%; ****25–50%.

TABLE 2. STATISTICAL ANALYSES OF SECRETION AMOUNT IN HEAD AND THORACIC GLANDS

	ANOVA	
	Head glands	Thoracic glands
Total	$P = 0.0004$	$P = 0.006$
Effect of age	$P = 0.15$	$P = 0.12$
Effect of task	$P < 0.0001$	$P = 0.03$

TABLE 3. TOTAL AMOUNT OF VOLATILE COMPOUNDS FROM HEAD AND THORACIC LABIAL GLANDS OF WORKER HONEYBEES BELONGING TO DIFFERENT AGE AND TASK GROUPS.

Type of worker	Volatile (μg/glands of 5 bees; mean $\pm$ SD)	
	Head Glands	Thoracic glands
From typical colony		
Foragers	8.1 ± 2.8 (4)[a] a[b]	2.7 ± 0.9 (4) ac
Nurses	2.8 ± 0.6 (4) b	2.4 ± 0.5 (4) a
From single cohort colony		
Old foragers	6.1 ± 1.8 (9) ac	3.2 ± 0.3 (9) bc
Young (precocious) foragers	5.8 ± 1.5 (9) cd	2.1 ± 0.4 (8) a
Old (overaged) nurses	4.0 ± 1.8 (9) b	2.4 ± 0.9 (9) a
Young nurses	4.4 ± 1.4 (9) bd	2.0 ± 0.5 (9) a

[a] (N)-number of replicates.
[b] Values accompanied by the same letter are not statistically different (ANOVA followed by Fisher's PLSD).

amounts of secretion than nurse bees that originated from the same cohort colonies, irrespective of age. While the differences between the old foragers and the two types of nurses were significant, secretion of precocious foragers was higher than that of old nurses, but not that of young nurses.

Typical colony and single-cohort colony nurse bees did not differ in secretionary quantities in the head gland (ANOVA Fisher's PLSD $P = 0.97$ and $P > 0.99$ for young and old nurses, respectively).

For the thoracic glands, there were no differences between typical colony foragers and foragers from the single-cohort colony, whether old or precocious foragers, in the amount of glandular secretion (ANOVA Fisher's PLSD $P = 0.87$ and $P = 0.82$ for old and precocious foragers, respectively). In contrast, there were differences in the thoracic gland secretion between nurses from a typical colony and old foragers from a cohort colony, but not from young or overaged nurses, ($P = 0.04$ for old foragers and $P = 0.38$ and $P = 0.67$ for young and old nurses, respectively).

Differences in the amounts of material in the head gland suggest that as bees begin to forage, their glands start to fill up with hydrocarbons. Although this process appears to be largely age independent, there does seem to be an effect of a maturation or experience maturity component. The somewhat lower amounts found in precocious foragers may be explained by the short time (just a few days) that they had spent as foragers. The tendency of task-related greater amount of secretion was also noticeable in thoracic gland, although it was not statistically different.

Honeybee labial glands were previously thought to be solely involved in processing sugar. The secretion was thought to be mostly water soluble and to

contain digestive saliva (Simpson, 1960). The presence of copious amounts of hydrocarbons in the glands, suggests that the glandular secretion may have additional functions. There are only a few studies pertaining to the chemistry and function of the labial glands in other bee species. The gland is intermittently developed in bees and its use as well as its chemistry may have evolved several times independently. The head gland in the mason bee, *Chalicodoma siculum*, possesses an array of hydrocarbons similar to that of honeybees, used in this species in nest construction. The secretion is mixed with saliva and provides the brood cells with a waterproof layer (Kronenberg and Hefetz, 1984). It may have a similar function in the honeybee, e.g., assisting the preforager bees to manipulate the wax while constructing the brood comb. We think that this function is unlikely since foragers had larger amounts than nurses, opposite to what is expected from the above function.

The hydrocarbons detected in the labial glands of the honeybee are also present on the epicuticule (Arnold et al., 1996, and personal observation). This raises the possibility of a link between these two body parts. If this is so, the honeybee labial gland may function in a manner comparable to the postpharyngeal gland (PPG) of some ant species in which it serves as a reservoir of hydrocarbons that arise both from internal sources and through exchanges with nestmates (Soroker et al., 1994; Soroker and Hefetz, 2000). The postpharyngeal gland in the ant *Cataglyphis niger* is the source of hydrocarbon nestmate recognition cues (Lahav et al., 1999). This implies a hydrocarbon exchange between the labial gland and the cuticle of individual bees, as well as interindividual exchanges via trophallaxis and allogrooming. Worker honeybees can discriminate members of the same subfamily (super sister) from workers of other subfamilies (half sisters) (Moritz and Hillesheim, 1990). This is thought to be based partly upon cuticular hydrocarbons (Page et al., 1991). Different studies have revealed that none of the major cuticular hydrocarbons gave a positive result in recognition bioassays, whereas hydrocarbons present in smaller quantities gave positive results. The complete recognition mixture probably contains minute amounts of hydrocarbons, perhaps along with other as yet unidentified components that can be either produced by the workers or acquired from external sources, e.g., flowers, the queen, or the wax comb (Breed, 1998).

This study shows that the labial glands constitute yet another set of exocrine glands that are affected by honeybee age polyethism. The results suggest that the glandular secretion has a role that is specific to foragers. One possibility is that the secretion may be involved in regulation of the age at which bees begin to forage. One regulatory model postulates the presence of an inhibitor (as yet unidentified) that inhibits the rate of behavioral development and delays the age and onset of foraging (Huang and Robinson, 1992, 1996). The inhibitor is hypothesized to be produced and/or transferred in greater amount by old bees (Huang and Robinson, 1992), and recent results indicate that this transfer is done by trophallaxis (Huang et al; 1998; Schultz et al., 1998). This idea fits with the hypothesis,

based on behavioral observations, that trophallaxis functions in communication as well as in food transfer (Korst and Velthuis 1982). Because the labial glands open to the buccal cavity, it is tempting to speculate that the secretion in foragers constitutes part of the hypothesized inhibitory system, but this awaits further experimentation.

Acknowledgments—We thank the Tzrifin apiary for assistance in establishing experimental hives, Dr. T. Simon for her advice, Roy Fayman for his technical assistance, Nomi Paz for editorial help, and A. J. Ross for expert technical assistance in Illinois. This research was supported in part by The Israel Science Foundation founded by the Israel academy of Science and Humanities to Abraham Hefetz and in part by NIH grant DC03008 to Gene E. Robinson. This research was partly a contribution by the Agricultural Research Organization, The Volcani Center, Israel (506/0A).

REFERENCES

ARNOLD, G., and DELGE-DERACHEN, B. 1978. Nouvelles données sur l'équipment enzimatique des glandes salivaries de l'ouvrier *d'Apis mellifera* (Hymenoptera Apidae). *Ann. Sci. Nat., Zool. Biol. Anim*. 20:401–422.

ARNOLD, G., QUENET, B., CORNUET, J. M., MASSON, C. DE SCHEPPER, B., ESTOUP, A. and GASQUI, P. 1996. Kin recognition in honey bees. *Nature* 379:498.

BERGMAN, P., and BERGSTRÖM, G. 1997. Scent marking, scent origin and species specificity in male premating behavior of two Scandinavian bumble bees. *J. Chem. Ecol*. 23:1235–1251.

BERGSTRÖM, G., SVENSSON, B. G., APPELGREN, M., and GROTH, I. 1981. Complexity of bumble bee marking pheromones: Biochemical, ecological and systematical interpretations, pp. 175–183, P. E. Howse, and J. L. Clement, (eds.). in *Biosystematics of Social Insects*. Academic Press, London.

BREED, M. D. 1998. Recognition pheromones of the honey bee. *BioScience* 48:463–470.

CRUZE LANDIM, C. 1967. Estudo comparativo de algumas glandulas das abelhas (Hymenoptera, Apoidea) a respectivas imlicacoes evolutivas. *Arg. Zool*. 15:177–290.

GENIN, E., JULLIEN, R., PEREZ, F., FONTA, C., and MASSON, C. 1984. Preliminary results on the chemical mediators of the bumble bee *Bombus hypnorum. C. Rd. Acad. Sci. III*. 299:297–302.

HEFETZ, A., TAGHIZADEH, T., and FRANCKE, W. 1996. The exocrinology of the queen bumble bee *Bumbus terrestris* (Hymenoptera: Apidae, Bombini). *Z. Naturforsch*. 51C:409–422.

HOVORKA, O., URBANOVA, K., and VALTEROVA, I. 1998. Premating behavior of *Bombus confusus* males and analysis of their labial gland secretion. *J. Chem. Ecol*. 24:183–193.

HUANG, Z. Y., and ROBINSON, G. E. 1992. Honey bee colony integration: Worker–worker interactions mediate hormonally regulated plasticity in division of labor. *Proc. Natl. Acad. Sci*. U.S.A. 89:11726–11729.

HUANG, Z. Y., and ROBINSON, G. E. 1996. Regulation of honey bee division of labor by colony age demography. *Behav. Ecol. Sociobiol*. 39:147–158.

HUANG, Z. Y., PLETTNER, E., and ROBINSON, G. E. 1998. Effects of social environment and worker mandibular glands on endocrine-mediated behavioral development in honey bees. *J. Comp. Physiol*. 183:143–152.

KINDL, J., HOVORKA, O., URBANOVA, K., and VALTEROVA, l. 1999. Scent marking in male premating behavior of *Bombus confusus. J. Chem. Ecol*. 25:1489–1500.

KORST, P. J. A. M., and VELTHUIS, H. H. W. 1982. The nature of throphallaxis in honeybees. *Insects Soc*. 29:209–221.

KRONENBERG, S., and HEFETZ, A. 1984. Role of labial glands in nesting behaviour of *Chalicodoma sicola* (Hymenoptera: Megachilidae). *Physiol. Entomol*. 9:175–179.

KULLENBERG, B., BERGSTRÖM, G., and STALLBERG-STENHAGEN, S. 1970. Volatile components of the cephalic marking secretion of male bumble bees. *Acta Chem. Scand.* 24:1481–1483.

KULLENBERG, B., BERGSTRÖM, G., BRINGER, B., CALBERG, B., and CEDERBERG B. 1973. Observation of scent marking by *Bombus* Latr. and *Psithyrus* Lep. males (Hym. Apidae) and localization of site of production of the secretion. *Zoom. Suppl.* 1:23–30.

LAHAV, S., SOROKER, V., HEFETZ, A., and VANDER MEER, R. 1999. Direct behavioural evidence for hydrocarbons as ant recognition discriminators. *NaturwissenSchaften* 86:246–249.

MINCKLEY, R. L., BUCHMAN, S. L., and WCISLO, W. T. 1991. Bioassay evidence for a sex attractant pheromone in the large carpenter bee, *Xylocopa varipuncta. J. Zool*. 224:285–291.

MORITZ, R. F., and HILLESHEIM E. 1990. Trophallaxis and genetic variance of kin recognition in honey bees, *Apis mellifera* L. *Anim. Behav*. 40:641–647.

PAGE, R. E., JR. METCALF, R. A., METCALF, R. L., ERICKSON E. H., JR,. and LAMPMAN, R. L. 1991. Extractable hydrocarbons and kin recognition in honey bee (*Apis mellifera* L.). *J. Chem. Ecol*. 17:745–756.

ROBINSON, G. E., PAGE, R. E., STRAMBI, C., and STRAMBI A. 1989. Hormonal and genetic control of behavioral integration in honey bees colonies. *Science* 246:109–112.

SCHULTZ, D. J., HUANG, Z. H., and ROBINSON, G. E. 1998. Effects of colony food shortage on behavioral development in honey bees. *Behav. Ecol. Sociobiol*. 42:295–303.

SIMPSON, J. 1960. The function of the salivary glands of *Apis mellifera. J. Insect. Physiol*. 4:107–121.

SOROKER, V., and HEFETZ A. 2000. Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger. J. Insect Physiol*. 46:1097–1102.

SOROKER, V., VIENNE, C., NOWBAHARI, E., and HEFETZ, A. 1994. The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger. NaturwissenSchaften* 81:510–513.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

SEGREGATION OF COLONY ODOR IN THE DESERT ANT *Cataglyphis niger*

SIGAL LAHAV,[1] VICTORIA SOROKER,[1,3] ROBERT K. VANDER MEER,[2] and ABRAHAM HEFETZ[1,*]

[1]*Department of Zoology*
George S. Wise Faculty of Life Sciences, Tel-Aviv University
Ramat Aviv 69978, Israel

[2]*Center for Medical Agricultural and Veterinary Entomology*
US Department of Agriculture, Agricultural Research Service
1600 SW 23 Drive, Gainesville, Florida 32608

(Received September 19, 2000; accepted January 15, 2001)

Abstract—There are two separate, and presumably opposing, processes that affect colony odor in the desert ant *Cataglyphis niger*: (1) biosynthesis and turnover of these chemicals by individual ants, and (2) homogenization of colony odor through exchange of cues. The first increases signal variability; the latter decreases it. The impact of these factors was tested by splitting colonies and monitoring the profile changes occurring in the postpharyngeal glands (PPG) and cuticular hydrocarbons.

From each of two polygynous nests four daughter colonies were formed, three monogynous and one queenless. Thereafter, 10 ants from each were randomly selected each month, for three successive months, for analyses of their PPG and cuticular hydrocarbons. From two colonies we also obtained ants from a known matriline. Over time, there was a shift in hydrocarbon profiles of both the PPG and cuticular washes in each of the tested colonies. Moreover, by subjecting selected hydrocarbon constituents to a discriminant analyses based on their relative proportions, all of the daughter colonies (queenright and queenless) were distinguishable from each other and from their respective mother colonies. In each of the queenright daughter colonies, the queen profile was indiscriminable from that of the workers and often was in the center of the group. Full sisters were clearly distinguishable from their nestmates, emphasizing the genetic versus environmental processes that govern colony odor. The effect of time was always superior to the separation effect in contributing to odor segregation. Comparison of the Mahalanobis distances indicated that the shift in hydrocarbon seems to proceed along parallel lines rather than in divergence. However, there was no

*To whom correspondence should be addressed.
[3]Current address: Department of Entomology, Institute of Plant Protection, Volcani Center, Beit Dagan, Israel.

0098-0331/01/0500-0927$19.50/0

overt aggression between ants that originated from the different subgroups in dyadic encounters. It appears that in this species a three-month separation period is not sufficient to change the hydrocarbon profile beyond the recognition threshold.

Key Words—Hydrocarbons, *Cataglyphys niger*, nestmate recognition, postpharyngeal gland, gestalt.

INTRODUCTION

Studies pertaining to nestmate recognition have focused in recent years on the nature of the recognition cues and the modes by which colony odor is obtained. In ants, and to some extent in termites, we have indirect, correlative evidence on the role of cuticular hydrocarbons (HCs) as recognition cues (Bonavita-Cougourdan et al., 1987a,b, 1993). Direct evidence for the specific involvement of HCs, but not the more polar cuticular lipids, in interspecific recognition was obtained for *Reticulitermes* (Bagnères et al., 1991), and for intraspecific nestmate recognition in *Cataglyphis niger* (Lahav et al., 1999).

The finding that cuticular HC composition appears congruent with that of the postpharyngeal gland (PPG) (Bagnères and Morgan, 1991), has focused research on the role of this gland in nestmate recognition. PPG extracts were indeed able to modify aggressive behavior in two phylogenetically remote species, *Manica rubida* (Hefetz et al., 1996) and *C. niger* (Soroker et al., 1994). Using HCs as a model for recognition cues and *C. niger* as a model ant, it was further demonstrated that the PPG acts as a "gestalt organ" that enables the admixing and rapid integration of odors from various sources (Soroker et al., 1994, 1995b). This provided empirical evidence supporting the "gestalt model" for creation and acquisition of a general colony odor (Crozier and Dix, 1979). At the individual ant level, there is an exchange between epicuticle and PPG HCs through self-grooming, which explains the chemical congruency between them. This was also confirmed for *Camponotus vagus*, where deposition of (*Z*)-9-tricosene on the cuticle of a worker resulted in its occurrence in the PPG (Meskali et al., 1995). At the colony level, mutual exchanges of cuticular and PPG HCs between nestmates takes place through trophallaxis, allogrooming, and physical contact. This was determined for several species belonging to remote ant subfamilies, as well as for artificially mixed species groups (Soroker et al., 1995b). Evidence for HC exchange was also observed in a species of termite, but the exchange was achieved only through physical contact (Vauchot et al., 1996, 1998). Blending of individual odors into a uniform colony odor seems to be a system common to social insects.

There are two separate processes that presumably govern the chemical nature of the recognition signal within ant colonies: (1) biosynthesis and turnover of these chemicals by each individual in the colony, and (2) the homogenization of colony

odor through continuous exchange of recognition cues. These opposing factors affect the variability of the chemical signal. Individual production increases variability, whereas signal exchange decreases it. Signal production by each individual is probably genetically based, and we can assume that it remains qualitatively constant, although it has been shown in other ant species that quantitative changes (relative amounts) in cues can occur over time (Vander Meer et al., 1989; Provost et al., 1993; Boulay et al., 2000). In polygynous colonies variability in signal composition is increased by the cohabitation of several matrilines (and possibly several patrilines within each matriline). Despite this individual variability in signal composition, a unified colony odor is maintained by mutual exchanges of the signal among members of the colony. Because population composition of a given colony changes with time, we hypothesized that colony odor is dynamic and also changes with time. We tested this hypothesis by following the changes in HC profiles with time in polygynous mother colonies of *C. niger*, as well as their monogynous or queenless daughter colonies.

METHODS AND MATERIALS

Collection and Maintenance of Ant Colonies. Two polygyne colonies of *C. niger*, A (with 11 queens) and B (with 18 queens), were collected from the Tel-Aviv area, transferred to artificial nests in the laboratory and reared under controlled conditions ($28 \pm 2^{\circ}$C, 8L : 16D photoperiod). All colonies were provided with an identical diet of sugar water and minced insects three times a week. Three monogyne fragments and one queenless (QL) group (daughter colonies) were created from each of the two polygyne colonies (mother colonies) (Figure 1). The mother and daughter colonies were then separated for three months. Each original mother colony consisted of about 3000 ants, and each daughter colony of 250 ants. Before separation a random sample of 10 ants was taken from each of the mother colonies for chemical analyses of the PPG and cuticular washes. Thereafter, once a month, for three months, 10 ants were sampled from the mother colonies and from one of the daughter colonies for chemical analyses. The three other daughter nests were sampled after three months of separation. From two monogyne daughter colonies (A1 and A3) matriline workers were obtained. These were separated from their respective colony as pupae and the emerging adults (manually assisted) were analyzed separately at the age of seven days.

Chemical Analysis. Detailed chemical analyses of PPG and cuticular HC profiles have been published previously (Soroker et al., 1995a; Soroker and Hefetz, 2000). A sample from each mother colony was analyzed by GC-MS to verify that HC profiles from workers of the two mother colonies were qualitatively identical to previously published results.

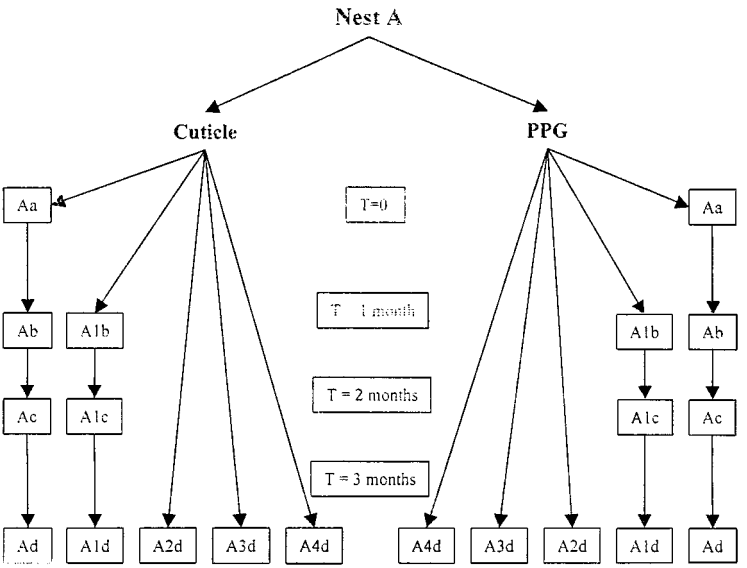


FIG. 1. Flow chart showing the procedures of nest separation and chemical analyses of PPG extract and cuticular washes of ants from a polygyne mother nest and its daughters' monogyne or queenless fragments. Abbreviations are as follows: Aa, mother nest at time 0, before the separation; Ab–Ad, mother nest after one, two, and three months, respectively; A1b–A1d, daughter nest after one, two, and three months, respectively; A2d, A3d, A4d, daughter colonies after three months of separation.

Extraction was accomplished by immersing dissected glands or isolated thoraces in 100 μl of pentane. Thoraces were extracted for 5 min only, to avoid internal contamination. Eicosane (750 ng) was added to each as an internal standard. Samples were run on a Varian 3700 gas chromatograph (Varian Analytical Systems, Walnut Creek, California) equipped with a split/splitless injector, a flame ionization detector, a Leap Technologies autosampler, and a fused silica capillary column (30-m DB-1, 0.32 mm ID, 0.25-μm film thickness capillary column; J&W Scientific Incorporated, Folsom, California). The oven was temperature programmed from 120°C to 285°C at 5°C/min. The carrier gas was hydrogen and the make-up gas nitrogen. Quantification of the various components was achieved by peak integration in comparison to the internal standard using the TurboChrome Workstation, software version 6.1.0.1:F04 (Perkin Elmer Corporation, Norwalk, Connecticut).

Statistical Analysis. To assure equal treatment of the data we selected 18 HC peaks (out of a total of 72) that could be accurately and reliably quantified (Figure 2) and performed a canonical discriminant analysis, using the relative proportions of each of the above-mentioned peaks. This analysis takes a classification variable (mother or daughter colony) and quantitative variables (the selected

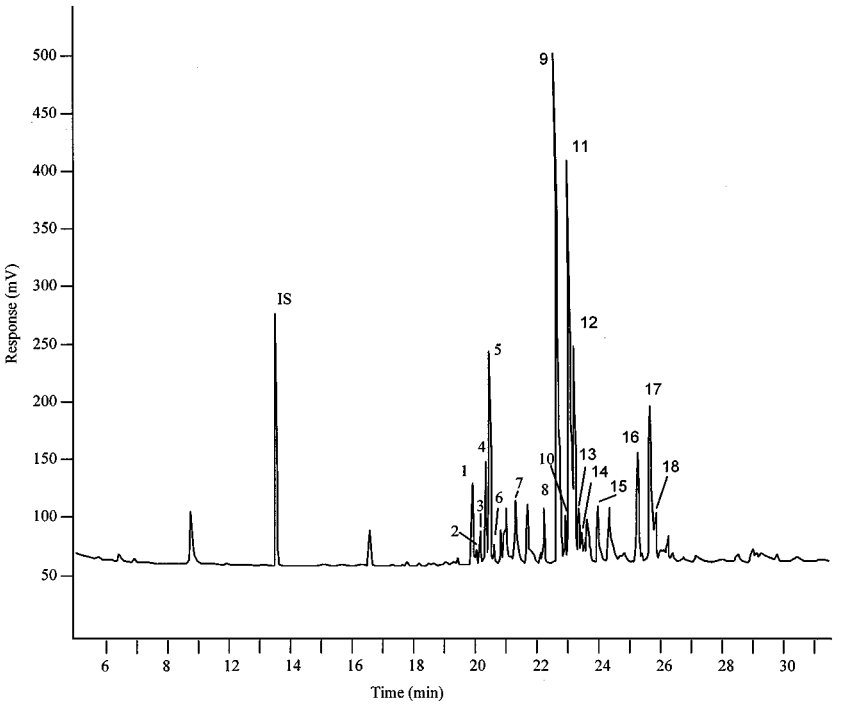


FIG. 2. Gas chromatogram of PPG secretion of *C. niger* workers delineating the peaks used in the discriminant analyses to assess the effects of time and social environment on hydrocarbon profile expression. Peak numbers are designated as follows: 1, 13- + 11-methylheptacosane; 2, 7-methylheptacosane; 3, 5-methylheptacosane; 4, 11,15-dimethylheptacosane; 5, 3-methylheptacosane; 6, 5, *x*-dimethylheptacosane; 7, 12-methyloctacosane; 8, *n*-nonacosane; 9, 9- + 11- + 13- + 15-methylnonacosane; 10, 5-methylnonacosane; 11, 11, 15- + 11, 17- + 11, 19- + 9, 13-dimethylnonacosane; 12, 3-methylnonacosane; 13, 5, 13-dimethylnonacosane + 5, 9-dimethylnonacosane; 14, *x*, *y*, *z*-trimethylnonacosane; 15, *x*, *y*-dimethyltriacontane; 16, 11- + 13- + 15-methylhentriacontane; 17, 11, 15-dimethylhentriacotane; 18, 7, 15-dimethylhentriacontane.

18 peaks from each chromatogram) and derives a canonical variable that summarizes between-class variation. The statistical analysis was performed using Statistica 5.0 for IBM.

Behavior Assays. Behavioral assays were carried out between workers of the subgroups after three months of separation, to test whether the separation had an affect on nestmate recognition. Dyadic encounters between workers from a daughter colony and workers from their respective mother colony were conducted ($N = 19$ for each colony). Encounters between nestmates from the daughter as well as the mother colony served as a control ($N = 15$ for each of the four respective

colonies). The behavior of the ants was registered every 3 min for the first 3 hr of the encounter. The protocols for registering the behavior and construction of the aggression index were as previously published (Lahav et al., 1999).

RESULTS

Figures 3–6 present the results of discriminant analyses performed on the HC profiles (based on the selected 18 peaks) for colonies A and B. In general, the within-group compositions of cuticular extracts were more heterogeneous than those of the PPG extracts. Consequently, the groups were less separated using cuticular hydrocarbons when compared to analyses using PPG extracts. This was expressed by the consistently higher values of Wilk's lambda (a measure of the extent of separation among the groups tested and displayed in each of the discriminant analysis graphs). Nonetheless, in all cases the between-group separation was significant ($P < 0.001$, ANOVA) for both the PPG and cuticular extracts. Figure 3 depicts the differences in HC profiles of PPG and cuticle, separately, of the mother colonies A and B and their daughter fragments. In both nests, regarding the HC profiles of PPG, the mother colonies were clearly separated from the daughter colonies. Separation between daughter colonies was less pronounced, with some overlap of the 95% confidence limit ellipsoids. The between-group separations, however, were significant and the centroids were clearly distinct. Two points should be emphasized: (1) the ants from the queenless daughter fragment were clustered and well separated from the queenright daughter fragments, suggesting that their homogeneity was not inferior to that of the queenright daughter fragments; and (2) queens (marked with an arrow and the letter Q in Figures 3, 5, and 6) of the queenright daughter colonies were often the closest to the group centroid (except for the queens of daughter colonies 1 and 2 for colonies A and B, respectively). Although the homogeneity of the cuticular profiles was less apparent, albeit the between-group separation was significant, the same trends as seen for the PPG occurred.

Figures 4 and 5 depict temporal changes in the HC pattern of both mother and daughter colonies for which ants were sampled and analyzed monthly. In these figures $T = 0$ is representative of the HC profile (PPG and cuticle, respectively) of the mother colonies A and B before the queenright and queenless fragments were created. Figure 4 shows a clear shift in PPG HC profiles of ants sampled from the mother colonies at each of the time points analyzed. Although the direction of changes can not be determined, it can be seen that the changes in colony A were of similar magnitude every month. In colony B the changes were not as symmetrical, and those between months 2 and 3 were slightly less pronounced. Cuticular profiles also changed with time in the mother colonies, but again this was less pronounced. Similar changes with time were observed in the two queenright daughter colonies (Figure 5) for which monthly samples of ants were analyzed.

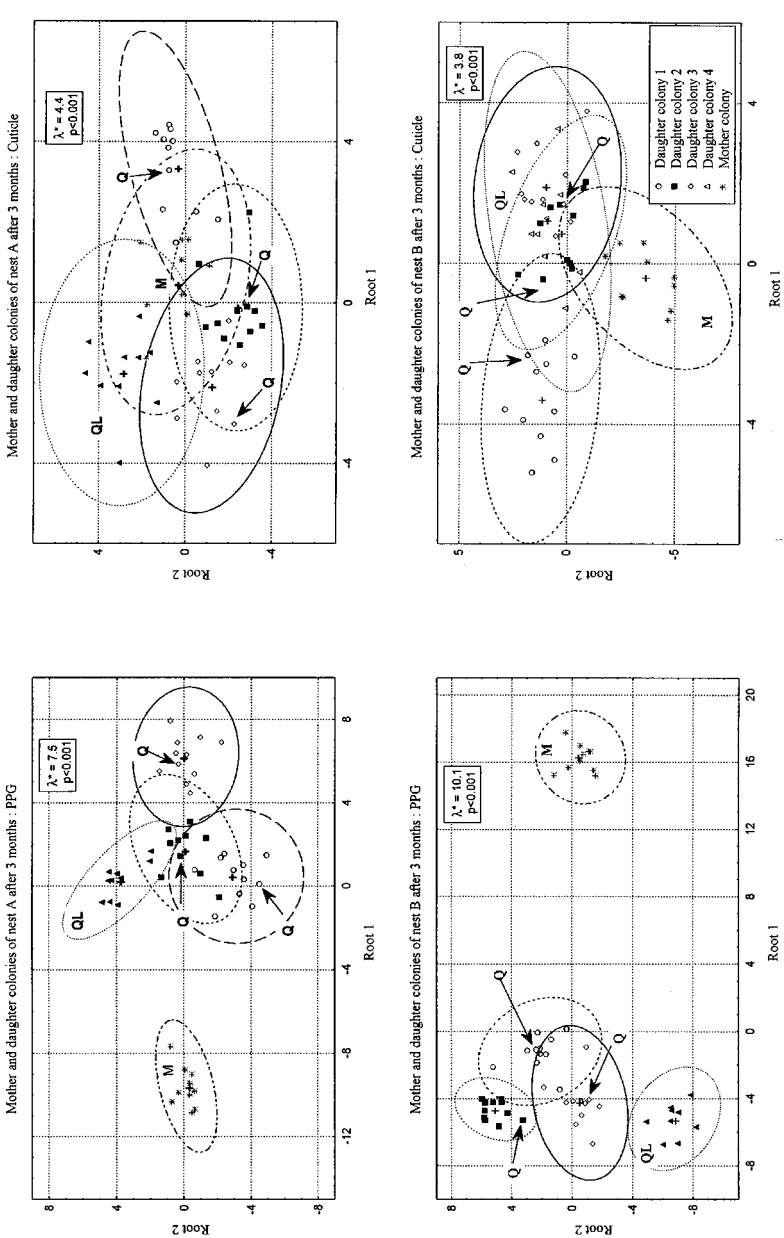


FIG. 3. Signal segregation estimated by a discriminant analysis based on hydrocarbon profiles of the polygyne mother nests and the monogyne queenright or queenless daughter colonies after three months of separation. The ellipses around each group delineate the 95% confidence limits. λ^* was calculated from Wilk's λ after Ln $1/\lambda$ transformation for linearity. The centroid of each group is marked by a plus sign. The HC profile of the queen in each of the queenright daughter groups is marked by an arrow and the letter Q. The queens were considered as a member of the appropriate daughter group for the discriminant analyses.

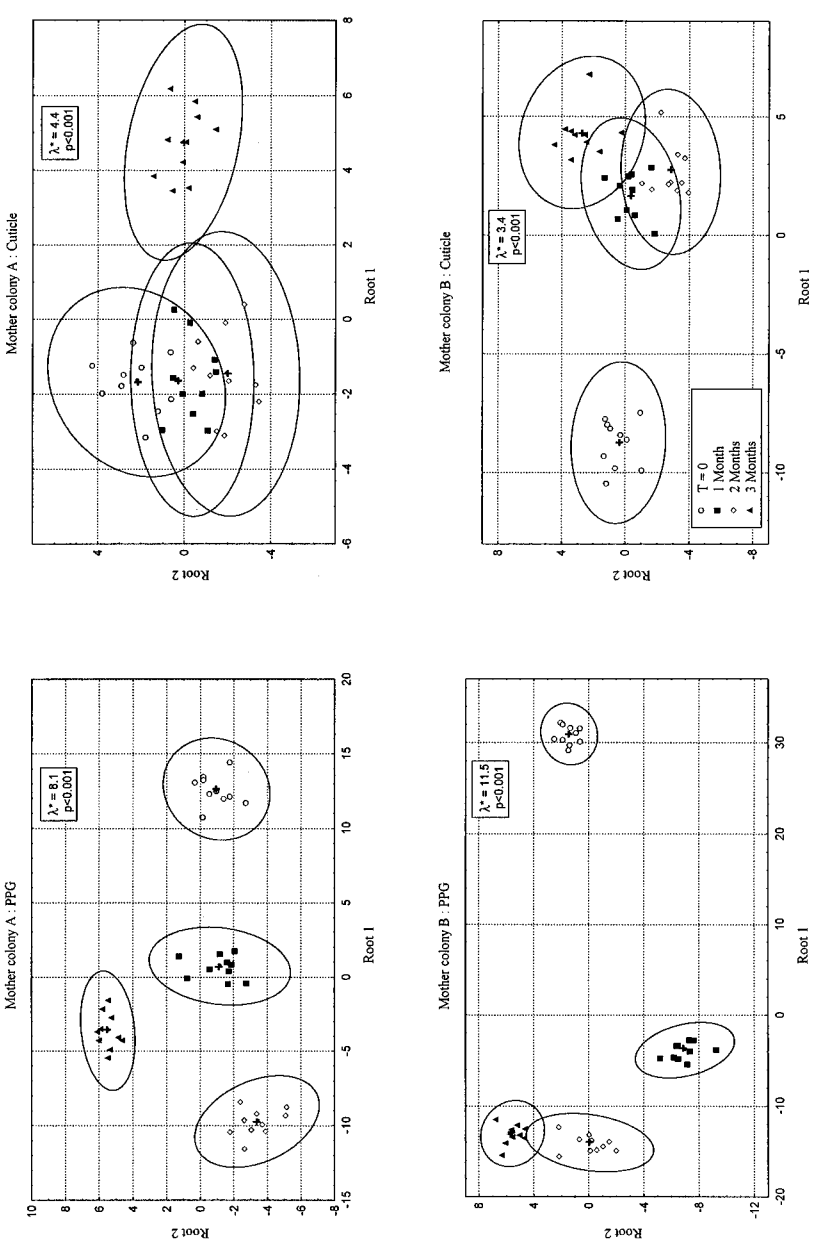


FIG. 4. Time-dependent signal segregation estimated by a discriminant analysis based on hydrocarbon profiles of the polygyne mother nests A and B. $T = 0$ represents the mother colony before it was split. Thereafter the mother nests were sampled after one, two, and three months. The centroid of each group is marked by a +.

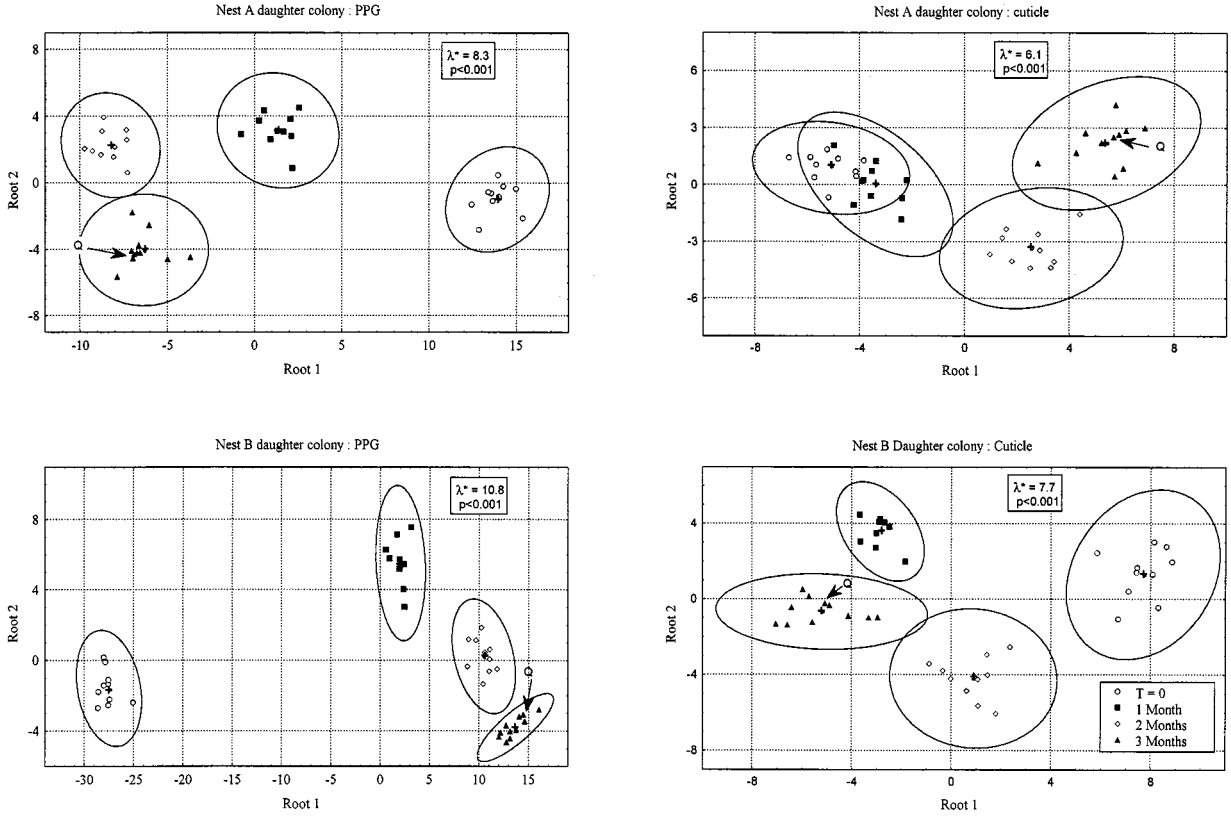


FIG. 5. Time-dependent signal segregation estimated by a discriminant analysis based on hydrocarbon profiles of the monogyne daughter nests. $T = 0$ represents a sample of the mother colonies at the onset of the experiment before separation. Thereafter, the daughter colonies were sampled after one, two, and three months. The centroid of each group is marked by a plus sign. The HCs profile of the queen in each of the queenright daughter groups is marked by an arrow and the letter Q. The queens were considered as a member of the appropriate daughter group for the discriminant analyses.

In this case separation of both PPG profiles and cuticular profiles was good. For these colonies the queen was killed at the end of three months. Both queen HC profiles (PPG and cuticle) were near the centroid (Figure 5).

Figure 6 shows HC profile similarities between a group of matrilines and their randomly selected nestmates. In this figure we include the profile of ants from the mother colonies at time 0 as a reference point. As can be seen, the matriline HC profiles are grouped together and are distinctly separated from their nestmate. For both cuticle and PPG HC profiles the corresponding colony queen profile of each colony was positioned in between the profiles of the matriline and colony worker groups.

The above discriminant analyses represent a combined effect of the changes with time and the separation from the mother colony. In order to investigate the relative impact of each of these factors we compared the mean squared Mahalanobis distance (MSMD) between the HC profile of one group and the centroid of a second group (Table 1). Greater MSMD corresponds to larger deviations of the HC profiles between the groups. For example, the MSMD between group Aa (mother colony at $T = 0$) and the centroid of group A1b (daughter colony 1 after 1 month) represent the divergence of the daughter colony profile after one month from that of the mother colony at $T = 0$. This represents the combined effect of colony separation and the time factor. On the other hand, comparing Aa with Ab (mother colony after one month) represents the effect of time only on profile divergence. Likewise, comparing the profiles of Ab and A1b (mother and daughter colonies respectively after one month) represents the effect of group separation on profile divergence. Table 1 presents the results of such analyses over the three-month period of the experiment for colonies A and B. For both colonies the MSMD was consistently highest when both time and separation were taken into account. Of the two factors, time was the most influential in all cases, as revealed by its larger MSMD, when compared to the separation factor. The fact that the physical separation between the colonies caused the lowest shift in HC profiles suggests that the profiles of each group changed in a similar way, i.e., it reflects the genetic similarity between the groups. To test this hypothesis, the MSMD was plotted as a function of the time of separation for these factors (time and separation) alone or combined. As depicted in Figure 7, the lowest slope is for the separation factor, and for colony B there was no slope at all. Although the sample size was small, these results suggest that, although each of the colony HC profiles changed with time, the magnitude of the changes was similar rather than divergent.

Behavioral Assays. In all the encounters performed, aggressive behavior was rare and limited to mandibular opening. The level of aggression of workers from the mother colony towards workers from the daughter colony was 0.08 ± 0.03. The aggression of workers from the daughter colonies towards workers from the mother colonies was similarly low, amounting to 0.11 ± 0.04. Neither was statistically

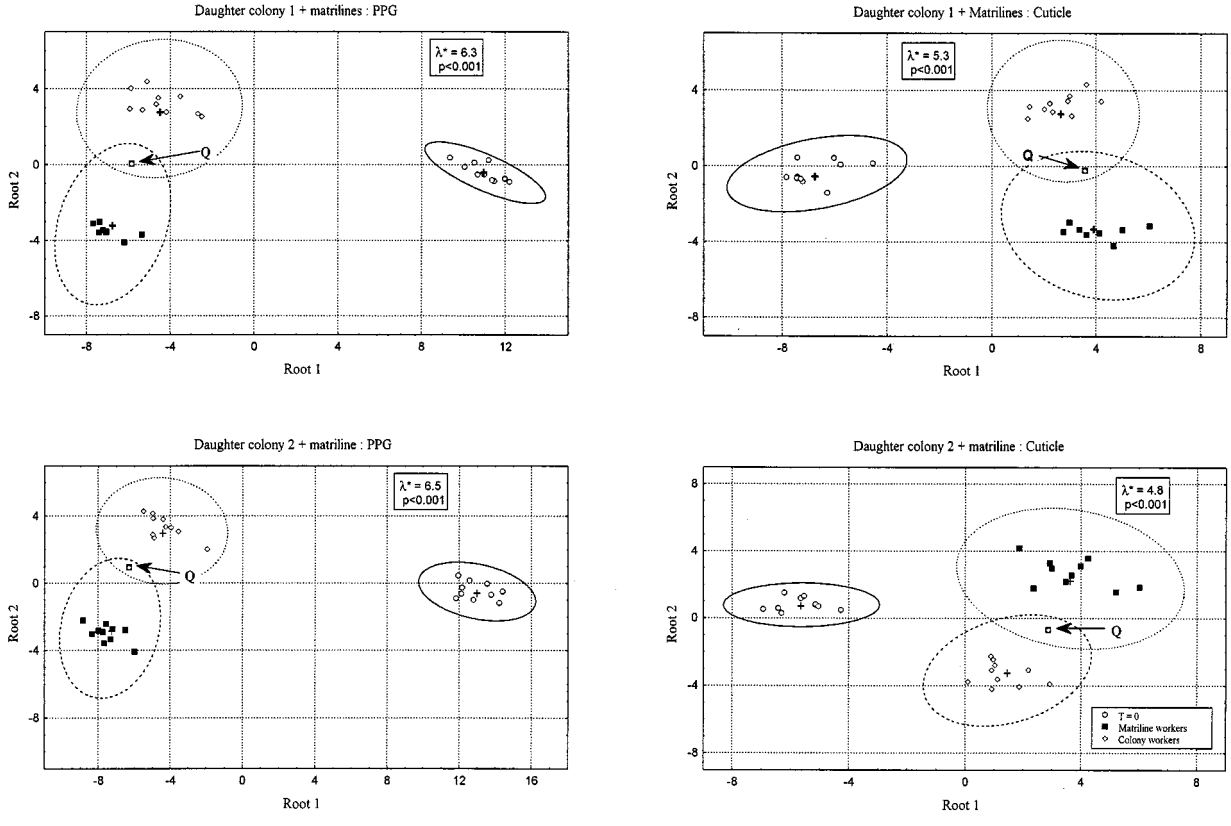


FIG. 6. Signal segregation estimated by a discriminant analysis based on hydrocarbon profile of the polygyne mother nest A at $T = 0$ and two of its monogyne daughter colonies (1 and 2, respectively). The daughter colonies constituted two groups—random workers that originated from the polygyne mother colony and matriline workers that were laid by the respective queens and that emerged in isolation.

TABLE 1. EFFECT OF GROUP SEPARATION AND TEMPORAL CHANGES ON HC PROFILE DIVERGENCE IN *C. niger* WORKERS[a]

Group	Factors affecting profile divergence	Time of separation (mo)	Mean squared Mahalanobis distance	
			PPG	Cuticle
Nest A				
Aa-Alb	Time + separation		139.7 ± 27.1	27.1 ± 10.1
Aa-Ab	Time	1	88.1 ± 17.4	24.1 ± 7.6
Ab-Alb	Separation		44.4 ± 12.4	33.9 ± 25.8
Aa-Alc	Time + separation		280.5 ± 23.5	33.6 ± 8.5
Aa-Ac	Time	2	222.2 ± 24.6	24.7 ± 10.0
Ac-Alc	Separation		124.7 ± 18.3	22.4 ± 10.8
Aa-Ald	Time + separation		236.5 ± 27.6	64.1 ± 11.6
Aa-Ad	Time	3	177.4 ± 22.3	41.8 ± 9.4
Ad-Ald	Separation		103.7 ± 6.3	30.8 ± 18.7
Nest B				
Ba-Blb	Time + separation		767.7 ± 52.6	64.1 ± 11.6
Ba-Bb	Time	1	474.5 ± 40.6	68.9 ± 23.7
Bb-Blb	Separation		83.3 ± 28.7	46.1 ± 16.6
Ba-Blc	Time + separation		1108.0 ± 56.9	67.3 ± 24.4
Ba-Bc	Time	2	852.9 ± 51.6	76.4 ± 23.5
Bc-Blc	Separation		54.7 ± 13.6	25.3 ± 9.1
Ba-Bld	Time + separation		1133.8 ± 58.6	101.4 ± 28.1
Ba-Bd	Time	3	869.9 ± 49.8	104.9 ± 30.0
Bd-Bld	Separation		71.2 ± 21.6	37.9 ± 23.7

[a] Values are expressed a mean squared Mahalanobis distance (MSMD), obtained from the discriminant analyses, between individuals of selected groups and the centroid of the appropriate second group.

different from aggression between nestmates, which equaled 0.13 ± 0.04 (pooled data from the mother and daughter colonies; Mann-Whitney $P = 0.38$).

DISCUSSION

The temporal chemical analyses revealed that HC profiles of colonies of *C. niger* appear to shift with time. This was true irrespective of number of queens in the colony, polygyne, monogyne, or queenless. The results corroborate earlier findings in several ant species, e.g., *Solenopsis invicta* (Vander Meer et al., 1989), *Leptothorax lichtensteini* (Provost et al., 1993), *Cataglyphis iberica* (Dahbi and Lenoir, 1998), and *Formica truncorum* (Liebig et al., 2000). Since the rearing conditions were constant during the three months of separation and only a few new workers emerged, we can exclude the possibility that changes in HC profiles over time are due to the physical environment and/or population changes. The ants were sampled at random and were all well matured workers, also excluding a possible

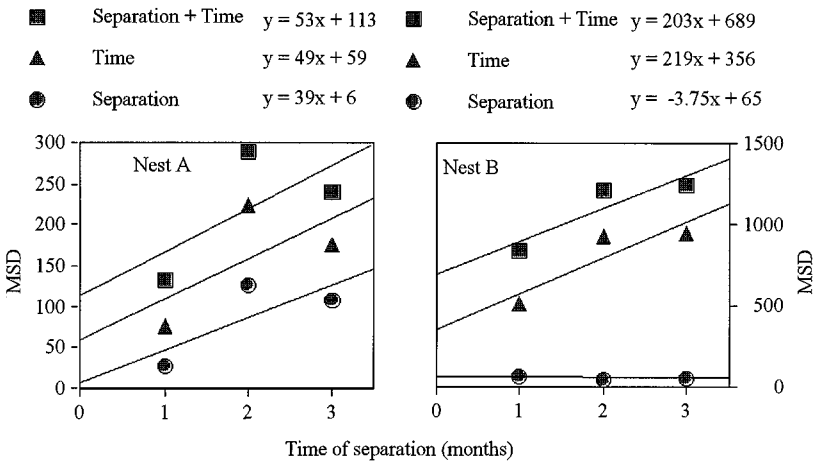


FIG. 7. Changes in Mahalanobis distance as a function of time of separation. The slopes delineate the effects of the factors time and separation combined, time alone, and separation alone.

age effect on the HC composition. We suggest, rather, that these changes are the result of genetically based changes in HC expression. These are not qualitative differences, i.e., disappearance or appearance of specific compounds, but quantitative changes expressed as shifts in the relative intensities of existing compounds. Because in polygyne *C. niger* genetic heterogeneity is large, it was predicted that individual differences in HC composition would create divergent odor compositions in the individuals within a group; however, within-group homogeneity was clear for all groups.

The PPG has been postulated to be the nestmate recognition "gestalt" organ (Soroker et al., 1994, 1995b), where heritable and environmental contributors to colony odor (including recognition cues) are continuously blended and dispersed through trophallaxis. Recent time-course experiments revealed that at any one time the PPG content is more mobile and changes faster than that of the cuticle and that the rate of PPG content change is dependent on the rate of trophallaxis exhibited by the species (Lenoir et al., 2001). Our data clearly support a "gestalt" organ function for the PPG. Similar results were obtained in *C. iberica*, where groups of workers separated from their mother nest for five months exhibited differences in their PPG HC profiles, while remaining homogenous within each group (Dahbi and Lenoir, 1998). It was also demonstrated in this species that when isolated groups of nestmates are reunited, trophallaxis between groups was significantly higher than trophallaxis within group (Dahbi et al., 1999), which would be expected to lead to homogenization of the recognition cues associated with each of the reunited groups.

A recent study corroborates the assumption that changes in HC compositions are individual and may lead to divergent odor composition. Worker *Camponotus fellah* ants that were isolated for up to 40 days showed a higher mean Euclidean distance between individual HC profiles than their nestmates that were reared in groups (Boulay et al., 2000). In our study the matriline ants were reared separately and each worker could not exchange HCs with any of the others. Nonetheless, they showed within-group homogeneity as good as groups that had the opportunity for social interactions (grooming and trophallaxis). The newly eclosed ants were reared under consistent conditions excluding all possible environmental influence. This suggests that, within the timeframe of our experiment (seven days after eclosion), heritable cuticular HC patterns change in parallel rather than at random. It is unknown whether *C. niger* mates with only one male or with several; however, our results suggest that matrilines in *C. niger* have high genetic relatedness.

The HC composition of the PPG was more homogenous within groups, and between-group separation was better than the corresponding cuticular HC samples. Quantitatively, the cuticle contains larger amounts of *n*-alkanes, in particular *n*-nonacosane, than are present in the PPG (Soroker and Hefetz, 2000). Thus, there is no direct quantitative correspondence between HCs derived from the cuticle and those derived from the PPG. To date, behavioral bioassays have shown a causative nestmate recognition role for total PPG HCs (Lahav et al., 1999), but the role of the specific types of HCs in nestmate recognition is unknown. Therefore, we can not assess whether or not the differences in PPG and cuticular homogeneity have any consequences on nestmate recognition. This indicates that the controlling mechanisms are different for the two systems. We suggest that interaction occurs between the PPG and cuticular lipids (monitored through HC analysis), analogous to an environment/gene interaction.

How the cuticular HC profiles are influenced by the PPG contents is complex and not well understood. There are external and internal mechanisms for the PPG to interact with cuticle chemical profiles. Transfer of material between body surfaces of nestmates is done mainly by allogrooming and/or by self-grooming that takes place after trophallactic exchanges (Soroker et al., 1995b). Internally, the PPG (the "gestalt" organ) has been shown to obtain hydrocarbons from the hemolymph (Soroker et al., 1995b), presumably via lipophorin transport; however, there may be active bidirectional exchange, thus permitting the hydrocarbon content of the PPG to influence the hydrocarbon pattern on the cuticle. The contents of the PPG as colony blend subject to constant updating can be considered as a continuous environmental impactor on the individual worker's heritable cuticular hydrocarbon profile. Our results demonstrate that in this environment (PPG)–genetic (an individual's heritable cuticular hydrocarbon profile) interaction is not perfect, since cuticular hydrocarbon variability is greater than PPG hydrocarbon variability; however, the observed variability is not great enough to cause a behavioral reaction (aggression). The "gestalt" PPG hydrocarbon pool influences, but does not dictate

the hydrocarbon composition on the cuticle. Qualitative and quantitative selectivity may occur with lipophorin transport and/or at the site of hydrocarbon uptake at the epicuticle, but the main point is that the cuticle hydrocarbon profile does not have to mimic the PPG composition. The position of the queen's profile in relation to that of her matriline workers and that of her randomly selected nestmates is especially interesting. In both cases the queen's profile was positioned in-between the two worker groups and was almost completely congruent with one of the matriline ants. This illustrates well the factors affecting colony odor composition in the polygyne *C. niger*. Genetic influences create within-matriline uniformity but multiple matrilines yield within-colony heterogeneity, while the continual nondiscriminatory cue exchanges between all workers eventually results in a uniform colony odor.

The contribution of the queen to the changes in HC profile can be deduced indirectly by comparing the monogyne and queenless daughter colonies. The results revealed homogeneity within each daughter colony, whether queenright or queenless. This finding differs from previously reported observations on the queen effect on HC uniformity in *Formica sp.* workers (Yamaoka and Kubo, 1990), and highlights interspecific variability and the dangers of generalization. In *C. niger* we have demonstrated by biochemical means that the direct impact of queen on the composition of colony odor and on nestmate recognition is minor. In fact, the queen donates smaller amounts of HCs than she gets in trophallactic exchanges, which, combined with the very low HC biosynthesis rate exhibited by queen, results in her always being in the center of the gestalt (Lahav et al., 1999). This hypothesis is supported by the present study's findings that queen profile was often in the center of the group.

Divergence in HC profiles did not affect nestmate recognition. Workers separated for three months still reacted to each other as nestmates despite measurable differences in HC patterns. This suggests that the observed differences are still within the cue variation that normally occurs in *C. niger* colonies. Interestingly, in encounters between workers from the separated subcolonies, there were some cases of mutual transport. This behavior was never observed between nonseparated nestmates, indicating that the separated workers may have sensed chemical differences that were not drastic enough to elicit aggression, but were sufficient to induce carrying behavior. A similar phenomenon was found in *C. iberica*, where, after hibernation, transport of callow workers could be correlated with chemical disparity (Dahbi et al., 1997).

Recognition mechanisms involve an interplay between the label (recognition cues) and the mechanism for its detection and interpretation, i.e., the template. It follows that the temporal changes in recognition cues (HCs in the case of *C. niger*) must also affect the characteristics of the template. While the nature of this template is still obscure, we can spectulate on the consequences of the above-shown change in HC profiles. Recognition based on matching self-odor or nestmate odor with the

odor of the encountered ant is presumably not affected by our observed temporal shifts in odor. The effective formation of a uniform colony odor on the resident as well as its nestmates buffers these changes, resulting in congruence between the individual odor and that of nestmates at all times. If the template is neural, the temporal changes in referent (cues), regardless of the referent source, self or nestmate, should also be reflected in neural plasticity with regards to the template. Although we only monitored the changes in HC profiles at monthly intervals, we can assume that these changes are continuous rather than abrupt. This, coupled with the continuous homogenization within the colony, should facilitate the constant updating of the experience-based neural template despite the odor shifts within the colony, as demonstrated in this work.

REFERENCES

BAGNÈRES, A. G., and MORGAN, E. D. 1991. The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 47:106–111.

BAGNÈRES, A. G., KILLIAN, A., CLÉMENT, J. L., and LANGE, C. 1991. Interspecific recognition among termites of the genus *Reticulitermes*: Evidence for a role for the cuticular hydrocarbons. *J. Chem. Ecol.* 17:2397–2420.

BONAVITA-COUGOURDAN, A., CLÉMENT, J .L., and LANGE, C. 1987a. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus* Scop. *J. Entomol. Sci.* 22:1–10.

BONAVITA-COUGOURDAN, A., CLÈMENT, J. L., and LANGE, C. 1987b. Subcaste discrimination in the ant *Camponotus vagus* Scop., p. 475, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Proceedings of the Tenth International Congress of the International Union for the Study of Social Insects, Munich, 1986. Verlag J. Peperny, Munich.

BONAVITA-COUGOURDAN, A., CLÈMENT, J. L., and LANGE, C. 1993. Functional subcaste discrimination (foragers and brood-tenders) in the ant *Camponotus vagus* Scop.: Polymorphism of cuticular hydrocarbon patterns. *J. Chem. Ecol.* 19:1461–1477.

BOULAY, R., HEFETZ, A., SOROKER, V., and LENOIR, A. 2000. *Camponotus fellah* colony integration: Worker individuality necessitates frequent hydrocarbon exchanges. *Anim. Behav.* 59:1127–1133.

CROZIER, R. H., and DIX, M. W. 1979. Analysis of two genetic models for the innate components of colony odour in social Hymenoptera. *Behav. Ecol. Sociobiol.* 4:217–224.

DAHBI, A., and LENOIR, A. 1998. Nest separation and dynamics of the Gestalt odor in the polydomous ant *Cataglyphis iberica* (Hymenoptera, Formicidae). *Behav. Ecol. Sociobiol.* 42:349–355.

DAHBI, A., CERDÁ, X., HEFETZ, A., and LENOIR, A. 1997. Adult transport in the ant *Cataglyphis iberica*: A means to maintain a uniform colonial odour in a species with multiple nests. *Physiol. Entomol.* 22:13–19.

DAHBI, A., HEFETZ, A., CERDA, X., and LENOIR, A. 1999. Trophallaxis mediates uniformity of colony odor in *Cataglyphis iberica* ants (Hymenoptera, Formicidae). *J. Insect Behav.* 12:559–567.

HEFETZ, A., ERRARD, C., CHAMBRIS, A., and LE NÈGRATE, A. 1996. Postpharyngeal gland secretion as a modifier of aggressive behavior in the myrmicine ant *Manica rubida*. *J. Insect Behav.* 9:709–717.

LAHAV, S., SOROKER, V., HEFETZ, A., and VANDER MEER, R. K. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaflen* 86:246–249.

LENOIR, A., CUISSET, D., and HEFETZ A. 2001. Effects of social isolation on hydrocarbons pattern and nestmate recognition in the ant *Aphaenogaster senilis. Insectes Soc.* In press.

LIEBIG, J., PEETERS, C., OLDHAM, N. J., MARKSTADTER, C., and HOLLDOBLER, B. 2000. Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? *Proc. Natl. Acad. Sci. U.S.A.* 97:4124–4131.

MESKALI, M., BONAVITA-COUGOURDAN, A., PROVOST, E., BAGNÈRES, A. G., DUSTICIER, G., and CLÉMENT, J. L. 1995. Mechanism underlying cuticular hydrocarbon homogeneity in the ant *Camponotus vagus* (Scop.) (Hymenopetra: Formicidae): Role of postpharyngeal glands. *J. Chem. Ecol.* 21:1127–1148.

PROVOST, E., RIVIERE, G., ROUX, M., MORGAN, E. D., and BAGNÈRES, A. G. 1993. Change in the chemical signature of the ant *Leptothorax lichtensteini* Bondroit with time. *Insect. Biochem. Mol. Biol.* 23:945–957.

SOROKER, V., and HEFETZ, A. 2000. Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger. J. Insect Physiol.* 46:1097–1102.

SOROKER, V., VIENNE, C., HEFETZ, A., and NOWBAHARI, E. 1994. The postpharyngeal gland as a "gestalt" organ for nestmate recognition in the ant *Cataglyphis niger. Naturwissenschaflen* 81:510–513.

SOROKER, V., HEFETZ, A., COJOCARU, M., BILLEN, J., FRANKE, S., and FRANCKE, W. 1995a. Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger. Physiol. Entomol.* 20:323–329.

SOROKER, V., VIENNE, C., and HEFETZ, A. 1995b. Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera, Formicidae). *J. Chem. Ecol.* 21:365–378.

VANDER MEER, R., SALIWANCHIK, D., and LAVINE, B. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta.* Implications for nestmate recognition. *J. Chem. Ecol.* 15:2115–2125.

VAUCHOT, B., PROVOST, E., BAGNÈRES, A. G., and CLÉMENT, J. L. 1996. Regulation of the chemical signatures of two termite species, *Reticulitermes santonensis* and *Reticulitermes lucifugus grassei*, living in mixed experimental colonies. *J. Insect Physiol.* 42:309–321.

VAUCHOT, B., PROVOST, E., BAGNÈRES, A. G., RIVIÈRE, G., ROUX, M., and CLÉMENT, J. L. 1998. Differential adsorption of allospecific hydrocarbons by cuticles of two termite species, *Reticulitermes santonensis* and *R. lucifugus grassei*, living in mixed colony. *J. Insect Physiol.* 44:59–66.

YAMAOKA, R., and KUBO, H. 1990. Identity of cuticular hydrocarbon profile among workers of the ant which is maintained by the presence of the queen would be the nestmate recognition cue. pp. 406–407, *in* G. K. Veeresh, B. Mallik and C. A. Viraktamath (eds.). Social Insects and the Environment: Proceeding of the 11th International Congress of the IUSSI. Oxford & IBH Publishing, New Delhi.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

ABSOLUTE CONFIGURATION OF ANABASINE FROM *Messor* AND *Aphaenogaster* ANTS

SABINE LECLERCQ,[1] SEBASTIEN CHARLES,[1] DESIRE DALOZE,[1] JEAN-CLAUDE BRAEKMAN,[1,*] SERGE ARON,[2] and JACQUES M. PASTEELS[2]

[1]*Laboratory of Bio-organic Chemistry*
Department of Organic Chemistry, CP 160/07
[2]*Laboratory of Cellular and Animal Biology, CP 160/12*
Free University of Brussels
Av. F. D. Roosevelt
50 - 1050 Brussels, Belgium

(Received June 28, 2000; accepted January 3, 2001)

Abstract—A method has been developed to assign the absolute configuration and enantiomeric excess of anabasine based on small amounts of material (in the microgram range), by derivatization with (+)-menthylchloroformate followed by capillary GC analysis of the resulting carbamate(s). This method was applied to three samples of anabasine isolated from *Messor* and *Aphaenogaster* ants. In *Messor sanctus*, only (2′*S*)-anabasine was present, whereas in *Aphaenogaster subterranea* and *A. miamiana* (2′*S*)-anabasine was determined to have an ee of 78 and 24%, respectively.

Key Words—Alkaloids, anabasine, ants, *Messor*, *Aphaenogaster*, absolute configuration.

INTRODUCTION

The alkaloid anabasine (**1**) has been isolated from several plant genera, such as *Anabasis*, *Nicotiana*, *Duboisia*, *Malacocarpus*, and *Marsdenia* (Leete, 1983). (−)-Anabasine ($[\alpha]_D^{20}$ −82.2, neat) from *Anabasis aphylla* L. was shown to have the (2′*S*) absolute configuration (Lukes et al., 1962) (Figure 1), but values of the optical rotation of anabasine from different sources vary considerably (from −82.2 to 0) (Leete, 1983). More recently, anabasine has also been reported from animal sources, e.g., in two of the 19 species of hoplonemertine worms surveyed

* To whom correspondence should be addressed. e-mail: braekman@ulb.ac.be

0098-0331/01/0500-0945$19.50/1

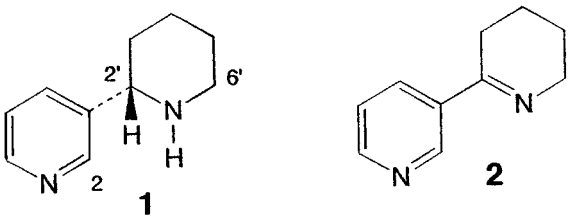


FIG. 1. Structures of (2′*S*)-(−)-anabasine [(2′*S*)-**1**] and anabaseine (**2**).

(Kem, 1988), in the poison gland of the ants *Messor ebeninus* (Coll et al., 1987) and *M. bouvieri* (Jackson et al., 1989), and as a minor component in *M. capensis* (Brand and Mpuru, 1993) and *Aphaenogaster rudis*, where it coexists with anabaseine (**2**) (Figure 1) (Attygalle et al., 1998).

From a biosynthetic point of view, it would be interesting to compare the absolute configuration of anabasine (**1**) isolated from plants and from animals (worms and ants). To this end, an effective and practical procedure to assign the absolute configuration of this alkaloid and applicable to quantities in the microgram range was needed. Although the separation of the enantiomers of anabasine as their trifluoroacetyl derivatives on a dipentyl β-cyclodextrin GC column has been reported (Armstrong et al., 1990), this method is not very convenient since this column is not commercially available. Our attempts to separate the anabasine enantiomers on two commercial chiral columns (CP-Chirasil Val and CP-Chirasil-Dex CB) were unsuccessful as the alkaloids did not elute from these columns at the temperature limit. In this paper, we have developed a method based on the reaction of (±)-anabasine with (+)-menthylchloroformate, affording the corresponding menthoxycarbonyl derivatives, followed by comparison of their retention time in capillary gas chromatography with that of a standard of known absolute configuration. This procedure allowed us to assign the absolute configuration and to measure the ee of anabasine (**1**), which was detected in three ant species: *Messor sanctus*, *Aphaenogaster subterranea*, and *A. miamiana* (Myrmicinae: Myrmicini).

METHODS AND MATERIALS

Sample Collection. Colonies of *Messor barbarus* and *M. sanctus* were collected at Port Leucate (southern France). Colonies of *Messor structor* were collected in the area of Toulouse (southern France). Colonies of *Aphaenogaster subterranea* were collected in Bouconne forest (Midi-Pyrénées), whereas *Aphaenogaster senilis* were collected at Consprons (Banyuls-sur-Mer, southern France). Colonies of *Aphaenogaster miamiana* were collected near Gainesville, Florida.

Analyses of Ants for Alkaloids. Ants were separated manually from the soil and dipped into MeOH. Five hundred ants of each species were then exhaustively

extracted with $CHCl_3$–MeOH (1 : 1). The extract was evaporated under vacuum and submitted to filtration on an alumina column, first eluting with CH_2Cl_2 (10 ml) to remove nonpolar compounds, then with CH_2Cl_2–MeOH (1 : 1; 20 ml) to yield a polar fraction which was analyzed by TLC and GC-MS for the presence of alkaloids.

Chemical Analyses. UV spectra were taken on a Philips PU 8700 UV-VIS spectrophotometer in MeOH. IR spectra were recorded on a Bruker IFS 25 instrument as a film on a NaCl disk. NMR spectra were recorded in $CDCl_3$ at 600 and 150.87 MHz (Varian Unity 600 instrument) or at 250 MHz (Bruker WM 250). Chemical shifts (δ) are reported in parts per million from internal TMS, and the coupling constants are given in hertz. Mass spectra were performed on a Fisons VG Autospec mass spectrometer in the electron impact (70 eV) mode. Optical rotations were measured on a Perkin Elmer 141 polarimeter (Na vapor lamp) in a 10-cm cell at room temperature. Flash liquid chromatography was performed over Macherey-Nagel Si gel (0.04–0.063 mm) and thin-layer chromatography analyses (TLC) on Polygram SilG/UV_{254} precoated plates (0.25 mm). The compounds were visualized under UV_{254} light, and/or by spraying with Dragendorff reagent. GC-MS analyses were carried out on a Fisons VG Autospec mass spectrometer in the electron impact (70 eV) mode, coupled to a Carlo Erba GC 8065 gas chromatograph equipped with a split injector, and with a 25-m × 0.25-mm CP-Sil 5CB fused silica column (Chrompack). The conditions were: 1 min at 150°C, then increased 10°C/min to 320°C, then maintained 2 min at 320°C. Carrier gas was helium, and injector and detector temperatures were 250°C. GC analyses were performed on a Varian gas chromatograph equipped with a 25-m × 0.53-mm CP-Sil 19CB fused silica column (Chrompack). The carrier gas was nitrogen, and oven and injector temperatures were maintained at 250°C and the detector temperature at 270°C.

*Derivatization of (±)-Anabasine with (+)-(1S,2R,5S)-Menthylchloroformate [(+)−**3**]*. To (±)-anabasine (1.0 mg, 0.0062 mmol) in a 300-μl Reacti-vial was added anhydrous THF (150 μl), NEt_3 (5 μl, 0.036 mmol) and finally (+)-**3** (4.0 μl, 0.018 mmol). After stirring at 60°C under argon for 2 hr, the reaction mixture was poured into 10% aqueous NaOH (2 ml), and the aqueous phase extracted with diethyl ether (3 × 1 ml). Drying and concentration of the combined organic extracts *in vacuo* afforded an oily residue, which was purified by flash chromatography on silica gel (CH_2Cl_2, then CH_2Cl_2–MeOH 9 : 1) to quantitatively afford a mixture of (2′*R*)-**4** and (2′*S*)-**4** (2.1 mg, 0.0061 mmol). They were separated by reverse-phase HPLC (Chromspher C_{18}, 5 μm, 10 × 250 mm, CH_3CN–H_2O, 65 : 35, flow: 5 ml/min).

(2′R)- [(1S,2R,5S)-Menthoxycarbonyl]anabasine [(2′*R*)−***4***]. $[\alpha]_D^{20}$ +110 (c 0.135, CH_2Cl_2); EI-MS (70 eV) m/z 344 ($M^{+\cdot}$, $C_{21}H_{32}N_2O_2$, 6), 216 (10), 207 (100), 206 (66), 189 (22), 162 (38), 138 (7), 133 (14), 128 (7), 92 (21), 83 (53), 69 (26), 55 (38); IR (film) 2928–2866, 1694, 1422, 1265, 1169, 1037, 810-714 cm^{-1}; UV (MeOH) λ_{max} (ε) 203 (6800), 262 (1900); GC: t_r = 13.0 min; ^{1}H NMR

(600 MHz, $CDCl_3$) δ 8.52 (m, 2 H, H-2, H-6), 7.57 (d, 8.0 Hz, 1 H, H-4), 7.32 (m, 1 H, H-5), 5.51 (bs, 1H, H-2′), 4.65 (td, $J = 11.0, 4.0$, 1 H, H-8′), 4.12 (bd, $J = 13.0$, 1 H, H-6′eq), 2.79 (td, $J = 14.0, 5.0$, 1 H, H-6′ax), 2.28 (bd, $J = 14.0$, 1 H, H-3′eq), 2.12 (bd, $J = 11.0$, 1 H, H-13′eq), 1.96 (tt, $J = 13.8, 4.8$, 1 H, H-3′ax), 1.67 (m, 4 H, H-4′eq, H-10′eq, H-11′eq, H15′), 1.62 (m, 1 H, H-5′eq), 1.52 (m, 2 H, H-5′ax and H-12′ax), 1.43 (bq, $J = 13.0$, 1 H, H-4′ax), 1.30 (m, 1 H, H-9′ax), 1.04 (m, 1 H, H-10′ax), 0.98 (q, $J = 11.8$, 1 H, H-13′ax), 0.91 (d, $J = 6.7$, 3 H, CH_3-14′), 0.86 (m, 1H, H-11′ax), 0.78 (d, $J = 5.0$, 3 H, CH_3-16′ or 17′), 0.73 (d, $J = 6.3$, 3 H, CH_3-16′ or 17′).

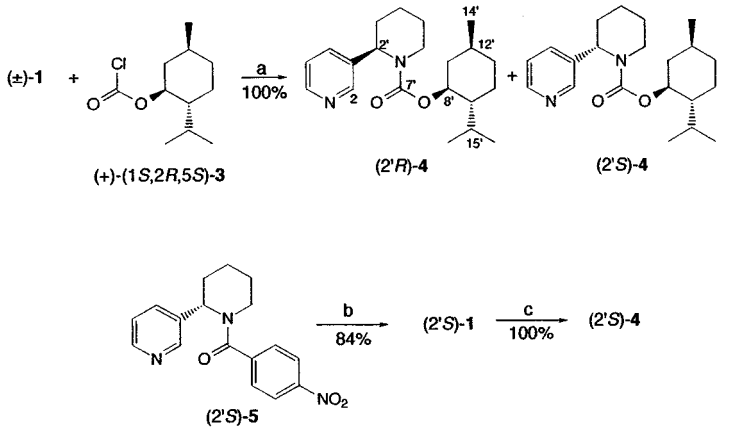
*(2′S)-[(1S,2R,5S)-Menthoxycarbonyl]anabasine [(2′S)-**4**].* $[\alpha]_D^{20}$ −100 (c 0.485, CH_2Cl_2); EI-MS (70 eV) m/z 344 ($M^{+\cdot}$, $C_{21}H_{32}N_2O_2$, 6), 216 (10), 207 (100), 206 (66), 189 (22), 162 (38), 138 (7), 133 (14), 128 (7), 92 (21), 83 (53), 69 (26), 55 (38); IR (film) 2956–2872, 1699, 1410, 1261, 1038, 963, 815-712 cm^{-1}; UV (MeOH) λ_{max} (ε) 203 (4600), 262 (1800); GC: $t_r = 13.5$ min; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.54 (m, 2 H, H-2, H-6), 7.62 (d, $J = 8.0$, 1 H, H-4), 7.36 (dd, $J = 8.0, 4.0$, H-5), 5.53 (bs, $J = bd$, 1 H, H-2′), 4.61 (td, $J = 11.0, 4.0$, 1 H, H-8′), 4.13 (bd, $J = 14.0$, 1 H, H-6′eq), 2.79 (ddd, $J = 14.0, 11.0, 4.0$, 1 H, H-6′ax), 0.92 (d, $J = 6.7$, 3 H, CH_3-14′), 0.79 (d, $J = 6.9$, 3 H, CH_3-16′ or -17′), 0.73 (d, $J = 6.9$, 3 H, CH_3-16′ or 17′). The derivatization procedure described above was applied to the alkaloid fractions coming from *M. sanctus*, *A. subterranea*, and *A. miamiana*. The menthoxycarbamates thus obtained were analyzed by capillary GC and by GC-MS.

*Hydrolysis of (2′S)-Anabasine-p-NO_2-Benzamide [(2′S)-**5**] into (2′S)-Anabasine.* (2′*S*)-**5** (Pfrengle and Kunz, 1989) was kindly provided by Prof. H. Kunz (University of Mainz). (2′*S*)-**5**, $[\alpha]_D^{20}$ −130 (c 3, MeOH) (19.6 mg, 0.063 mmol) was dissolved in a mixture of 33% HBr–AcOH (0.6 ml) in a 1 ml Reacti-vial. The mixture was stirred at 80°C for 45 hr and was then poured into NH_4OH (8 M) and the aqueous phase quickly extracted with CH_2Cl_2. The combined organic extracts were concentrated *in vacuo* to afford an oily residue that was purified by flash chromatography on a silica gel column (CH_2Cl_2–MeOH 9 : 1) to afford (8.5 mg) (2′*S*)-anabasine in an 84% yield.

*(2′S)-Anabasine [(2′S)-**1**].* $[\alpha]_D^{20}$ −21 (c 0.5, MeOH); ^{1}H NMR data of *(2′S)*-**1** were identical to those of our synthetic material and to literature data (Yang and Tanner, 1997). Treatment of this sample with (+)-**3** as described above furnished (2′ S)-[(1S,2*R*,5*S*)-menthoxycarbonyl]anabasine [(2′S)-**4**], which gave a single peak at $t_r = 13.5$ min in capillary GC.

RESULTS

Development of a Procedure to Assign the Absolute Configuration of Anabasine. We first applied to a sample of (±)-anabasine [(±)-**1**], synthesized according



SCHEME 1. Derivatization of (±)-**1** with (+)-(1*S*,2*R*,5*S*)-menthylchloroformate, and synthesis of (2′*S*)-**4**. Reagents and conditions: (a) NEt_3, THF, 60°C, 2 hr; (b) 33% HBr–AcOH, 80°C, 45 hr; (c) (+)-(1*S*,2*R*,5*S*)-**3**, NEt_3, THF, 60°C, 2 hr.

to Deo and Crooks (1996), the procedure that was developed for the solenopsins (Leclercq et al., 1994), but we could not separate the resulting diastereomeric MTPA amides by capillary GC or by HPLC. In contrast, treatment of (±)-**1** with an excess of (+)-menthylchloroformate [(+)-**3**] quantitatively furnished the diastereomeric carbamates (2′*R*)-**4** and (2′*S*)-**4** (Scheme 1), which could be separated by reverse-phase HPLC on a C_{18} column. Moreover, their retention times in capillary GC on a CP-Sil 19CB column were also different (Figure 2). The two diastereomers were identified by GC comparison with a reference sample of (2′*S*)-**4**, which was prepared from (2′*S*)-anabasine-*p*-nitrobenzamide [(2′*S*)-**5**] (Pfrengle and Kunz, 1989) (Scheme 1). Under our GC conditions, the retention time of (2′*R*)-**4** was 13.0 min and that of (2′*S*)-**4**, 13.5 min (Figure 2). We have also assigned all the proton signals of (2′*R*)-**4** by a 2D NMR study at 600 MHz.

Detection of Anabasine and Anabaseine in Messor *and* Aphaenogaster *Ants*. Five hundred ants of each species were exhaustively extracted, and each extract was checked for the presence of anabasine (**1**) by TLC and GC-EI-MS. Compound **1** was present as the sole alkaloid in two of the six species, *Messor sanctus* and *Aphaenogaster miamiana*. In TLC, the alkaloidic fraction of *A. senilis* exhibited one major spot less polar than anabasine. GC-EI-MS analysis revealed the presence of one major peak identified as anabaseine (**2**) (Wheeler et al., 1981). No trace of anabasine was detected in this species. In contrast, the two compounds were present in the venom of *A. subterranea*, in an approximately 60 : 40 ratio. Finally, neither anabasine nor anabaseine could be detected in the venom of *M. barbarus* and *M. structor*. The relative amounts of anabasine (**1**) and anabaseine (**2**) identified in the venom of *Messor* and *Aphaenogaster* ants are reported in Table 1.

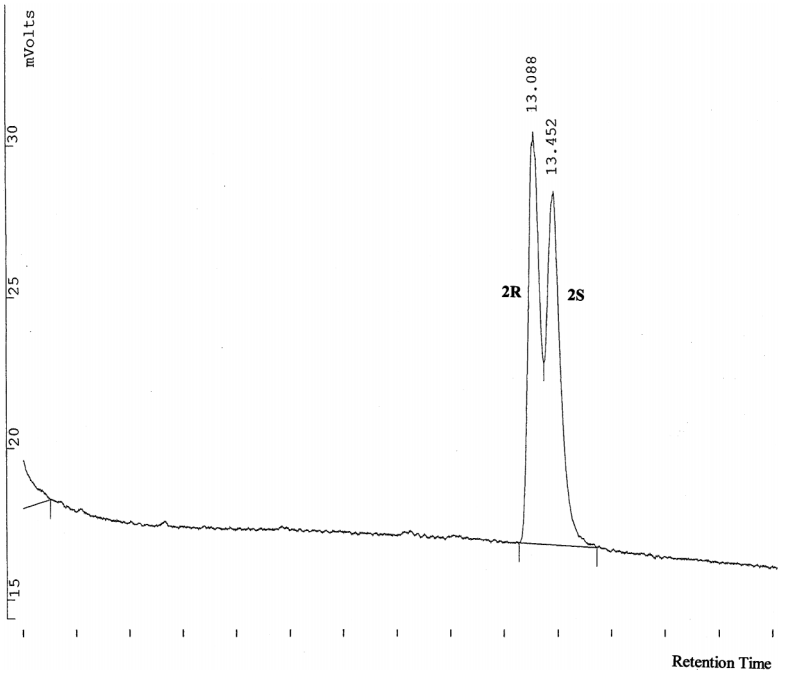


FIG. 2. GC (FID) separation of (2′*S*)-**4** and (2′*R*)-**4**.

Absolute Configuration and Enantiomeric Excess of Anabasine from Messor *and* Aphaenogaster *Ants*. The procedure described above was applied to determine the absolute configuration of anabasine isolated from the venom of *M. sanctus*, *A. subterranea*, and *A. miamiana*. In *M. sanctus*, only one diastereoisomer was present after derivatization, whose retention time corresponded to that of synthetic

TABLE 1. RELATIVE AMOUNTS OF **1** AND **2** IN VENOMS OF *Messor* AND *Aphaenogaster* ANTS

	Anabasine (**1**)	Anabaseine (**2**)
M. sanctus	100	0
M. barbarus	0	0
M. structor	0	0
A. subterranea	60[a]	40[a]
A. senilis	0	100
A. miamiana	100	0

[a] Approximate figures, due to peak superimposition.

(2′*S*)-**4**. Thus, anabasine is present in the venom of *M. sanctus* as a single enantiomer having the 2′*S* configuration.

In *A. subterranea* and *A. miamiana*, anabasine was present as a mixture of *R* and *S* enantiomers, with the *S* enantiomer predominating. Since we have checked that no kinetic resolution occurs during conversion of anabasine into diastereoisomers **4**, the enantiomeric excess of (*S*)-anabasine from *A. subterranea* and *A. miamiana* could be determined to be 78% and 24%, respectively, from the (2′*S*)-**4**/(2′*R*)-**4** ratio.

DISCUSSION

The data reported here on the ee of anabasine from ants are too limited to make any generalization. However, it should be pointed out that our results are similar to those obtained with plants, where anabasine either exists as a single enantiomer with the *S* configuration or as *S*/*R* mixtures, with the *S* enantiomer being generally predominant (Leete, 1983). Several hypotheses can be put forward to explain the wide range of enantiomeric purity observed for anabasine samples isolated from both plants and ants: (1) poor stereochemical control of the enzymatic reaction leading to the formation of the (2′*S*)-stereogenic carbon, (2) co-occurrence of two different biosynthetic routes for the (+) and (−) enantiomers, and (3) chemical or enzymatic isomerization of enantiomerically pure anabasine. The relative contribution of these different possibilities is not known yet. In any case, the detection of a single anabasine enantiomer in *M. sanctus* shows that our method of analysis does not lead to any racemization.

The presence of anabasine and anabaseine in the genus *Aphaenogaster* could be of taxonomic significance, since it supports the current classification of these ants with both genera being placed within the same tribe Pheidolini (Bolton, 1995); the genus *Messor* was even once considered as a subgenus of *Aphaenogaster* (Forel, 1890). However, it is striking that two of the three *Messor* species analyzed in this study do not contain alkaloids, at least in amounts sufficient to be detected by TLC and CG-MS.

Acknowledgments—We are grateful to Prof. Luc Passera (University of Toulouse) for collecting *M. structor* ants and to Dr. R. K. Vander Meer (USDA, Gainesville, Florida) for collecting *A. miamiana* ants. We also thank Mr. S. Rossay for his participation in the preliminary aspects of this work, Dr. M. Luhmer for the NMR spectra and Mr. C. Moulard for the mass spectra. S. A. and S. L. thank the Fonds National de la Recherche Scientifique of Belgium for financial support.

REFERENCES

ARMSTRONG, D. W., LI, W., STALCUP, A. M., SECOR, H. V., IZAC, R. R., and SEEMAN, J. I. 1990. Capillary gas chromatographic separation of enantiomers with stable dipentyl-α-, β-and γ-cyclodextrin-derivatized stationary phases. *Anal. Chim. Acta* 234:365–380.

ATTYGALLE, A. B., KERN, F., HUANG, Q., and MEINWALD, J. 1998. Trail pheromone of the myrmicine ant *Aphaenogaster rudis* (Hymenoptera: Formicidae). *Naturwissenschaften* 85:38–41.

BOLTON, B. 1995. A New General Catalogue of the Ants of the World. Harvard University Press, Cambridge, Massachusetts.

BRAND, J. M., and MPURU, S. P. 1993. Dufour's gland and poison gland chemistry of the myrmicine ant, *Messor capensis* (Mayr). *J. Chem. Ecol.* 19:1315–1321.

COLL, M., HEFETZ, A., and LLOYD, H. A. 1987. Adnexal glands chemistry of *Messor ebeninus* Forel (Formicidae: Myrmicinae). *Z. Naturforsch.* 42c:1027–1029.

DEO, N. M., and CROOKS, P. A. 1996. Regioselective alkylation of *N*-(diphenylmethylidine)-3-(aminomethyl)pyridine: A simple route to minor tobacco alkaloids and related compounds. *Tetrahedron Lett.* 37:1137–1140.

FOREL, A. 1890. Fourmis de Tunisie et de l'Algérie orientale récoltées et décrites par Aug. Forel *Ann. Soc. Entomol. Belg.* 34:61–76.

JACKSON, B. D., WRIGHT, P. J., and MORGAN, E. D. 1989. 3-Ethyl-2,5-dimethylpyrazine, a component of the trail pheromone of the ant *Messor bouvieri. Experientia* 45:487–489.

KEM, W. R. 1988. Pyridine alkaloid distribution in the hoplonemertines. *Hydrobiologia* 156:145–151.

LECLERCQ, S., THIRIONET, I., BROEDERS, F., DALOZE, D., Van DER MEER, R., and BRAEKMAN, J. C. 1994. Absolute configuration of the solenopsins, venom alkaloids of the fire ants. *Tetrahedron* 28:8465–8478.

LEETE, E. 1983. "Biosynthesis and metabolism of the tobacco alkaloids." pp. 85–152, *in* S.W. Pelletier (ed.). Alkaloids. Chemical and Biological Perspectives, Vol. 1. John Wiley, New York.

LUKES, R., AROJAN, A. A., KOVAR, J., and BLAHA, K. 1962. Zur Konfiguration Stickstoffhaltiger Verbindungen XV. Bestimmung der absolute Konfiguration von Anabasin und Anatabin. *Collect. Czech. Chem. Commun.* 27:751–756.

PFRENGLE, W., and KUNZ H. 1989. Hetero-Diels-Alder reactions on a carbohydrate template: Stereoselective synthesis of (*S*)-anabasin. *J. Org. Chem.* 54:4261–4263.

WHEELER, J. W., OLUBAJO, O., STORM, C. B., and DUFFIELD, R. M. 1981. Anabaseine: Venom alkaloid of *Aphaenogaster* ants. *Science* 211:1051–1052.

YANG, C. M., and TANNER, D. D. 1997. A simple synthesis of (±)-1,2,3,6-tetrahydro-2,3′-bipyridine (anatabine) and (±)-3-(2-piperidinyl)pyridine (anabasine) from lithium aluminium hydride and pyridine. *Can. J. Chem.* 75:616–620.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

ALLOMONAL AND HEPATOTOXIC EFFECTS FOLLOWING METHYL EUGENOL CONSUMPTION IN *Bactrocera papayae* MALE AGAINST *Gekko monarchus*

SUK-LING WEE* and KENG-HONG TAN

School of Biological Sciences
Universiti Sains Malaysia
Minden 11800, Penang, Malaysia

(Received January 12, 2000; accepted January 10, 2001)

Abstract—Methyl eugenol (ME), is converted into two major phenylpropanoids, 2-allyl-4,5-dimethoxyphenol and *trans*-coniferyl alcohol, following consumption by the male fruit fly *Bactrocera papayae*. Chemical analysis of wild male *B. papayae* rectal glands, where the compounds are sequestered, revealed the presence of ME metabolites in varying quantities. These phenylpropanoids are shown to be involved in the fruit fly defense both in no-choice and choice feeding tests against the Malayan spiny gecko, *Gekko monarchus*. After being acclimatized to feeding on fruit flies, geckos consumed significantly fewer ME-fed male flies than controls that consumed all the ME-deprived male flies offered throughout a two-week period. Diagnosis of dissected livers from geckos that consumed ME-fed male flies revealed various abnormalities. These included discoloration and hardening of liver tissue, whitening of the gallbladder, or presence of tumor-like growths in all geckos that consumed ME-fed male flies. Control geckos fed on ME-deprived male flies had healthy livers. When given an alternative prey, geckos preferred to eat untreated house flies, *Musca domestica* to avoid preying on ME-fed fruit flies.

Key Words—Methyl eugenol, 2-allyl-4,5-dimethoxyphenol, *trans*-coniferyl alcohol, *Bactrocera papayae*, allomone, hepatotoxicity, *Gekko monarchus.*

INTRODUCTION

The fruit fly, *Bactrocera papayae* Drew and Hancock (Diptera: Tephritidae), is a serious pest to fruit production in Malaysia and its neighboring countries. Sexually mature males of *B. papayae* are strongly attracted to, and voraciously feed on, methyl eugenol (ME). ME is attractive to males of several *Bactrocera* species

* To whom correspondence should be addressed.

0098-0331/01/0500-0953$19.50/0

and occurs naturally in at least 200 plant species from 32 families (Tan and Toong, 1993). It is usually contained in the leaves, blossoms, and fruits where it is a component of the essential oil.

ME has been shown to act as a precursor of the male sex pheromone (Tan and Nishida, 1998). After consumption of ME, males of *B. papayae* produced 2-allyl-4,5-dimethoxyphenol (DMP), *trans*-coniferyl alcohol (CF), and trace quantities of (*Z*)-3,4-dimethoxycinnamyl alcohol (DCA) (Nishida et al., 1988a,b). These phenylpropanoids were released by ME-fed males during the courtship period to form lek (male aggregation) and to boost their sexual competitiveness in mating (Tan and Nishida, 1998).

ME was recently found to play a role in the fruit fly's defense strategy by deterring predation by the Asian house gecko, *Hemidactylus frenatus* Dumeril and Bibron (Tan and Nishida, 1998). However, nothing is known about the presence of ME metabolites and their levels in wild male fruit flies, or about their pheromonal and allomonal roles. Furthermore, little is known about the physiological significance of the aversion effect by ME-fed males on predators, i.e., their toxicity. This information could help shed light on the tritrophic plant–fruit fly–predator relationship. In addition, the amount of ME a male is able to consume and, subsequently, the phenylpropanoids produced were also investigated.

Here we report on the presence and variation of ME metabolites in the rectal gland of wild *B. papayae* males, on the allomonal effect of the male pheromone of *B. papayae* on the Malayan spiny gecko, *Gekko monarchus* Dumeril and Bibron (Squamata: Gekkonidae), and on induced hepatotoxicity in the gecko after consumption of ME-fed male fruit flies.

METHODS AND MATERIALS

Chemicals. ME (1-allyl-3,4-dimethoxybenzene; 99.8% purity) was obtained from International Pheromone System Limited, England. Authentic compounds, CF (96.0% *trans*) and DMP (>96.0% purity), for peak identification and quantification using gas chromatography were supplied by R. Nishida (Kyoto University, Japan).

Flies. B. papayae was cultured in the laboratory using artificial medium and adult diet as described by Tan and Nishida (1998). Flies were sexed within five days of emergence. Virgin male and female flies were maintained in separate cages.

The common house fly, *M. domestica*, was cultured using moistened rat food pellet as larval medium. Adults were given water, milk powder (as a source of protein), and a mixture of sucrose and vitamin B complex. Flies were kept in the insectary at $30 \pm 2^{\circ}$C and relative humidity $73 \pm 5\%$ with a 12L : 12D photoperiod.

Malayan Spiny Gecko. G. monarchus was captured from the Universiti Sains Malaysia campus in Penang. Geckos were acclimatized to laboratory conditions

and offered live untreated fruit flies (male and female) for a minimum period of one month. Geckos that consumed a minimum of 10 flies/day were selected for feeding tests.

ME Consumption by an Individual Male. The mean quantity of ME consumed by a male *B. papayae* was determined as follows. Sexually mature virgin males (14–19 days old) were lightly anesthetized with carbon dioxide and placed ventrally with plasticine fixed on the wings to reduce struggling. A 1-μl microcapillary pipet (32 mm long) (Drummond) containing ME was placed 3–5 mm away from a male. Upon sensing the ME, the male would reach for the lure by stretching out its proboscis. The fly was then allowed to consume the ME from the microcapillary pipet mounted on a micromanipulator stand. The source was removed when the male stopped stretching out its proboscis or when it moved its proboscis away. The amount of ME consumed was then recorded with the help of a micrometer calibrating glass slide. Most males stopped feeding within 5 mins. This experiment was conducted in the morning (08:00–11:00 hr) during the fly's peak period of response to ME (Tan, 1985). Twenty males were used on each day of feeding. A total of five replicates were performed, each using flies from a different cohort.

Accumulation of Phenylpropanoids in Rectal Gland. ME (20 μl) was dispensed onto a piece of Whatman No. 1 filter paper (2.5 $\times$ 0.5 cm^2) with a 50-μl Hamilton syringe. ME was allowed to be adsorbed by the filter paper before being presented to the males. A group of 10 males was allowed to feed *ad libitum* on the filter paper containing ME for 15 min. A similar procedure was employed to prepare ME-fed fruit fly males [one day after treatment (1-DPT)] for subsequent choice and no-choice feeding tests.

The fruit fly's rectal gland was extracted the following day (1-DPT). The extracted rectal gland was placed into a narrow V-shaped glass tube (5 mm diam.) containing 20 μl or appropriate quantity of redistilled ethanol containing an internal standard (50 ng/μl of dodecanol). The content was then thoroughly homogenized with a fine glass rod. After sonicating for 5 min, 1 μl was then injected into the gas chromatographic column for separation and detection of volatile components.

Quantification of the volatile components was done on a Shimadzu GC 14A using a HP Ultra-1 column (nonpolar; 25 m $\times$ 0.25 mm $\times$ 0.33 μm fused silica column coated with cross-linked bonded dimethylpolysiloxane). Temperature was programmed from 80°C (approx. 1 min holding time) to 220°C at a rate of 10°C/min. Quantities were determined by comparing the FID intensities with standards of known concentration using a Shimadzu C-R6A Chromatopac reporting integrator.

Chemical Analysis of Wild Male Rectal Gland. ME-baited traps were set in three regions of Penang Island (i.e., northern region = Tanjung Bungah, central region = Ayer Itam, southern region = Minden and Greenlane). All sites were located near hillside forests. Traps were designed to prevent attracted males from feeding on the ME source. Trapping was conducted from June to July 2000 on

different days. Some of the male fruit flies were caught using specimen vials before the flies entered the traps. Trapped male fruit flies were transported back to the laboratory, and the extraction of individual male rectal glands was carried out within 6 hr of trapping. The male rectal glands were processed in the similar manner as described above.

No-Choice Feeding Test. Ten geckos were randomly assigned to two groups with five (**A**–**E**) as treatments and five as controls. Geckos were individually caged and fed with fruit flies to satiety daily. Prior to the feeding experiment, they were starved for three days to ensure a similar degree of hunger (a fully satiated gecko could stop feeding for two days) and their individual weights were determined. The effectiveness of ME-fed males as a deterrent was then evaluated by offering 10 ME-fed males per day to treatment geckos and the same number of untreated males to the control geckos for a period of two weeks. Consumed or dead flies were replaced daily. Daily consumption of each gecko was recorded. Comparison of the mean number of flies consumed per gecko per day was done using the Student's *t* test (one-tailed) at $P = 0.01$.

At the end of the experiment, both groups of geckos were weighed individually. Geckos were killed by exposing them to ethyl acetate vapor for 10 min or longer, and then they were dissected for observation of liver abnormalities.

Choice Feeding Test. To examine the effectiveness of the fruit fly allomone in the presence of a palatable prey (similar in size but without aposematic coloration as the fruit fly), the house fly, *M. domestica* was used. To show that geckos had no intrinsic preference for the house fly after being acclimatized to feed on fruit flies, each gecko was simultaneously offered 15 house flies and 15 untreated male fruit flies in the pretreatment feeding. Consumption for each category of fly was recorded. After three days of fly deprivation, geckos were offered eight ME-fed male fruit flies and eight untreated house flies daily for one week. Dead and consumed flies were replaced daily and fly consumption by each gecko was noted. Ten geckos were used in this experiment. Data was analyzed using paired *t* test ($P = 0.01$).

RESULTS

Ninety-four geckos from four common species of Malaysian gecko, were caught including the Asian house gecko (*Hemidactylus frenatus*), Malayan spiny gecko (*Gekko monarchus*), flat-tailed gecko, (*Cosymbotc platyurus*), and *Gehyra mutilatus* (which has translucent body skin). Among the 94 geckos, *G. monarchus* was the only species that consumed 10 or more fruit flies and thus was the only species selected for further testing.

Geckos are often used as a model predator to study prey–predator relationships. In this experiment, not all geckos readily fed on fruit flies. Insectivorous geckos are not a common predator of fruit flies. The daily activity rhythm of

G. monarchus and *B. papayae* are asynchronous, i.e., the former is an indoor nocturnal predator, while the latter is an outdoor diurnal insect. This may partly explain the low consumption rate of fruit flies by this gecko. It is likely also that geckos may have learned from previous bad experience that fruit flies are not palatable or that the aposematic coloration of the fruit fly, which mimics a wasp, may result in aversion.

The average quantity of ME (mean $\pm$ SE) consumed by each male was 0.58 ± 0.02 μl. Most flies consumed a minimum of 0.1 μl ME. Approximately 22% of the males consumed below 0.2 μl while two individuals consumed as much as 2.3 and 2.7 μl of ME.

Accumulation of Phenylpropanoids in Laboratory-Reared Males. When laboratory-reared males were fed ME, two major phenylpropanoids, DMP and CF, were detected in their rectal glands. These were absent from the rectal glands of ME-deprived males. The average contents (mean $\pm$ SE) of DMP and CF in the rectal glands of ME-fed males one day after ME consumption, was 5.9 ± 1.1 and 12.7 ± 2.8 μg/male, respectively. The quantity of CF was consistently higher than DMP.

Chemical Analysis of Wild Male Rectal Gland. Among 78 male *Bactrocera* spp. trapped (Ayer Itam: 36, Tanjung Bungah: 23, Minden: 12, and Greenlane: 7), all were *B. papayae* except two *B. carambolae* (one each from Ayer Itam and Minden).

All wild male fruit flies contained varying quantities of ME metabolites in their rectal glands ranging from a trace to as high as 55.1 μg for DMP and 47.5 μg for CF in a single male. Overall, 34 of 76 males (45%) contained at least 5 μg of either DMP or CF in their rectal gland.

The ratio of DMP:CF in wild fruit flies was different from that of the laboratory strain. Forty-one of 76 males (54%) contained almost equal quantities of DMP and CF. Thirteen males (17%) contained at least 1.5 times more DMP than CF, and 22 males (29%) contained at least 1.5 times more CF than DMP.

No-Choice Feeding Test. Treatment geckos substantially reduced their consumption of ME-fed males compared to control geckos ($t = 7.35$; $P < 0.0005$) (Figure 1). Consumption of ME-fed males by geckos was consistently low throughout the two-week period. Some of the ME-fed flies were regurgitated by geckos. This was indicated by the remains of body parts of ME-fed flies offered. Control geckos consumed all the fruit flies offered to them.

A significant increase of body weight (445.4 ± 61.1 mg) was observed in control geckos at the end of experiment ($t = 7.29$; $P < 0.005$). However, the decrease in the weight (3.8 ± 112.0 mg) of treated geckos was not significant ($t = 0.03$, $P > 0.05$) even though the consumption rate was significantly reduced compared to control geckos (Figure 1).

Morphological examination of control geckos indicated that their livers were healthy without any abnormality (Figure 2a). However, all treated geckos had

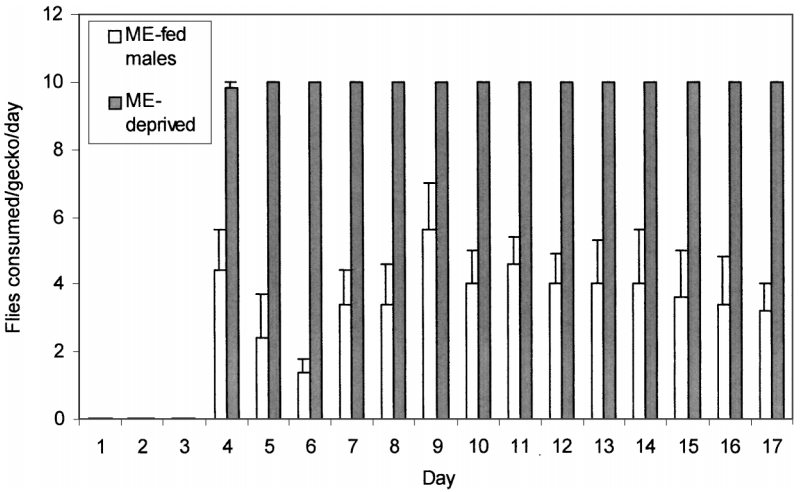


FIG. 1. Deterrent effect of methyl eugenol-fed male *Bactrocera papayae* against *Gekko monarchus* in a no-choice feeding test (bar = SE; $P < 0.005$). After deprivation: each treatment gecko was offered 10 ME-fed males; each control gecko was offered 10 ME-deprived males daily for two weeks.

various abnormalities, ranging from anemia to hardening of, and tumor growth in, the liver (Figure 2b–2f).

Table 1 shows the total ME-fed *B. papayae* males and the estimated doses of phenylpropanoids consumed by each gecko. Gecko **B** consumed the fewest ME-fed males and had light discoloration in its liver (Figure 2b). As the consumption

TABLE 1. TOTAL METHYL EUGENOL (ME) -FED MALE *Bactrocera papayae* CONSUMED BY EACH GECKO, *Gekko monarchus*, IN TWO WEEKS AND ESTIMATED DOSES OF T*rans*-CONIFERYL ALCOHOL (CF) AND 2-ALLYL-4,5-DIMETHOXYPHENOL (DMP) CONSUMED IN RESPECTIVE GECKOS

Gecko	Body weight (mg)		ME-fed males consumed	Estimated dose consumed (μg)[a]	
	Initial weight	Weight change		CF	DMP
B	4421.9	−144.4	18	229	106
D	5651.0	−184.0	38	483	224
E	5714.2	−81.5	53	673	313
A	5317.0	−43.8	57	724	336
C	4008.9	+434.8	91	1156	537

[a]The estimated phenylpropanoid doses consumed per gecko was based on the results obtained from gas chromatographic analysis on the individual rectal gland of *B. papayae* males ($N = 9$; a group of 10 males was allowed to feed on 20 μl of ME dispensed on a filter paper for 15 min).

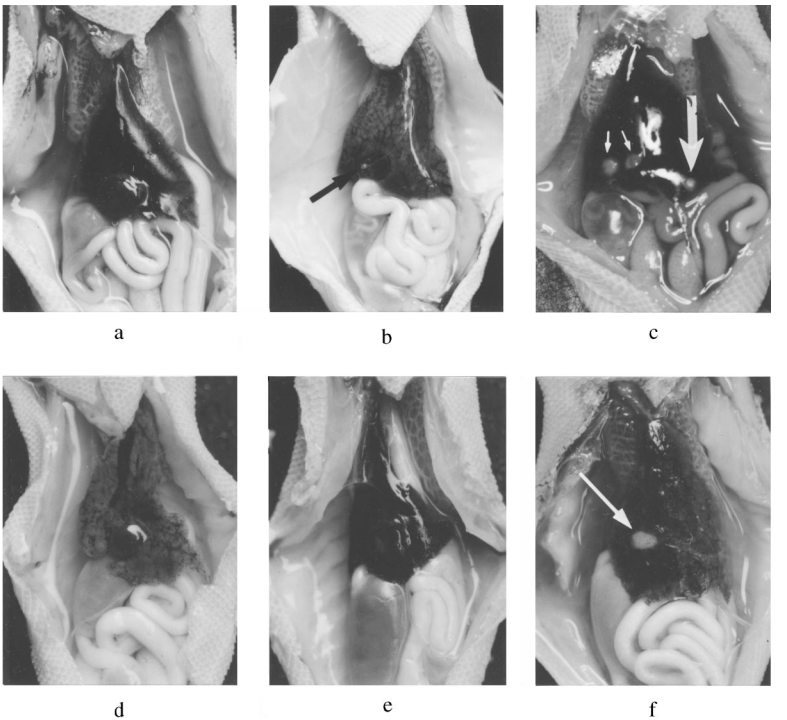


FIG. 2. Photos show liver lobes (*in situ*) of geckos: (a) healthy liver lobes of a control gecko; (b) gecko **B** with anemic liver lobes—arrow shows whitish tumorlike growth; (c) arrows show whitish tumorlike structures in gecko **D**; (d) advanced discoloration on the liver lobes of gecko **E**; (e) gecko **A** with darkened and hardened liver lobes; and (f) gecko **C** with a hardened dark liver—arrow shows whitish gallbladder.

of ME-fed males increased, more discoloration and/or whitish tumorlike growths were observed in geckos **D**, **E**, and **A** (Figure 2c, 2d, and 2e). Gecko **C** consumed the highest number of ME-fed males and had a liver that was hardened and darkened with a whitish gall bladder (Figure 2f).

Choice Feeding Test. Geckos preferred to prey on fruit flies rather than house flies after a month of acclimatization feeding on untreated fruit flies ($t = 5.77$; $P < 0.001$). However, prey preference was immediately reversed when ME-fed male fruit flies were offered to them instead of untreated males ($t = 3.54$; $P < 0.01$) (Figure 3). After their first encounter with the treated fruit flies, geckos were observed to prey on significantly more house flies than ME-fed fruit flies throughout the experimental period ($P < 0.001$).

Besides decreased consumption of treated fruit flies, undigested ME-fed fruit fly bodies were excreted together with uric acid (Figure 4; labeled **P**). All house

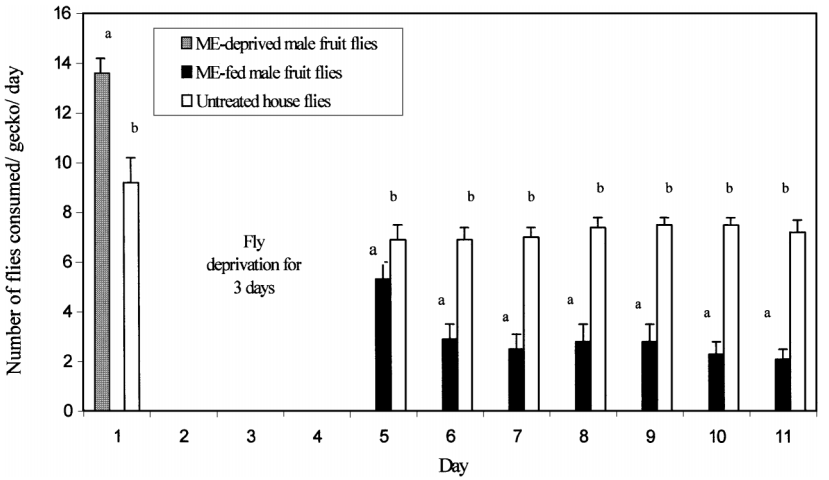


FIG. 3. Deterrent effect of methyl eugenol (ME) -fed male *Bactrocera papayae* against *Gekko monarchus* in the presence of an alternative prey, *Musca domestica* in a choice feeding test (bar = SE; bars with different letters are significantly different at $P = 0.01$, paired *t* test). Before deprivation, geckos were offered 15 flies each of fruit and house flies. After deprivation, each gecko was offered 8 ME-fed fruit flies and 8 untreated house flies daily.

flies offered to control geckos were consumed and there were no undigested body parts in their excrement (Figure 4; labeled **K**). There was a significant increase in gecko body weight before and after the feeding test (259.0 ± 44.0 mg) ($t = 5.88$; $P < 0.001$). Observation of the livers showed no abnormalities.

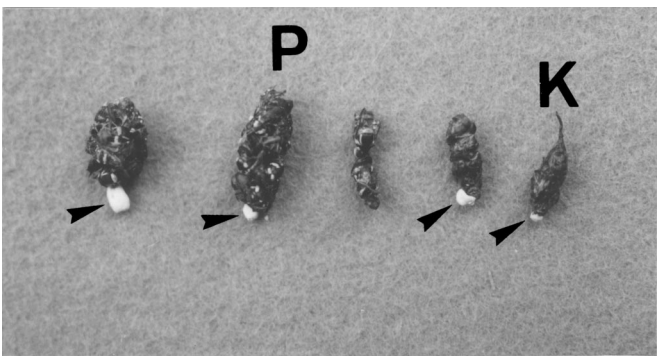


FIG. 4. Gecko excrement showing undigested methyl eugenol-fed fruit flies (labeled with **P**). Arrowheads indicate the uric acid portion of the excrement. Control is labeled with **K**.

DISCUSSION

Males of *B. papayae*, after consumption of methyl eugenol, deterred geckos. Our results showed that ME-fed males 1-DPT produced high amounts of DMP and CF in their rectal gland. These large amounts of ME metabolites function as allomones to deter the geckos. Results from no-choice and choice feeding tests point to the allomonal property of ME metabolites and are consistent with Tan and Nishida (1998) working with the Asian house gecko, *H. frenatus.* ME-fed *B. papayae* were also found to deter the toad, *Bufo asper*, in a recent study (Tan, unpublished observation).

Field trapping results revealed the presence of ME metabolites in all wild male fruit fly rectal glands. However, the quantity of phenylpropanoids varied tremendously between individuals. The fact that over 45% of wild males contained high amounts of DMP and CF in their rectal glands, similar to the levels found in laboratory-ME-fed males, strongly supports claims that these compounds have a defensive function in nature, as well as a pheromonal role in the wild population (Nishida et al., 1988b; Tan and Nishida, 1996, 1998; Shelly, 2000). In addition, Tan and Nishida (1998) also reported that 83% of *B. papayae* males trapped in the wild contained ME metabolites in their rectal glands.

The high variability of ME metabolites in individual wild males is consistent with the findings of Nishida et al. (1988b). This variability might result from differences in the quantity of ME consumed by individual male fruit flies in the wild. Individual laboratory-reared male flies consumed 0.1–2.7 μl of ME. It is possible that males had previously consumed ME from different plant sources with varying levels of ME. ME consumption as high as 4 μl in *B. papayae* males was reported by Tan and Nishida (1998). Cunningham (1989) observed that a *B. dorsalis* male continued to feed on pure ME until its crop was filled and it subsequently died. This amazing affinity to ME and compulsive feeding behavior indirectly reflects the ecological significance of ME to *Bactrocera* males.

It is possible that repeated feeding on ME occurs in the wild. The fact that males respond to ME-baited traps while the rectal gland still contains substantial quantities of ME metabolites is self-explanatory. Laboratory experiments showed that ME metabolites can be detected in the rectal gland of *B. papayae* males 20 days after feeding on 0.1 μl ME (unpublished data). Furthermore, ME occurs naturally in at least 200 plant species (Tan and Toong, 1993). In view of the rich floral diversity in the tropics, there could be more ME-containing plant species that await discovery.

The ratio of DMP and CF contents in wild males as compared to lab-reared strains may be of ecological significance. By treating grains before offering them to the Japanese sparrow, DMP was observed to deter birds better than ME, followed by CF (Nishida and Fukami, 1990). In addition, DMP was reported to be a weak sex attractant to *B. papayae* females while CF was found to attract significantly

more male and female *B. papayae* (Hee and Tan, 1998; Tan and Nishida, 1996). Comparing the two compounds, DMP (i.e., a phenolic compound) is a more potent deterrent; CF is a better sex pheromone component. In the laboratory, sex pheromones are more crucial than allomones where the need to deter predators is greatly reduced. Fruit flies may be adapted to produce higher CF than DMP in the laboratory. Therefore, it is suggested that the production and ratio of DMP to CF may be indirectly related to the contemporary needs of the individual fly.

Tan and Nishida (1998) reported that after an initial exposure and distasteful experience, Asian house geckos totally avoid feeding on ME-fed *B. papayae* males. Therefore, further investigation on the toxic effect of ME metabolites was conducted. In this experiment, using a different predator model, *G. monarchus*, the antipredation effect of ME-fed *B. papayae* males together with the induced hepatotoxic effect on predatory geckos were demonstrated. ME-fed *B. papayae* males were unpalatable to geckos, as indicated by regurgitation and reduced consumption in no-choice tests. However, as the spiny geckos continued to consume a small number of ME-fed flies each day to overcome hunger during the two-week experimental period, the diagnosis of the liver abnormality was made possible.

While the carcinogenic activities of DMP and CF are not known, formation of phenolic compounds from ring oxidation of ME or metabolism of the methoxy substituents might be involved in determining toxicity, i.e., the induced hepatotoxicity. Furthermore, to demonstrate the role of ME in fruit fly–predator relationships, ME-fed *B. papayae* males were offered as whole flies to geckos. The possible ME residue in the fruit fly body might also contribute to the results obtained. Methyl eugenol residue was detected in the crop, but not in the rest of the alimentary canal including the rectal gland of *B. papayae* males following ME consumption (Tan, unpublished data). In mouse liver, ME has been reported to be as active a carcinogen as safrole (1-allyl-3,4-methylenedioxybenzene) and estragole (1-allyl-3,4-methoxybenzene) (Miller et al., 1983). Therefore, the combination of ME, DMP, and CF may have synergistic effects in the antifeeding test as well as the induced hepatotoxicity of geckos. The toxicity of individual chemicals on the predator certainly warrants further investigation.

In choice-feeding tests, geckos learned through experience that ME-fed male fruit flies were not palatable and would search for alternative prey. The yellow markings on the fruit fly's thorax serve as very effective aposematic coloration to predators. It is obvious that feeding on the limited number of house flies provided was not sufficient, and therefore, geckos would attempt to feed on ME-fed male flies to satisfy their hunger. However, undigested ME-fed male bodies were excreted. This may account for healthy livers found in these geckos upon dissection.

Many local farmers refer to the fruit fly as "fruit fly wasp/bee." The reason being the distinctive yellow marking (aposematic color) on the thorax, petiolate abdomens, as well as a jerky, wasp-like walk of most of the tropical fruit flies, i.e., *B. papayae*, *B. carambolae*, and *B. cucurbitae* all resemble wasps. Therefore,

the fruit fly may be considered as a Batesian mimic and hence might gain some protection against vertebrate predators that have had a bad experience with the wasp. However, after pharmacophagy of ME, *B. papayae* males offered a better protection via automimicry to both ME-deprived and conspecific females, as they do not produce an allomone to deter geckos (Tan and Nishida, 1998).

ME-fed males may also serve as a model for many other fruit fly species through Mullerian mimicry. For instance, in the case of the male melon fly, *B. cucurbitae*, which is attracted to cue-lure (CL) and raspberry ketone (RK), it produces an endogenous compound, 1,3-nonanediol when it attains sexual maturity. This compound acts as a potent deterrent against gecko predation (Tan, 2000).

Similarly, the Oriental fruit fly, *B. dorsalis* (nonendemic in Malaysia) after pharmacophagy of ME produces the same phenylpropanoids as in *B. papayae* males and stores it in their rectal glands (Tan and Nishida, 1996). Therefore, it is not surprising that ME-fed *B. dorsalis* males are also able to deter gecko predation apart from being sexually more competitive than ME-deprived males as demonstrated by Tan and Nishida (1998).

Sequestration of allomonal components originating from nonhost plants and incorporation into their defensive repertoire by pharmacophagus insects was documented in a few insect orders. These include several lepidopterans associated with pyrrolizidine alkaloids, such as danaid butterflies (Meinwald et al., 1969; Boppré, 1978; 1986) and arctiid moths (Schneider et al., 1982). Such tritrophic associations also exist between turnip sawflies, *Athalia rosae* (Hymenoptera) and clerodendrins (Nishida and Fukami, 1990), dibroticite leaf beetles, *Diabrotica speciosa* (Coleoptera) and cucurbitacins (Nishida and Fukami, 1990), and between fruit flies, *Bactrocera* spp., and methyl eugenol (Nishida and Fukami, 1990; Tan and Nishida, 1998). Predators were either deterred/repelled from preying on the pharmacophagus insects or vomited/regurgitated to rid themselves from the ill effect of allomones. This experiment has taken a further step in understanding the underlying physiological significance of the allomone to the predator. It has been shown that allomones are indeed hazardous to the predator (geckos in this instance). Thus, it can be concluded that ME and its metabolites play a significant role in the fruit fly's anti-predation strategy.

Acknowledgments—This work is supported by the Malaysian R & D Grant MPKSN/IRPA 01-02-05-6004.

REFERENCES

BOPPRÉ, M. 1978. Chemical communication, plant relationships, and mimicry in the evolution of danaid butterflies. *Entomol.Exp. Appl.* 24:64–77.

BOPPRÉ, M. 1986. Insects pharmacophagously utilizing defensive plant chemicals (pyrrolizidine alkaloids). *Naturwissenschaften* 73:17–26.

CUNNINGHAM, R. T. 1989. Parapheromones, pp 221–228, *in* A. S. Robinson, and G. Hooper (eds.). Fruit Flies: Their Biology, Natural Enemies and Control, Volume 3A. Elsevier, Amsterdam.

HEE, A. K. W., and TAN, K. H. 1998. Attraction of female and male *Bactrocera papayae* to conspecific males fed with methyl eugenol and attraction of females to male sex pheromone components. *J. Chem. Ecol.* 24:753–764.

MEINWALD, J., MEINWALD, Y. C., and MAZZOCCHI, P. H. 1969. Sex pheromone of queen butterfly: Chemistry. *Science* 164:1174–1175.

MILLER, E. C., SWANSON, A. B., PHILIPS, D. H., FLETCHER, T. L., LIEM, A., and MILLER, J. A. 1983. Structure–activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.* 43:1124–1134.

NISHIDA, R., and FUKAMI, H. 1990. Sequestration of distasteful compounds by some pharmacophagus insects. *J. Chem. Ecol.* 16:151–164.

NISHIDA, R., TAN, K. H., and FUKAMI, H. 1988a. *Cis*-3,4-Dimethoxycinnamyl alcohol from the rectal glands of male fruit fly, *Dacus dorsalis. Chem. Express* 3:207–210.

NISHIDA, R., TAN, K. H., SERIT, M., LAJIS, N. H., SUKARI, S., TAKAHASHI, S., and FUKAMI, H. 1988b. Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis. Experentia* 44:534–536.

SCHNEIDER, D., BOPPRÉ, M., ZWEIG, J., HORSLEY, S. B., BELL, T. W., MEINWALD, J., HANSEN, K., and DIEHL, E. W. 1982. Scent organ development in *Creatonotos* moth: Regulation by pyrrolizidin alkaloids. *Science* 215:1264–1265.

SHELLY, T. E. 2000. Flower-feeding affects mating performance in male oriental fruit flies *Bactrocera dorsalis. Ecol. Entomol.* 25:109–114.

TAN, K. H. 1985. Estimation of native populations of male *Dacus* spp. by using Jolly's stochastic method using a new designed attractant trap in a village ecosystem. *J. Plant Prot. Trop.* 2:87–95.

TAN, K. H. 2000. Sex pheromonal components in the defense of the Melon fly, *Bactrocera cucurbitae* against the Asian house gecko, *Hemidactylus frenatus. J. Chem. Ecol.* 26(3):697–704.

TAN, K. H., and NISHIDA, R. 1996. Sex pheromone and mating competition after methyl eugenol consumption in the *Bactrocera dorsalis* complex, pp. 147–153, *in* B.A. MCPheron, and G. J. Steck (eds.). Fruit Fly Pest—A World Assessment of Their Biology and Management. St. Lucie Press, Delray, Florida.

TAN, K. H., and NISHIDA, R. 1998. Ecological significance of male attractant in the defense and mating strategies of the fruit fly, *Bactrocera papayae. Entomol. Exp. Appl.* 89:155–158.

TAN, K. H., and TOONG, Y. C. 1993. The role of methyl eugenol in community ecology—an overview. Joint PSU-USM-USU Seminar, Hat Yai, Thailand.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

EFFECTS OF ISOFLAVONOIDS FROM *Cicer* ON LARVAE OF *Heliocoverpa armigera*

MONIQUE S. J. SIMMONDS[1,*] and PHILIP C. STEVENSON[1,2]

[1]*Royal Botanic Gardens*
Kew, Richmond, Surrey TW9 3AB, UK

[2]*Natural Resources Institute*
University of Greenwich
Chatham Maritime, Kent ME4 4TB, UK

(Received October 6, 2000; accepted January 14, 2001)

Abstract—Four recently identified isoflavonoids, isolated from wild relatives of chickpea, *Cicer arietinum*, were shown to deter larval feeding by *Heliocoverpa armigera* at 100 ppm, judaicin and maackiain retained their antifeedant activity at 50 ppm and 10 ppm, respectively. The isoflavonoids were tested in combinations and with chlorogenic acid; the combinations containing judaicin and maackiain were most active, and chlorogenic acid enhanced the antifeedant activity of all four isoflavonoids. *H. armigera* was the only one of four noctuids to be deterred by all four isoflavonoids. *Spodoptera littoralis* was deterred by judaicin alone and *S. frugiperda* by maackiain alone. *Heliothis virescens* and *S. exigua* were not deterred from feeding by any of the isoflavonoids. When incorporated into a diet, isoflavonoids decreased the weight gain of early stadia larvae of *H. armigera* more than they did later stadia, and maackiain and judaicin were most potent. We conclude that the isoflavonoids, especially maackiain and judaicin, could play a role in decreasing the susceptibility of *Cicer* to attack by *H. armigera.*

Key Words—*Heliocoverpa armigera, Cicer arietinum,* isoflavonoids, chlorogenic acid, *Heliothis virescens, Spodoptera littoralis, Spodoptera exigua, Spodoptera frudiperda,* maackiain, judaicin, antifeedant.

INTRODUCTION

The chickpea, *Cicer arietinum* L., is an important staple food resource in semi-arid tropical regions. The plant is susceptible to attack by a range of pathogens and insects, especially by the noctuid caterpillar, *Heliocoverpa armigera*. Previous studies have shown that the foliar and root isoflavonoids, medicarpin and

* To whom correspondence should be addressed.

0098-0331/01/0500-0965$19.50/0

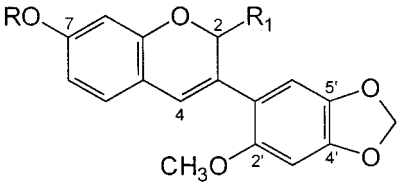
maackiain, contribute to the resistance of chickpea to attack by fungi (Daniel and Barz, 1990; Stevenson et al., 1997; Stevenson and Haware, 1999). Other recent studies have shown that isoflavenes and arylbenzofurans from the roots of wild relatives of chickpea contribute to *Fusarium* resistance in these species (Stevenson and Veitch, 1998a), but as yet the role these compounds play in insect–plant interactions is unclear.

Heliocoverpa armigera larvae exhibit differential responses when feeding on different genotypes of chickpea (Cowgill and Lateef, 1996), and these responses have been attributed to the presence of organic acids on the pods and leaf surfaces (Yoshida et al., 1995). For example, exudates from the trichomes on chickpea leaves contain high levels of malic acid (Lazzaro and Thomson, 1995). Many legumes also contain caffeoyl-quinic acids, which inhibit the development of *H. armigera* and other Heliothine larvae when consumed at high concentrations (Isman and Duffey, 1982; Kimmins et al., 1995). Despite this knowledge about potential resistance mechanisms in chickpeas, little progress has been made in breeding *H. armigera*-resistant chickpeas, and *Heliocoverpa* continues to be the number one insect pest of chickpea.

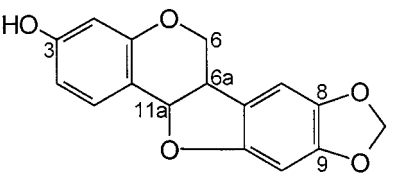
In this study, we have tested four recently identified isoflavonoids that occur in the wild relatives of chickpea, *C. bijugum*, *C. judaicum*, and *C. pinnatifidum* (Stevenson and Veitch, 1998b), to see whether they could influence the behavior and development of *H. armigera*. The objectives were to investigate the effect of: (1) isoflavonoids on the feeding behavior of *H. armigera* in choice and no-choice tests; (2) combinations of isoflavonoids on feeding behavior; (3) isoflavonoids in combination with the phenylpropanoid chlorogenic acid on feeding behavior; (4) isoflavonoids on the development of different stadia of *H. armigera*; and (5) isoflavonoids on the feeding behavior of a range of other polyphagous noctuids.

METHODS AND MATERIALS

Compounds. Judaicin 7-*O*-glucoside **(1)**, judaicin **(3)**, and maackiain **(4)** were isolated from the roots of *Cicer judaicum* Boiss. (Stevenson and Veitch, 1996) and 2-methoxyjudaicin **(2)** was isolated from the roots of *C. bijugum* Rech.f. (Veitch and Stevenson, 1997) (Figure 1). Freeze-dried root material was ground in methanol and extracted at 4°C for 18 hr. The extract was filtered and the filtrate evaporated to dryness and then taken up in methanol to provide an extract equivalent to 1 g/ml. Aliquots (150 μl) were injected directly onto a Spherisorb 5 ODS column (10.0 mm ID × 250 mm). A Waters HPLC system consisting of a 717 autosampler, a LC 600E pump, and a 996 photodiode array detector was used in semipreparative gradient elution mode where A = 2% acetic acid and B = 2% acetic acid in acetonitrile. A = 50% at $t = 0$ min; A = 40% at $t = 20$ min; and A = 20% at $t = 30$ min at 4.7 ml/min. Chlorogenic acid was purchased from Sigma Chemical Company Ltd.



1 R = Glc, R_1 = H
2 R = H, R_1 = OCH_3
3 R = R_1 = H



4

FIG. 1. Structures of isoflavonoids from *Cicer*: judaicin 7-*O*-glucoside **(1)**, 2-methoxy-judaicin **(2)**, judaicin **(3)**, and maackiain **(4)**.

Plant Material. *Cicer judaicum* ICCW 89 and *C. bijugum* ICCW 42 were obtained from the Genetic Resources Unit, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. Vouchers of the plants are retained within ICRISAT.

Insects. *H. armigera* were originally from Hyderabad, India, and had been in a laboratory culture at Kew for two to four generations. The other noctuids were from established laboratory cultures in Britain. For example, the culture of *S. littoralis* from Egypt has been in culture at Kew for 28 generations. All species were reared on a wheat-based diet (Simmonds et al., 1992). The behavioral bioassays were all undertaken on larvae 36–48 hr in their final stadium. They were deprived of food for 2–4 hr prior to the experiments. The developmental bioassay used insects 8–12 hr into the stadium. The numbers of replicates varied among experiments and are presented in the Tables.

Behavioral Tests. A choice bioassay that used glass-fiber disks was used to calculate the feeding index $[(C - T)/(C + T)] \times 100$, an index used to compare the effect of compounds on insects feeding (Simmonds et al., 1990). Glass-fiber disks (2.1 cm dia., Whatman) were treated with 100 μl of sucrose and left to dry. The treatment disks were then treated with 100 μl of one of the test compounds at

either 100 ppm (0.28 μg/cm^2), 50 ppm (0.14 μg/cm^2), 10 ppm (0.028 μg/cm^2), or 1 ppm (0.0028 μg/cm^2) in methanol. The disks were dried, weighed, and placed in pairs, one control and one treatment disk, into a Petri dish (9.1 cm diam.). Prior to placing the standardized final stadium larvae into the dishes, the disks were each treated with 100 μl distilled water to make them more palatable to the larvae. To avoid the bioassay changing from a choice to no-choice bioassay, the larvae were removed after 8 hr, the time taken for the larvae to eat about 50–70% of either disk. After removing the larvae the disks were left to dry and reweighed. The feeding index was calculated by using the amount of control (C) and treatment (T) disks eaten: a positive value indicates an antifeedant, a negative value a phagostimulant.

The choice bioassay was used to compare the effect of different concentrations of the compounds against *H. armigera* and to test the compounds at 100 ppm against a range of noctuids. The bioassay was also used to evaluate whether applying two compounds to the treated disk would result in additive or synergistic increases in activity. Due to the limited amounts of compounds available for the combination experiments, we did not make fresh solutions with the compounds combined in a solution. We used the original 50 ppm solutions. For example, the treatment disks were treated with 100 μl of one compound at 50 ppm, left to dry, and then treated again with 100 μl of another compound at 50 ppm. The isoflavonoids were also tested in combination with chlorogenic acid. In this test, a 100-μl aliquot of chlorogenic acid (50 ppm) was applied to the treatment disks, which were then dried before being treated with 100 μl of one of the isoflavonoids at 50 ppm. In these combination experiments, the second solution was applied with a zigzag movement across the disk to avoid the compounds accumulating at the outer edge of the disk.

A no-choice glass-fiber bioassay was used to evaluate the effect of the compounds on rejection behavior and to evaluate whether, if active, the insects would habituate to the compounds. The disks were treated with sucrose and a test compound at 100 ppm, as outlined above. However, in this experiment the disks were placed singly in the center of a Petri dish and pinned to the dish. The dishes were then placed onto a square of blue card. The disks were pinned down to prevent them being moved by the larvae during the course of the experiment, and the blue background was to provide contrast for the disk and the larvae to enable us to record the movement of the larvae onto a computer.

H. armigera fifth stadium larvae were placed singly in each Petri dish; their behavior was observed continuously for 120 min and then recorded by camera onto a computer running Ethovision software (video tracking, motion analysis and behavior recognition system software v 1.7; Tracksys Ltd. and Noldus Ltd.) for a further 6 hr. During the first part of the experiment, the behavior of eight larvae was input manually into a computer that was running Observer software (Noldus, 1991). During the first observation period, records were made of the larvae contacting the disk and feeding on the disk. Feeding included both biting and rasping the disk.

Whether insects accepted or rejected a disk at first contact, the time taken to accept a disk and then time spent in contact with a disk were calculated. However, because over 75% of the larvae rejected both the control and treatment disks when they first encountered it, the behavior on first contact was not used as a measure of palatability. Instead the latency (to feeding or sampling) before the insects were observed to bite and consume part of the disk was measured. Sampling a disk was defined as when the larvae contacted and removed a section either by biting or rasping. The behavior of the eight insects was recorded over a 6-hr period and the time spent on disks calculated. The larvae were removed and the disks dried before they were reweighed to calculate the amount eaten. Contact with a disk was recorded if any part of the insect was in contact with the disk. However, this does not mean that the insects were feeding, as the mouthparts of the insect were often obscured from the camera, and the insects spent time stationary on the disks as if "resting." The number of replicates recorded at a session was eight: this consisted of two treatments of either four disks from two isoflavonoids or an isoflavonoid and a control.

Development Bioassay. Larvae were placed individually on diet (1.5 ml/well) in wells of a Bioserve bioassay tray (C-D International Inc.) treated with 100 μl of one of the test compounds at 100 ppm. First and second stadia larvae, 8–12 hr into the stadium were placed on the diet and weighed after five days. Third, fourth, and fifth stadia larvae were weighed, and those that weighed within the range of ± 1 SEM were then placed on the diet for five days and reweighed. In all cases, the weights of larvae are compared with those of their respective control, which had been exposed to control diets treated with 100 μl of the solvent methanol. This type of bioassay has been criticized by Freedman et al. (1979), as they reported that insects can avoid ingesting the test extract by boring under the treated diet. However, this bioassay is frequently used in industry to evaluate the activity of plant extracts or compounds. Earlier stadia larvae are usually tested as they are more responsive to compounds than later stadia. In our experience, if the surface of the diet is scored with a knife before the test solution is applied, it will penetrate the surface and disperse through the 1.5-ml aliquot of diet.

Behavioral Bioassays Analysis. The Wilcoxon matched-pairs test was used to analyse the data in the choice bioassays and the Mann-Whitney U test in the no-choice bioassay.

Development Bioassay Analysis. The *t* test was used to test whether the actual weights of the larvae on the compound-treated diets differed from the weights of the larvae exposed to the control diet.

RESULTS

Effect of Isoflavonoids on Feeding Behavior of H. armigera *in Choice and No-Choice Tests*. In the choice bioassay, all four isoflavonoids showed significant

antifeedant activity at 100 ppm, the highest concentrations tested (Table 1, Part A). The isoflavonoids all showed a dose-dependent decrease in activity, with maackiain retaining its antifeedant activity down to 10 ppm and judaicin down to 50 ppm. Similarly, all four isoflavonoids decreased the amount eaten by *H. armigera* larvae in the no-choice bioassay when tested at 100 ppm (Table 1, Part B). Although all four isoflavonoids showed antifeedant activity in both the choice and no-choice bioassay, the observations made during the no-choice bioassay indicated differences in their mode of action. For example, judaicin 7-*O*-glucoside and maackiain deterred the larvae from sampling the isoflavonoid-treated disks (latency to sampling) for longer than 2-methoxyjudaicin or judaicin. However, data from the whole 8-hr experiment showed that maackiain was the only isoflavonoid to decrease the amount of time larvae spent in contact with treated disks. When the behavior of the insects was videotaped, it was not possible to measure ingestion patterns with accuracy because the insects would often position themselves with their mouthparts out of view of the camera. Therefore, we could not accurately record the time spent feeding or the amount consumed per hour. However, despite this limitation, the results suggest that insects were deterred from feeding on maackiain-treated disks from the start of the experiment, and although they did eat some of the maackiain-treated disk, the insects did not habituate to the compound during the course of the experiment. In contrast, the initial deterrent activity of judaicin-7-*O*-glucoside decreased, and the insects spent as much time on the disks treated with this compound as they did on the control disks or disks treated with 2-methoxyjudaicin or judaicin. Thus, although the judaicin-based isoflavonoids deter *H. armigera* larvae from feeding, they are not as active as maackiain.

Effect of Combinations of Isoflavonoids on Feeding Behavior. The feeding index was highest for combinations that had either maackiain or judaicin, and the highest indices were obtained from those combinations that contained judaicin with either judaicin-7-*O*-glucoside or maackiain (Table 1, Part C). The feeding indices recorded did not reflect a consistent interaction among the isoflavonoids. For example, the feeding indices for the combinations of maackiain (50 ppm) with judaicin (50 ppm) and of judaicin-7-*O*-glucoside (50 ppm) with judaicin (50 ppm) were higher than those recorded for the compounds tested singly at 50 ppm. This suggests additive interactions. In contrast, the feeding index for the combination of 2-methoxyjudaicin with judaicin-7-*O*-glucoside was similar to that recorded for 2-methoxyjudaicin at 50 ppm, but lower than that recorded for judaicin-7-*O*-glucoside at 50 ppm. This suggests an antagonist interaction between the compounds. These differences in behavioral responses to the combinations could reflect differences in the molecular interactions between the compounds and the receptors on the larvae's maxillary taste sensilla. For example, the judaicin-type isoflavonoids could be competing for similar receptor sites that differ from those

TABLE 1. EFFECT OF COMPOUNDS ON FEEDING BEHAVIOR OF FIFTH STADIUM LARVAE OF *Helicoverpa armigera*

Part A. Choice bioassay: different concentrations (ppm) of four isoflavonoids tested against of *H. armigera* ($N = 10$)

	Feeding index (mean ± SEM)[a]			
	Judaicin 7-*O*-glucoside	2-Methoxy judaicin	Maackiain	Judaicin
100	35 ± 4.2*[b]	32 ± 8.1*[b]	53 ± 5.6**[b]	49 ± 11.5**[b]
50	20 ± 7.3	12 ± 3.4	30 ± 7.1*[b]	41 ± 9.4*
10	5 ± 6.7	10 ± 5.9	33 ± 6.6*[b]	15 ± 5.5
1	−3 ± 8.4	3 ± 4.1	21 ± 6.3	5 ± 3.6

Part B. No-choice bioassay: effect of isoflavonoids (100 ppm) on latency (min) to feeding, amount eaten (%) and time (%) spent by larvae in contact with the disks ($N = 8$)

	Mean ± SEM				
	Control	Judaicin 7-*O*-glucoside	2-Methoxy judaicin	Maackiain	Judaicin
Latency (min)	25 ± 2.3	52 ± 5.4*[c]	38 ± 4.9	57 ± 5.8*[c]	45 ± 6.7
Amount eaten(%)	62 ± 3.8	29 ± 3.8**[c]	25 ± 3.8**[c]	15 ± 2.7**[c]	26 ± 7.4**[c]
Time on disk(%)	39 ± 3.3	30 ± 3.2	28 ± 3.1	19 ± 2.9**[c]	25 ± 4.6

Part C. Choice bioassay: Treatment disk (T) treated with two isoflavonoids each at 50 ppm ($N = 10$)

	Feeding index (mean ± SEM) isoflavonoids[a]		
In combination with	2-Methoxy judaicin	Maackiain	Judaicin
Judaicin-7-*O*-glucoside	14 ± 4.5	43 ± 5.2*[b]	67 ± 6.3**[b]
2-Methoxyjudaicin		37 ± 5.0*[b]	39 ± 4.5*[b]
Maackiain			59 ± 8.4*[b]

Part D. Choice bioassay: treatment disk (T) treated with an isoflavonoid (50 ppm) and chlorogenic acid ((50 ppm) $N = 10$)

	Feeding index[a] (mean ± SEM)[a]			
Judaicin-7-*O*-glucoside	2-Methoxy judaicin	Maackiain	Judaicin	Chlorogenic acid
18 ± 7.7	32 ± 8.4*[b]	49 ± 9.4**[b]	51 ± 4.8**[b]	18 ± 12.4

[a] [(C − T)/(C + T)] %.
[b] **$P < 0.01$, *$P < 0.05$, Wilcoxon matched pairs test.
[c] **$P < 0.05$, **$P < 0.01$ Mann-Whitney U test:isoflavonoids compared with control.

stimulated by maackiain. If this were the case, then more receptors could be stimulated by combinations that contained maackiain and judaicin-type isoflavonoids, than combinations that contained two judaicin-type compounds. However, this theory would not support the additive antifeedant activity recorded for the judaicin and judaicin-7-*O*-glucoside combination (Table 1, Part C).

Effect on Feeding of Isoflavonoids in Combination with Chlorogenic Acid. When tested in combination with sucrose, chlorogenic acid did not elicit an antifeedant response from *H. armigera* larvae in the choice experiment (Table 1, Part D). However, when chlorogenic acid was tested in combination with an isoflavonoid at 50 ppm, an antifeedant response was recorded from three of the isoflavonoids; 2-methoxyjudaicin, maackiain, and judaicin. The antifeedant activity of 2-methoxyjudaicin and maackiain in this combination was greater than when the compounds were tested singly (Mann-Whitney U test, $P < 0.01$). Thus, chlorogenic acid increased the potency of these two isoflavonoids.

Effect of Isoflavonoids on Development of Different Stadia of H. armigera. All four isoflavonoids decreased the weight gain of first stadium larvae, over a five-day exposure, whereas the weight gain of second, third, and fifth stadium larvae was only reduced by maackiain and judaicin (Table 2). None of the isoflavonoids decreased the weight gain of fourth stadium larvae. Overall, judaicin had the greatest effect on larval development.

Effect of Isoflavonoids on Feeding Behavior of a Range of Polyphagous Noctuids. *H. armigera* was the only one of the four species of noctuids to be deterred from feeding by all four isoflavonoids (Table 3). *S. littoralis* was deterred from feeding by judaicin, and *S. frugiperda* by maackiain, whereas none of the isoflavonoids elicited an antifeedant response from *H. virescens* or *S. exigua*.

TABLE 2. EFFECT OF ISOFLAVONOIDS ON DEVELOPMENT OF DIFFERENT STADIA LARVAE OF *H. armigera* WHEN OVERLAID (100 ppm) ONTO LARVAL DIET ($N = 25$)

	% weight gain relative to control (100%) mean ± SEM[a]			
Stadium	Judaicin 7-*O*-glucoside	2-Methoxy-judaicin	Maackiain	Judaicin
1	$50 \pm 9.6^{**a}$	$72 \pm 18.6^{*}$	$59 \pm 13.7^{**}$	$45 \pm 16.6^{**}$
2	94 ± 16.5	94 ± 2.8	$72 \pm 17.6^{*}$	$55 \pm 4.9^{**}$
3	88 ± 21.7	91 ± 7.7	$59 \pm 3.6^{*}$	$50 \pm 15.8^{**}$
4	100 ± 3.4	108 ± 2.7	83 ± 15.7	79 ± 23.7
5	98 ± 4.8	100 ± 2.3	$77 \pm 13.8^{*}$	$71 \pm 8.6^{*}$

[a] $^{*}P < 0.05$, $^{**}P < 0.01$: t test undertaken on weights of larvae after five days of exposure to the treated diets.

TABLE 3. EFFECT OF ISOFLAVONOIDS ON FEEDING BEHAVIOR OF RANGE OF NOCTUID LARVAE ($N = 10$)

	Feeding Index (mean ± SEM)[a]			
	Judaicin 7-*O*-glucoside	2-Methoxy judaicin	Maackiain	Judaicin
H. armigera	$35 \pm 4.2^{*b}$	$32 \pm 8.1^{*}$	$53 \pm 5.6^{**}$	$49 \pm 11.5^{**}$
H. viresecens	3 ± 5.8	8 ± 1.9	5 ± 7.2	22 ± 6.2
S. littoralis	-6 ± 10.5	11 ± 7.1	1 ± 6.6	$39 \pm 6.9^{*}$
S. exigua	-3 ± 3.4	-2 ± 3.9	1 ± 4.5	-4 ± 3.9
S. frugiperda	-8 ± 2.5	4 ± 2.8	$45 \pm 7.2^{*}$	23 ± 4.1

[a] $[(C - T)/(C + T)]$ %, $^{**}P < 0.01$, $^{*}P < 0.05$, Wilcoxon matched pairs test.

DISCUSSION

In the last decade, there has been a steady increase in our understanding of the defense mechanisms in plants. This is due in part to advances in genomics and related technologies that have increased our knowledge about the functional basis of plant defenses. However, unequivocal evidence that a specific mechanism or compound stops an insect from damaging a plant is rare. This could be due to the complexity of the interactions involved in plant–insect relationships and our tendency to concentrate on evaluating the activity of a narrow group of stimulating "sign" compounds present in host plants rather than the level of specific deterrents in nonhost plants (Bernays and Chapman, 1994). A role for sign compounds in host-selection behavior of monophagous or oligophaguos species is easier to justify than in polyphagous species such as *H. armigera.* It is clear, however, that individual polyphagous insects show preferences for plants, but the factors influencing these preferences are still unclear (Simmonds and Blaney, 1990; Bernays and Chapman, 1994).

Wild relatives of the cultivated chickpea contain a higher diversity of isoflavonoids (Stevenson and Veitch, 1998b); many might have been lost from cultivars in breeding for high yield and palatable crop plants. If we can identify antiinsect compounds in wild relatives of crops that are either missing or at low levels in the crop plant, then manipulating the level of these compounds in a crop plant could decrease the susceptibility of that crop to damage by polyphagous pests. This is a traditional approach and was used recently to screen wild relatives for trypsin inhibitors (Patankar et al., 1999). The diversity of protein inhibitors was greater in wild relatives than in cultivated species, but *H. armigera* had adapted to the inhibitors. Thus, before manipulating crop plants either via traditional breeding techniques or via molecular techniques, it is important that we have knowledge about the insects' responses to the target compounds.

This study has shown that four of the isoflavonoids that occur in wild relatives of chickpea modulate the feeding behavior and development of larvae of *H. armigera*. Overall, the most potent of the isoflavonoids tested was judaicin and maackiain, although their activity varied depending on the bioassay and the stadium being tested. Previous studies have shown that both isoflavonoids inhibit germination of the spores of *Fusarium oxysporum* f.sp. *ciceri* (Stevenson and Veitch, 1998a), but the antifungal activity of maackiain varies among pathogens (Gordon et al., 1980).

In the no-choice feeding bioassay, the fact that a high proportion (75%) of *H. armigera* larvae rejected the sucrose-treated control glass-fiber disks at first contact could indicate that sucrose at 0.05 M was not a potent stimulant on the disks. Sucrose is known to stimulate *H. armigera* larvae to feed on glass-fiber disks over a 18 to 24-hr bioassay (Simmonds et al., 1990), but the influence of sucrose on the acceptance or rejection behavior of sucrose-treated wet glass-fiber disks by larvae has not been previously reported. However, the fact that two of the isoflavonoids extended the duration of time taken before the insects fed on the disks, relative to the sucrose-treated disks, would suggest that these compounds decreased the palatability. This could be because they modulate the ability of the larvae to perceive sucrose, or they stimulate deterrent neurons in the insects' taste sensilla. The differences in activity among the isoflavonoids could reflect differences in the magnitude of the interaction with sucrose, the magnitude of the response of deterrent neurons, or the ability of the larvae to adapt to the compounds (Schoonhoven et al., 1998).

None of the compounds totally deterred the insects from feeding or contacting the disks, and often the insects remained motionless for periods of time before either moving off the disk or feeding. There was no evidence of acute postingestive toxicity in either the behavioral (Glendinning and Slansky, 1995) or development bioassays, as no larval mortality was recorded during the experiments. The reduction in growth could result from the antifeedant activity of the compounds or some interaction of the compounds with the utilization of nutrients (Felton and Gatehouse, 1996).

When the isoflavonoids were applied to a diet, the weights gained over the five-day experiment by early stadia larvae were affected more by the isoflavonoids than the later stadia. This could reflect an ability of the later stadia to tolerate or detoxify them. In this experiment, one concentration (100 ppm) was applied to the diet, so the ratio of compound to insect weight would differ among the stadia, and the early stadia would be exposed to a higher dose (larval weight relative to concentration on diet) than the later stadia. Despite these differences, the weights of second, third, and fifth stadia larvae exposed to maackiain- and judaicin-treated diets were lowered. Whether this would have resulted in larval mortality or just an increased duration of larval development before pupation was not investigated. Any compound that extends larval development has the potential to decrease larval

fitness and increase the probability of predation. However, the interactions between phenolics, such as the isoflavonoids, and plant oxidative enzymes in chickpeas could modulate the effect on insect development as well as decrease the efficacy of microbial agents, such as baculoviruses that are increasingly being used to control lepidopterous pests (Hoover et al., 1998).

The behavioral responses to the isoflavonoids varied among the different noctuids. *H. virescens* was not deterred by any of the isoflavonoids, and of the three species of *Spodoptera* tested, only *S. littoralis* and *S. frugiperda* were deterred by judaicin and maackiain, respectively. In fact, *H. armigera* was the only species to be responsive to the four isoflavonoids. The fact that the insects differ in their behavioral responses to the compounds suggests that specificity of the receptors in the peripheral sensory system varies among species. Because of our lack of knowledge about the specificity of the taste receptors on the gustatory neurons of these insects, we can not predict how a species will respond to a group of compounds. For example, previous experiments with azadirachtin-derived compounds showed that *S. littoralis* was more responsive to a range of the derivatives than heliothines, and *H. virescens* was more responsive than *H. armigera* (Blaney et al., 1990). In contrast, Simmonds et al. (1990) showed that *H. armigera* was more responsive to polyhydroxyalkaloids than polyphagous species of *Spodoptera* and *H. virescens*. Thus, modulating the levels of isoflavonoids in chickpeas might decrease the palatability of the plants to target insects such as *H. armigera* and susceptibility to fungal pathogens, but further experiments are needed to evaluate the activity of the isoflavonoids against other pests of *Cicer*.

This is the first study to have evaluated the role of these isoflavonoids in the resistance of chickpeas to *H. armigera*. We suggest that isoflavonoids in *Cicer* could play an important part in the ecology of *Cicer*–herbivore interactions and that the isoflavonoids accumulated in wild relatives of chickpea, if expressed in chickpeas, could decrease susceptibility to target pests and pathogens.

Acknowledgments—Insect bioassays were undertaken at RBG, Kew, under a MAFF licence. We acknowledge GlaxoWellcome for financial support to purchase the video equipment used in this study. The isoflavonoids were isolated as part of a Darwin Initiative Grant to P.S.

REFERENCES

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York.

BLANEY, W. M., SIMMONDS, M. S. J., LEY, S. V., ANDERSON, J. C., and TOOGOOD, P. L. 1990. Antifeedant effects of azadirachtin and structurally related compounds on lepidopterous larvae. *Entomol. Exp. Appl.* 55:149–160.

COWGILL, S. E., and LATEEF, S. S. 1996. Identification of antibiotic and antixenotic resistance to *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *J. Econ. Entomol.* 89:224–229.

DANIEL, S., and BARZ, W. 1990. Elicitor induced metabolic changes in cell cultures of chickpea cultivars resistant and susceptible to *Aschochyta rabiei*. *Planta* 182:279–286.

FELTON, G. W., and GATEHOUSE, J. A. 1996. Antinutritive plant defence mechanisms, pp. 373–416, *in* M. J. Lehane and P. F. Billingsley (eds.). Biology of the Insect Midgut. Chapman and Hall, London.

FREEDMAN, B., NOWAK, L. J., KWOLEK, W. F., BERRY, D. C., and GUTHRIE, W. D. 1979. A bioassay for plant-derived pest control agents using the Eurpean corn borer. *J. Econ. Entomol.* 72:541–545.

GLENDINNING, J. L., and SLANSKY, D. 1995. Consumption of a toxic food by caterpillars increases with dietary exposure: Support for a role of induced detoxification enzymes. *J. Comp. Physiol. A* 176:337–345.

GORDON, M. A., LAPA, E. W., FITTER, M. S., and LINDSAY, M. 1980. Susceptibility of zoopathogenic fungi to phytoalexins. *Antimicob. Agents Chemother.* 17:120–123.

HOOVER, K., STOUT, M. J., ALANIZ, S. A., HAMMACK, B. D., AND DUFFEY, S. S. 1998. Influence of induced plant defences in cotton and tomato on the efficacy of baculoviruses on noctuid larvae. *J. Chem. Ecol.* 24:253–271.

ISMAN, M. B., and DUFFEY, S. S. 1982. Toxicity of the tomato phenolic compounds to the fruitworm *Heliothis zea*. *Entomol. Exp. Appl.* 31:370–376.

KIMMINS, F. M., PADGHAM, D. E., and STEVENSON, P. C. 1995. Growth inhibition of the cotton bollworm larvae by caffeoyl quinic acids from the wild groundnut *Arachis paraguariensis*. *Insect Sci. Appl.* 16:363–368.

LAZZARO, M. D., and THOMSON, W. W. 1995. Seasonal variation in hydrochloric acid, malic acid, and calcium ions secretered by the trichomes of chickpea (*Cicer arietinum*). *Physiol. Plant.* 94:291–297.

NOLDUS, L. P. J. J. 1991. The observer: a software system for collection and analysis of observational data. *Behav. Res. Methods Instrum. Comput.* 23:415–429.

PATANKAR, A. G., HARSULKAR, A. M., GIRI, A. P., GUPTA, V. S., SAINANI, M. N., RANJEKER, P. K., and DESHPANDE, V. V. 1999. Diversity in inhibitors of trypsin and *Helicoverpa armigera* gut proteinases in chickpea (*Cicer arietinum*) and its wild relatives. *Theor. Appl. Genet.* 99: 3–4.

SCHOONHOVEN, L. M., JERMY, T., and VAN LOON, J. J. A. 1998. Insect–Plant Biology from Physiology to Evolution. Chapman & Hall, London.

SIMMONDS, M. S. J., and BLANEY, W. M. 1990. Gustatory codes in lepidopterous larvae. *Symp. Biol. Hung.* 39:17–27.

SIMMONDS, M. S. J., BLANEY, W. M., and FELLOWS, L. E. 1990. Behavioural and electrophysiological study of antifeedant mechanisms associated with polyhydroxyalkaloids. *J. Chem. Ecol.* 16:3167–3196.

SIMMONDS, M. S. J., BLANEY, W. M., and SCHOONHOVEN, L. M. 1992. Effects of larval diet and larval age on the responsiveness of taste neurones of *Spodoptera littoralis* to sucrose. *J. Insect Physiol.* 38:249–257.

STEVENSON, P. C., and HAWARE, M. P. 1999. Maackiain in *Cicer bijugum* associated with resistance to Botrytis Grey Mould. *Biochem. Syst. Ecol.* 27:761–767.

STEVENSON, P. C., and VEITCH, N. C. 1996. Isoflavenes from the roots of *Cicer judaicum*. *Phytochemistry* 43:695–700.

STEVENSON, P. C., and VEITCH, N. C. 1998a. A 2-arylbenzofuran from roots of *Cicer bijugum* associated with fusarium wilt resistance. *Phytochemistry* 48:947–951.

STEVENSON, P. C., and VEITCH. N .C. 1998b. The distribution of isoflavonoids in *Cicer*. *Phytochemistry* 48:995–1001.

STEVENSON, P. C., TURNER, H. C., and HAWARE M. P. 1997. Phytoalexin accumulation in the roots of chickpea seedlings associated with resistance to fusarium wilt. *Physiol. Mol. Plant Pathol.* 50:167–178.

VEITCH, N. C., and STEVENSON, P. C. 1997. 2-Methoxyjudaicin, an isoflavene from the roots of *Cicer bijugum*. *Phytochemistry* 44:1587–1589.

YOSHIDA, M., COWGILL, S. E., and WIGHTMAN, J. A. 1995. Mechanism of resistance to *Helicoverpa armigera* (Lepidoptera, Noctuidae) in chickpea—role of oxalic-acid in leaf exudates as an antibiotic factor. *J. Econ. Entomol.* 88:1783–1786.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

RESPONSE OF BROWN TREESNAKES (*Boiga irregularis*) TO MAMMALIAN BLOOD: WHOLE BLOOD, SERUM, AND CELLULAR RESIDUE

DAVID CHISZAR,[1,*] THOMAS M. DUNN,[3] PATRICK STARK,[1] and HOBART M. SMITH[2]

[1]*Department of Psychology*
[2]*E.P.O. Biology*
University of Colorado
Boulder, Colorado 80309-0345
[3]*Department of Psychology*
University of Northern Colorado
Greeley, Colorado, 80639

(Received September 22, 1999; accepted December 17, 2000)

Abstract—Brown treesnakes (*Boiga irregularis*) responded with more tongue flicks to blood (from rabbits, rats, and mice) than to water. When rat blood was centrifuged at 3500 rpm for 5 min, separating serum from cellular residue, snakes responded strongly to serum but not to cellular residue.

Key Words—Predation, foraging, blood, serum, cellular residue, brown treesnakes, bait, attractant, Guam, traps.

INTRODUCTION

Problems on Guam subsequent to the introduction of the brown treesnake (*Boiga irregularis*) near the end of World War II have been described by several authors (e.g., Engbring and Fritts, 1988; Fritts, 1988; Savidge, 1987). Consequently, great attention has been given to *B. irregularis* in an attempt to measure and reduce its populations on Guam and to prevent the spread of this snake to other Pacific islands (Fritts, 1987; Fritts and Scott, 1985; Rodda and Fritts, 1992). Part of this effort required study of the diet and predatory behavior of *B. irregularis* (Greene, 1989; Fritts and McCoid, 1991; Rodda, 1992; Savidge, 1988) and the development of baits to lure snakes to traps (Chiszar et al., 1988, 1999; Fritts et al., 1989;

*To whom correspondence should be addressed. e-mail: chiszar@clipr.colorado.edu

0098-0331/01/0500-0979$19.50/0

Rodda et al., 1992, 1999). In this context it, was discovered that *B. irregularis* responded strongly to mammalian blood in laboratory tests (Chiszar et al., 1992, 1993). Whole blood was used in those tests. Here we report experiments that separate the effects of serum and cellular residue.

Because serum contains blood proteins, especially albumin and globulins, and because these materials are biologically active in attracting predators and scavengers and as allergens (e.g., Chen, 1967; Goodman, 1958; Gurin and Carr, 1971), we expected serum to be responsible for the effects of whole blood seen in our earlier studies.

METHODS AND MATERIALS

Preliminary Experiment. By using the methods of Burghardt (1970), 10 *B. irregularis* from Guam were presented on alternate days with cotton-tipped applicators soaked in tap water, rabbit serum, or cellular residue (in random order; one trial with each chemical), and we counted the number of tongue flicks (TF) during the 60 sec immediately after the presentations. The mean TFs for water, serum, and residue presentations were 15.2, 31.4 and 15.8, respectively (pooled SE $= 7.5$). The mean for serum presentations was significantly higher than the means for either of the other presentations by paired *t* tests ($t = 2.97$, 2.55, respectively, $df = 9$, $P < 0.05$). These results encouraged us to conduct additional studies.

Experiment 1. Although cotton-tipped applicators are quite effective in laboratory tests, especially with small snakes and lizards (see Burghardt, 1970; Cooper, 1994; Halpern, 1992; for reviews), we wanted a technique that could also be used to present chemical cues in traps on Guam. Tampons can absorb considerable quantities of liquid (6–15 g) and can easily be incorporated into traps. Since specimens of *B. irregularis* attacked and ingested tampons soaked with human menstrual blood (Chiszar et al., 1993), tampons were used as vehicles in experiment 1. Our first step, then, was to demonstrate that rodent blood in tampons was a salient stimulus for *B. irregularis*.

Tampax tampons, super absorbency size, were used to present chemical cues. Two tampons were presented simultaneously to snakes, each wrapped in hardware mesh bags to prevent ingestion, but to permit chemical diffusion and inspection by snakes. One tampon contained 1.0 cc of tap water, the other contained 1.0 cc of whole blood from either mice (*Mus musculus*) or rats (*Rattus norvegicus*). Blood was collected through cardiocentesis by Albert Petkus, DVM and chairperson of the University of Colorado Animal Care Committee, and was stored at 6°C until used (within 48 hr). The mesh bags containing tampons were fastened to a plywood base (23 × 19 cm), and this apparatus was gently placed into a snake's cage for 5 min, during which we recorded TFs directed at each bag. This method of presenting objects to snakes has been used successfully in previous studies

(e.g., Duvall et al., 1980). Five *B. irregularis* were observed with water and rat blood and five additional snakes were observed with water and mouse blood. All snakes were adult, long-term captives, maintained at 26°C on a 12L:12D photoperiod with the photophase centered at noon. Snakes had been deprived of food (mice and rats) for one week prior to all tests. Water and blood were warmed to 37°C prior to being absorbed into tampons. Following each trial, tampons were discarded and the mesh baskets were washed, rinsed thoroughly, and dried.

Experiment 2. Having shown that tampons can be used as vehicles for whole rodent blood, our next step was to partition blood into serum and cellular residue, presenting each of these substances to *B. irregularis* as in experiment 1.

Tampons were prepared with either 1.0 cc of water, 1.0 cc of rat serum, or 1.0 cc of rat cellular residue. Only the cellular residue produced red colored tampons. Five *B. irregularis* received tampons containing cellular residue and water, and five additional snakes received tampons containing serum and water. The tampons were placed in the same wire mesh baskets as in experiment 1, and trials were again 5 min long. Tongue flicks directed at each of the two tampons were recorded with hand-held counters. Rat blood was collected by cardiocentesis as in experiment 1 and was centrifuged at 3500 rpm for 5 min. Supernatant and residue were separated and the cuvettes were stored at 6°C until used (within 48 hr). As in experiment 1, materials were warmed to 37°C prior to being absorbed into tampons. Tampons were used only once, and the mesh baskets were washed, rinsed, and dried prior to the next trial.

RESULTS

Experiment 1. Table 1 presents mean TFs during the 5-min tests, where it can be seen that significantly more TFs were directed at blood-soaked tampons than at water-soaked ones. Relative effectiveness of mouse and rat blood was assessed by a 2×2 mixed analysis of variance (ANOVA) in which donor species was a between-subjects factor since different snakes responded to mouse and to rat blood,

TABLE 1. TONGUE FLICKS BY BROWN TREESNAKES TO TAMPONS CONTAINING WATER AND WHOLE RODENT BLOOD[a]

Source of blood	Snakes observed (N)	Tongue flicks/5 min (mean ± SEM)		Paired t tests ($df = 4$)
		Water	Whole blood	
Mice	5	93.6 ± 28.5	280.2 ± 53.3	2.85[a]
Rats	5	48.0 ± 4.5	169.2 ± 32.7	4.21[a]

[a] $P < 0.05$.

TABLE 2. TONGUE FLICKS BY FIVE BROWN TREESNAKES TO TAMPONS CONTAINING WATER AND CELLULAR RESIDUE FROM RAT BLOOD OR TAMPONS CONTAINING WATER AND RAT SERUM

	Tongue flicks/5 min (mean ± SEM)		
Content of experimental tampon	Control (water)	Experimental	Paired t tests ($df = 4$)
Cellular residue	49.8 ± 20.3	31.0 ± 9.1	0.67
Serum	26.8 ± 10.6	63.8 ± 10.5	2.89[a]
t tests for independent groups ($df = 8$)	0.98	3.01[a]	

[a] $P < 0.05$.

and experimental (blood) versus control (water) trials were treated as a repeated measures factor as each snake responded to both conditions. Number of TFs aimed at each tampon during the 5-min tests was the dependent variable. This analysis revealed a strong effect of experimental versus control trials ($F = 41.76$, $df = 1$, 8, $P < 0.01$), but neither the main effect of donor species nor the interaction of donor species × experimental versus control trials was reliable ($Fs = 1.99$, 1.87, respectively, $df = 1$, 8, $P > 0.05$). Hence, the snakes responded strongly to blood, and the source of the blood exerted no significant effect.

Experiment 2. Table 2 presents a summary of the data, and it is clear that the snakes' response to cellular residue did not differ from their response to water, but the snakes responded significantly more strongly to serum than to water. These assertions are confirmed by the t tests for paired comparisons reported in the right column of Table 2. We also compared responses to the two control tampons and to the two experimental tampoons (see t tests for independent groups reported in the last row of Table 2). Mean number of TFs aimed at the two control tampons did not differ significantly; but, the mean number of TFs aimed at the tampon containing serum exceeded the value aimed at the tampon contaning cellular reside.

Hence, based on the paired comparisons as well as the independent ones, we conclude that response to serum was stronger than response to cellular residue.

DISCUSSION

Most previous tests of *B. irregularis* response to blood have used cotton-tipped applicators to present whole blood. Now we show that another methodology produces convergent results, revealing that positive responses seen earlier were not restricted to the protocol used in those experiments. Further, we demonstrated that serum, rather than cellular residue, contained the components responsible for the snakes' response to blood.

Similar results were obtained by Gurin and Carr (1971) with the marine snail, *Nassarius obsoletus*, a deposit feeder and a facultative carrion feeder. Snails extended their proboscises to a variety of substances arising from dead animals (Copeland, 1918; Carr, 1967). Subsequent research showed that human plasma elicited a strong response, and the active components had molecular weights of 1000 or greater. Lipoproteins and fibrinogen had relatively little stimulative capacity (DeLalla and Gofman, 1954), whereas serum albumin had strong effects on the snails, accounting for up to 70% of the total activity of plasma (Gurin and Carr, 1971). Accordingly, we intend to try similar experiments with *B. irregularis* in our attempt to identify attractants that can increase the success of trapping efforts on Guam (see Burghardt et al., 1988; Halpern et al., 1984; Liu et al., 1999; for cognate experiments on garter snakes).

REFERENCES

BURGHARDT, G. M. 1970. Chemical perception in reptiles, pp. 241–308, *in* J. W. Johnston, Jr., D. C. Moulton, and A. Turk (eds.). Advances in Chemoreception I. Communicaton by Chemical Signals. Appleton-Century-Crofts, New York.

BURGHARDT, G. M., GOSS, S. E., and SCHELL, F. M. 1988. Comparison of earthworm- and fish-derived chemicals eliciting prey attack by garter snakes (*Thamnophis*). *J. Chem. Ecol.* 14:855–881.

CARR, W. E. S. 1967. Chemoreception in the mud snail, *Nassarius obsoletus*. I. Properties of stimulatory substances extracted from shrimp. *Biol. Bull.* 132:90–105.

CHEN, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* 242:173–181.

CHISZAR, D., KANDLER, K., LEE, R., and SMITH, H. M. 1988. Stimulus control of predatory attack in the brown tree snake (*Boiga irregularis*). 2. Use of chemical cues during foraging. *Amphibia-Reptilia* 9:77–88.

CHISZAR, D., FOX, K., and SMITH, H. M. 1992. Stimulus control of predatory behavior in the brown tree snake (*Boiga irregularis*). IV. Effect of mammalian blood. *Behav. Neural Biol.* 57:167–169.

CHISZAR, D., DUNN, T. M., and SMITH, H. M. 1993. Response of brown tree snakes (*Boiga irregularis*) to human blood. *J. Chem. Ecol.* 19:91–96.

CHISZAR, D., DUNN, T. M., and SMITH, H. M. 1999. Predatory behavior of brown treesnakes (*Boiga irregularis*): Laboratory studies of chemical attractants, pp. 187–195, *in* G. H. Rodda, Y. Sawai, D. Chiszar, and H. Tanaka (eds.). Problem Snake Management: The Habu and the Brown Treesnake. Comstock Publishing Assoc., Ithaca, New York.

COOPER, W. E., JR. 1994. Chemical discrimination by tongue-flicking in lizards: A review with hypotheses on its origin and its ecological and phylogenetic relationships. *J. Chem. Ecol.* 20:439–487.

COPELAND, M. 1918. The olfactory reactions and organs of the marine snails *Alectrion obsoleta* (Say) and *Busycon canaliculatum* (Linn). *J. Exp. Zool.* 25:177–227.

DELALLA, O. F., and GOFMAN, J. W. 1954. Ultracentrifugal analysis of serum lipoproteins. *Methods Biochem. Anal.* 1:459–478.

DUVALL, D., SCUDDER, K. M., and CHISZAR, D. 1980. Rattlesnake predatory behavior: Mediation of prey discrimination and release of swallowing by cues arising from envenomated mice. *Anim. Behav.* 28:674–683.

ENGBRING, J., and FRITTS, T. H. 1988. Demise of an unsular avifauna: The brown tree snake on Guam. *Trans. West. Sect. Wildl. Soc.* 24:31–37.

FRITTS, T. H. 1987. Movements of snakes via cargo in the Pacific region. *Elapaio* 47:17–18.

FRITTS, T. H. 1988. The brown tree snake, *Boiga irregularis*, a threat to Pacific islands. Biol. Rep. 88(31). US Fish and Wildlife Service, Washington, D.C.

FRITTS, T. H., and MCCOID, M. J. 1991. Predation by the brown tree snake on poultry and other domesticated animals in Guam. *The Snake* 23:75–80.

FRITTS, T. H., and SCOTT, N. J., JR. 1985. The brown tree snake on Guam: Studies of its ecology, control, and threats to other islands. US Fish and Wildlife Service., Portland, Orlando.

FRITTS, T. H., SCOTT, N. J., JR., and SMITH, B. E. 1989. Trapping *Boiga irregularis* on Guam using bird odors. *J. Herpetol*. 23:189–192.

GOODMAN, D. S. 1958. The interaction of human serum albumin with long-chain fatty acid anions. *J. Am. Chem. Soc*. 80:3892–3898.

GREENE, H. W. 1989. Ecological, evolutionary, and conservation implications of feeding biology in old world cat snakes, genus *Boiga* (Colubridae). *Proc. Calif. Acad. Sci*. 46:193–207.

GURIN, S., and CARR, W. E. 1971. Chemoreception in *Nassarius obsoletus*: The role of specific stimulatory proteins. *Science* 174:293–295.

HALPERN, M. 1992. Nasal chemical senses in reptiles: Structure and function, pp. 423–523, *in* C. Gans and D. Drews (eds.). Biology of the Reptilia, Vol. 18, Hormones, Brain and Behavior. University of Chicago Press, Chicago.

HALPERN, M., SCHULMAN, N., SCRIBANI, L., and KIRSCHENBAUM, D. M. 1984. Characterization of vomeronasally-mediated response-eliciting components of earthwork wash-II. *Pharmacol. Biochem. Behav*. 21:655–662.

LIU, J., CHEN, P., WANG, D., and HALPERN, M. 1999. Signal transduction in the vomeronasal organ of garter snakes: Ligand-receptor binding-mediated protein phosphorylation. *Biochim. Biophys. Acta* 1450:320–330.

RODDA, G. H. 1992. Foraging behaviour of the brown tree snake, *Boiga irregularis. Herpetol. J*. 2:110–114.

RODDA, G. H., and FRITTS, T. H. 1992. Sampling techniques for an arboreal snake, *Boiga irregularis. Micronesica* 25:23–40.

RODDA, G. H., RONDEAU, R. J., FRITTS, T. H., and MAUGHAN, O. E. 1992. Trapping the arboreal snake, *Boiga irregularis. Amphibia-Reptilia* 13:47–56.

RODDA, G. H., FRITTS, T. H., CLARK, C. S., GOTTE, S. W., and CHISZAR, D. 1999. A state-of-the-art trap for the Brown Treesnake, pp. 268–305, *in* G. H. Rodda, Y. Sawai, D. Chiszar, and H. Tanaka (eds.). Problem Snake Management: The Habu and the Brown Treesnake. Comstock Publishing Company, Ithaca, New York.

SAVIDGE, J. A. 1987. Extinction of an island forest avifauna by an introduced snake. *Ecology* 68:660–668.

SAVIDGE, J. A. 1988. Food habits of *Boiga irregularis*, an introduced predator on Guam. *J. Herpetol.* 22:275–282.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

HOST FINDING AND OVIPOSITION BEHAVIOR IN A CHRYSOMELID SPECIALIST—THE IMPORTANCE OF HOST PLANT SURFACE WAXES

CAROLINE MÜLLER[1,2,*] and MONIKA HILKER[1]

[1]*Institut für Biologie*
Freie Universität Berlin
D-12163 Berlin, Germany

[2]*Institute of Evolutionary and Ecological Sciences*
University of Leiden
P.O. Box 9516, 2300 RA Leiden, The Netherlands

(Received June 5, 2000; accepted January 15, 2001)

Abstract—Although several studies have elucidated the role of plant epicuticular waxes in host recognition and oviposition by herbivorous insects, there is little known about this subject in Chrysomelidae. In the present study, chemical cues for host recognition behavior and oviposition by the monophagous chrysomelid species *Cassida stigmatica* were investigated with special regard to surface waxes of the host plant, *Tanacetum vulgare* (Asteraceae). After hibernation in the soil, adults of this species must climb the plant's petioles, which emerge from the ground in spring. The response of adult *C. stigmatica* to contact and volatile cues from petioles and leaves of *T. vulgare* was investigated in a "stem arena," in which differently treated petioles and petiole dummies were offered. Volatile and contact cues of *T. vulgare* petioles served as well for host recognition. The contact cues were isolated from the petioles and leaves by hexane extraction and by cellulose acetate treatment, which removed cuticular waxes. The attractive volatiles were not extracted sufficiently by hexane. To examine the role of cuticular waxes of the host leaf surface in oviposition, female *C. stigmatica* were offered intact leaflets and leaflets from which cuticular waxes had been stripped by cellulose acetate treatment. Females did not discriminate between intact and stripped leaflets when only the upper leaf surfaces were offered. However, when the lower leaf surfaces that are generally used as oviposition sites were offered, *C. stigmatica* preferred to lay eggs on intact leaflets. We conclude that waxes of the lower leaf surface contain crucial information for oviposition in *C. stigmatica*.

Key Words—*Cassida stigmatica*, Chrysomelidae, *Tanacetum vulgare*, Asteraceae, surface waxes, oviposition, host finding.

* To whom correspondence should be addressed. e-mail: roca2911@hotmail.com

0098-0331/01/0500-0985$19.50/0

INTRODUCTION

Herbivorous insects are attracted to their host plants by visual and volatile cues. Upon contact with the host plant, surface morphology and chemistry offer numerous additional stimuli for host recognition and acceptance by the insect. The influence of plant epicuticular waxes on insect host plant acceptance has become an area of increasing interest (Bernays et al., 1976, 1985; Städler and Roessingh, 1990; Eigenbrode and Espelie, 1995; Spencer, 1996; Eigenbrode et al., 1998, 1999). Plant waxes can affect arthropod herbivores (and also their natural enemies) in different ways. The morphology of the cuticle influences the ability of the insect to attach to the host plant via its tarsi (Stork, 1980; Schoonhoven et al., 1998). Extracts of surface waxes, mainly composed of *n*-alkanes, alkyl esters, free fatty alcohols, and free fatty acids (Eigenbrode and Espelie, 1995), can evoke arresting behavior (Klingauf, 1971; Varela and Bernays, 1988) and stimulate feeding (Bernays et al., 1976; Eigenbrode and Pillai, 1998; Lin et al., 1998) and oviposition of insects (Städler, 1986; Renwick et al., 1992). Morphological and chemical characteristics differ with respect to the upper and lower leaf surfaces (Premachandra et al., 1993; Eigenbrode and Espelie, 1995; Eigenbrode et al., 1998, 1999). The effects of these differences on host plant acceptance by insects have yet to be investigated.

The removal of cuticular waxes from plants has traditionally been carried out by dipping the plant material in various solvents for a few seconds (Bohm and Constant, 1990; Renwick et al., 1992; Brooks et al., 1996). This procedure chemically alters the plant material. Oxidative enzymatic reactions in the leaf tissue cause the treated leaves to turn brown and wilt (Whitaker and Lee, 1995). To remove the surface waxes without further damage of the plant tissue, Baker et al. (1983) developed the cellulose acetate stripping method, which removes the epicuticular waxes, but keeps the remaining plant tissue intact.

The role of plant surface waxes in host finding and oviposition of herbivorous insects has been investigated mainly in species of the taxa Orthoptera, Lepidoptera, Diptera, and Aphidinae. To our knowledge, only two studies examined the effect of plant surface waxes on feeding behavior of Chrysomelidae (Adati and Matsuda, 1993; Lin et al., 1998). Possible impacts of plant waxes on chrysomelid oviposition behavior have not yet been considered.

The monophagous chrysomelid species *Cassida stigmatica* Suffr. is specialized on tansy (*Tanacetum vulgare* L., Asteraceae) (Koch, 1992; Müller, 1999). Adults of *C. stigmatica* must find and recognize their host plant when they emerge in spring from the soil after hibernation. Their host plant, *T. vulgare*, is usually in the initial stages of development at this time. The young leaves with petioles and 10–20 leaflets have emerged from the ground, but the plant stem is not yet developed. The adults climb the petioles of the young leaves searching for feeding and oviposition sites.

This study focuses on the olfactory and contact surface cues, in particular plant surface waxes, that influence the host plant recognition process of adult *C. stigmatica*. The beetle's choice of petiole to climb and leaflet on which to oviposit was investigated by laboratory bioassays.

METHODS AND MATERIALS

Insects. Adults of *C. stigmatica* were collected at ruderal sites in Berlin, Germany, in May 1998, and placed in plastic containers (20 × 20 × 6 cm) with a gauze lid (120 μm mesh). The bottom of each container was covered with filter paper and the adults were fed on leaves of *T. vulgare*. Insects were reared at 20°C, 75% relative humidity, and 16L : 8D.

Extract of T. vulgare. To isolate components of low polarity from petioles and leaves of *T. vulgare*, material was harvested from young plants and lyophilized (Christ, alpha 2–4) for 24 hr. Two grams of dry leaf material was extracted with hexane in a Soxhlet apparatus (100 ml) for 8 hr. The extract was concentrated to 8 ml, using a rotary evaporator.

Removal of Plant Cuticular Waxes. To obtain plant material of *T. vulgare* without surface waxes, the cuticular waxes were removed from the surface of leaflets and petioles using the cellulose acetate stripping method (Baker et al., 1983). Cellulose acetate was dissolved in acetone (5–10% w/v) by ultrasonic treatment for 2 hr. leaflets or petioles of young leaves were dipped for 1 sec in the solution. After evaporation of the acetone, the white film that appeared on the surface of the sample material was carefully removed with forceps.

General Procedure of Host Plant Recognition Bioassays. In order to examine the role of petiole stimuli in the host finding of adult *C. stigmatica*, the "stem arena" described by Müller and Hilker (2000) was employed in a modified form. Petioles (2.5 cm) of freshly sprouted, field-collected *T. vulgare* and differently treated, wooden petiole dummies (toothpicks) were offered vertically to adult *C. stigmatica* in an open Petri dish (5.5 cm dia. × 1 cm high). Four test and four control petioles were placed in alternating sequence and equidistant near the outer edge of the dish. The bottom of the Petri dish was filled with soil and covered by filter paper to fix the petioles and dummies in place. Bioassays were conducted at 25°C under a central spotlight (55 cm above the arena, 1200 lux) in a dark room.

Adult females were starved for a period of 3 hr prior to testing. An individual was placed in the center of the arena. After 2 min of acclimatization, the time spent by the beetle walking (climbing) on test and control petioles during a 10-min period was recorded using the software Noldus Observer program 3.0 (Wageningen, Netherlands) (Noldus et al., 1991). Each bioassay was replicated 20 times. Solvents used were allowed to evaporate for 2 min prior to each bioassay. All petioles and treated petiole dummies were renewed for every trial. Females

that were inactive for more than 50% of the observation time were excluded from the analysis. Durations of walking (climbing) on test and control petioles were statistically compared by the Wilcoxon signed-rank test for paired differences.

Host Plant Recognition Bioassay of Volatile Cues. To study the females' response to volatiles, test and control petioles were offered wrapped with perforated filter paper tubes (dia. 0.5 cm) to prevent direct contact. The perforation of the filter paper was done with a needle (20 holes/cm^2; hole diameter: 0.5 mm). The following samples were offered to a female: (a) petioles of *T. vulgare* (test) and untreated petiole dummies (control), (b) test petiole dummies with 15 μl hexane extract of *T. vulgare* (see above) (15 μl is equivalent to 40 mg fresh plant material, the average weight of a 2.5-cm-long petiole) and control petiole dummies with 15 μl hexane.

Host Plant Recognition Bioassay of Contact Cues. To test the females' response to a combination of volatile and contact cues, the following samples were offered allowing direct contact (without filter paper tubes): (a) petioles of *T. vulgare* (test) and untreated petiole dummies (control), (b) petiole dummies with 15 μl hexane extract of *T. vulgare* (test) and petiole dummies with 15 μl hexane (control), (c) petioles of *T. vulgare* with intact wax cover (untreated) and petioles of *T. vulgare* after cellulose acetate treatment.

Oviposition Bioassays of Plant Cuticular Waxes. To test the influence of leaf surface characteristics for oviposition, a gravid female *C. stigmatica* was offered two young field-collected adjacent leaflets of *T. vulgare* in a Petri dish (5.5 cm dia. $\times$ 1 cm high). The dish was lined with moistened filter paper (200 μ water) to keep the leaflets turgid. One of the leaflets was offered untreated (with intact wax layer), while the other was treated with cellulose acetate. Both leaflets were offered either with their upper surface up or with their lower surface up. Prior to testing, females were kept without plants for 2 hr. After 9 hr, the number of eggs was counted on both leaflets and statistically compared by the Wilcoxon signed-rank test for paired samples.

RESULTS

Host Plant Recognition Bioassay of Volatile Cues. Volatiles from young petioles serve females of *C. stigmatica* as cues to recognize the host plant. The beetles significantly preferred wrapped *T. vulgare* petioles with no access to petiole contact cues over wrapped petiole dummies. The duration of walking was longer on the wrapped petioles than on petiole dummies (Figure 1, treatment A). Dummies treated with a hexane extract of *T. vulgare* petioles and leaflets and then wrapped with perforated filter paper were not attractive to the females (Figure 1, treatment B). The duration of walking on test and control petioles did not differ significantly. Thus, the attractive volatiles of petioles could not be isolated by our hexane extraction of host leaves with petioles and leaflets included.

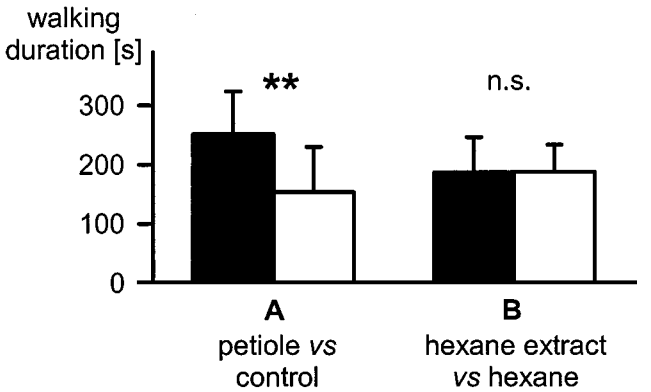


FIG. 1. Effect of volatile stimuli of the host plant *Tanacetum vulgare* on females of *Cassida stigmatica* (seconds, Duration of walking mean ± SD) on differently treated petioles during an observation period of 10 min is given. Test and control petioles were wrapped with filter paper to prevent contact. Mean duration of walking: (A) on petioles of *T. vulgare* (black bars) and petiole dummies (white bars); (B) on dummies treated with a hexane extract of *T. vulgare* (black bars) and dummies with the solvent only (white bars); $N = 20$ replicates per bioassay. $^{**}0.01 > P > 0.001$); n.s. = not significant; Wilcoxon signed-rank test for paired differences, two-sided.

Host Plant Recognition Bioassay of Contact Cues. When both unwrapped petioles that provided access to contact cues and petiole dummies were offered, the females preferred the host petioles (Figure 2, treatment A). The contact cues enhancing the walking activity could be isolated by hexane extraction of young leaves (petioles plus leaflets) of *T. vulgare*. On petiole dummies treated with this hexane extract the females walked significantly longer than on control dummies when contact was allowed (Figure 2, treatment B). Furthermore, the adults were observed to walk for a longer time on leaf petioles with an intact wax layer compared to petioles from which waxes had been removed (Figure 2, treatment C).

Oviposition Bioassays of Plant Cuticular Waxes. When the upper surface of the leaves was offered for oviposition, females of *C. stigmatica* did not discriminate between leaflets with an intact wax layer and leaflets treated with cellulose acetate. However, when the lower surface of leaves was offered with and without the wax layer, 2.5 times more eggs were laid on the leaves from which no waxes had been removed (Table 1).

DISCUSSION

Host plant recognition in *C. stigmatica* was shown in this study to be guided by both volatile and hexane-soluble, most probably waxy, contact cues of

TABLE 1. IMPORTANCE OF UPPER (ADAXIAL) AND LOWER (ABAXIAL) LEAF SURFACE WAXES OF *Tanacetum vulgare* FOR OVIPOSITION OF *Casssida stigmatica*[a]

Leaf side offered	Leaves with intact wax layer	Leaves after wax removal	*P*
Upper	2.50 ± 2.21	1.95 ± 2.04	NS
Lower	3.40 ± 1.73	1.40 ± 1.43	**

[a]Removal of waxes from treated leaves by the cellulose acetate stripping method (Baker et al., 1983). Numbers of eggs (mean ± SD) laid on treated and untreated leaves in dual choice bioassays after 9 hr are given; $N = 20$ replicates. $^{**}0.01 > P > 0.001$; NS = not significant; Wilcoxon signed-rank test for paired differences, two-sided.

T. vulgare petioles and leaflets (Figure 1, treatment A; Figure 2). Attraction by host volatiles has been shown in several other chrysomelid species (Feeny et al., 1970; Anderson and Metcalf, 1986; Visser, 1986; Mitchell, 1994; Müller and Hilker, 2000). The attractive host volatiles for chrysomelids identified so far are known to be both common plant volatiles such as the so-called general green leaf volatiles (e.g., Visser, 1986) and very host-plant-specific chemicals such as allyl isothiocyanate in Brassicaceae (e.g. Vincent and Stewart, 1984). *C. stigmatica* might respond to both the pattern of green leaf volatiles of *T. vulgare* and typical

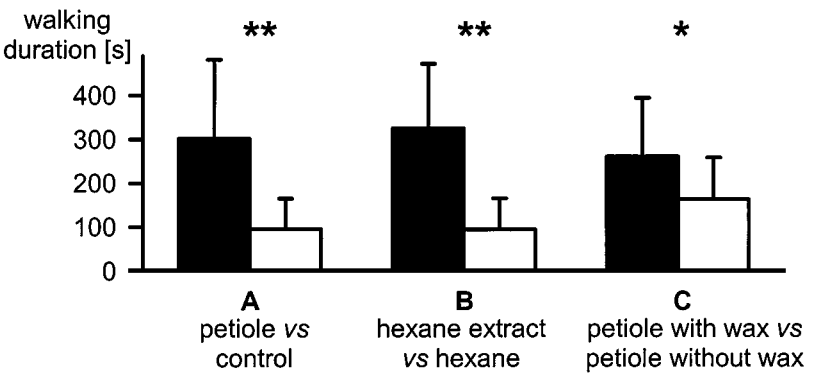


FIG. 2. Effect of volatile and contact stimuli of the host plant *Tanacetum vulgare* on females of *Cassida stigmatica* (seconds, Duration of walking mean ± SD) on differently treated petioles during an observation period of 10 min is given. Test and control petioles were offered for direct contact in the petiole arena. Mean duration of walking: (A) on petioles of *T. vulgare* (black bars) and petiole dummies (white bars); (B) on dummies treated with a hexane extract of *T. vulgare* (black bars) and dummies with the solvent only (white bars), and (C) on petioles of *T. vulgare* with an intact wax layer (black bars) and petioles of *T. vulgare* whose waxes had been removed (white bars); $N = 20$ replicates per bioassay. $^{**}0.01 > P > 0.001$; $^{*}0.05 > P > 0.01$; Wilcoxon signed-rank test for paired differences, two-sided.

volatiles of *T. vulgare* such as monoterpenes (e.g., thujone) and their derivatives (Teuscher and Lindequist, 1994). The hexane extraction used in this study did not sufficiently isolate the volatiles attractive to *C. stigmatica*. The concentration of possibly attractive compounds might have been too low or compounds might have been lost due to fast evaporation. Other extraction methods and behavioral studies of adult *C. stigmatica* are needed to identify the plant volatiles that serve as host recognition cues for this chrysomelid species.

Considering the role of contact cues for host recognition in *C. stigmatica*, two bioassay results strongly indicate that cuticular waxes of the petioles are important. These cues could be isolated from the petioles by the cellulose acetate stripping method, and the active compounds are soluble in hexane. However, with the methods we used we can not rule out the possibility that minute amounts of nonwaxy compounds were removed in addition to the plant waxes to elicit the beetle's response.

Two explanations are possible for these oviposition results:

1. The wax load is larger on upper leaf surfaces in several plant species (Premachandra et al., 1993). The cellulose acetate treatment removes only loose wax crystals and the outermost amorphous layer of waxes, as scanning electron microscopic investigations of *T. vulgare* leaves have shown (Müller, 1999). Therefore, while the waxes were most probably completely removed from the lower surfaces after cellulose treatment, some may have remained on the upper surface. These remaining waxes may have been sufficient to stimulate oviposition.
2. The upper surface offers suboptimal oviposition conditions resulting in faliure of *C. stigmatica* to differentiate between a suboptimal leaf surface with or without waxes. Numerous insects, including leaf beetles, are known to prefer the lower leaf surface for egg deposition (Kosior, 1975; C. Müller, personal observations). Ovipositions on upper leaf surfaces expose eggs to sunlight and, thus, unfavorable microclimatic conditions (Willmer, 1986). Furthermore, eggs are exposed to visually oriented egg predators and parasitoids. In the field, the upper leaf surface may be recognized by orientation of the leaf in space. However, in our bioassay, the leaves were placed horizontally onto a filter paper so that *C. stigmatica* could only encounter either of the offered leaf surfaces.

Females of *C. stigmatica* may have recognized the upper leaf surface by its texture and/or its wax composition. The upper leaf surface of *T. vulgare* has a different texture from the lower one. Size and frequency of glands and trichomes on upper and lower leaf surface differ in *T. vulgare*, as in several other Asteraceae (Gershenzon and Croteau, 1991; Müller, 1999). The trichomes were not removed by the cellulose acetate treatment. The wax composition of the upper leaf surface of *T. vulgare* may differ from that of the lower surface. Several other studies showed

that the chemical composition of the plant epicuticular waxes are different on the ab- and adaxial leaf surfaces (Jeffree, 1986; Premachandra et al. 1993; Yang et al., 1993; Eigenbrode et al. 1998, 1999).

Chemical stimuli present in wax have been shown to elicit oviposition in several other insect species. Surface extracts of cabbage plants contain oviposition stimulants for *Pieris rapae* L. (Lepidoptera: Pieridae) (Renwick et al., 1992) and *Delia floralis* (F.) (Diptera: Anthomyiidae) (Hopkins et al., 1997). In *Delia radicum* (L.), chemoreceptors on the tarsi are sensitive to the cabbage surface extracts (Städler and Roessingh, 1990). In the legume podborer *Maruca testulalis* (Geyer), contact chemoreceptors of the ovipositor respond to leaf washes (Waladde and Ochieng, 1990). 1-Octacosanal and 6-methoxy-2-benzoxazolinone (MBOA) elicit oviposition in the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae) (Morris et al., 2000).

Acknowledgments—This study was financially supported by a grant from the Universities of Berlin. We thank Sanford Eigenbrode, Johannes Steidle, Joachim Ruther, and two anonymous reviewers for their critical and helpful comments on an earlier version of the manuscript.

REFERENCES

ADATI, T., and MATSUDA K. 1993. Feeding stimulants for various leaf beetles (Coleoptera: Chrysomelidae) in the leaf surface wax of their host plants. *Appl. Entomol. Zool*. 28:319–324.

ANDERSON, J. F., and METCALF, R. L. 1986. Identification of a volatile attractant for *Diabrotica* and *Acalymna* spp. from blossoms of *Cucurbita maxima* Duchesne. *J. Chem. Ecol*. 12:687–699.

BAKER, E. A., HUNT, G. M., and STEVENS, P. J. G. 1983. Studies of plant cuticle and spray droplet interactions: A fresh approach. *Pestic. Sci*. 14:645–658.

BERNAYS, E. A., BLANEY, W. M., CHAPMAN, R. F., and COOK, A. G. 1976. The ability of *Locusta migratoria* L. to perceive plant surface waxes, pp. 35–40, *in* T. Jermy (ed.). The Host Plant in Relation to Insect Behavior and Reproduction. Plenum, New York.

BERNAYS, E. A., WOODHEAD, S., and HAINES, L. 1985. Climbing by newly hatched larvae of the spotted stalk borer *Chilo partellus* to the top of sorghum plants. *Entomol. Exp. Appl*. 39:73–79.

BOHM, B. A., and CONSTANT, H. 1990. Leaf surface terpenoids of *Eriodictyon tricholcalyx*. *Biochem. Syst. Ecol*. 18:491–492.

BROOKS, J. S., WILLIAMS, E. H., and FEENY, P. 1996. Quantification of contact oviposition stimulants for black swallowtail butterfly, *Papilio polyxenes*, on the leaf surfaces of wild carrot, *Daucus carota. J. Chem. Ecol*. 22:2341–2357.

EIGENBRODE, S. D., and ESPELIE, K. E. 1995. Effects of plant epicuticular lipids on insect herbivores. *Annu. Rev. Entomol*. 40:171–194.

EIGENBRODE, S. D., and PILLAI, S. K. 1998. Neonate *Plutella xylostella* responses to surface wax components of a resistant cabbage (*Brassica oleracea*). *J. Chem. Ecol*. 24:1611–1627.

EIGENBRODE, S. D., WHITE, C., RHODE, M., and SIMON, C. J. 1998. Epicuticular wax phenotype of the wel mutation and its effect on pea aphid populations in the greenhouse and in the field. *Pisum Genet.* 29:13–17.

EIGENBRODE, S. D., TIPTON, J. L., and WHITE, M. 1999. Differential cutting by leaf-cutter bees (Megachilidae: Hymenoptera) on leaves of eastern redbud and on Mexican redbuds with different surface waxes. *J. Kans. Entomol. Soc*. 72:73–81.

FEENY, P., PAAUWE, K. L., and DEMONG, N. J. 1970. Flea beetles and mustard oils: Host plant specificity of *Phyllotreta cruciferae* and *P. striolata* adults (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am*. 63:832–841.

GERSHENZON, J., and CROTEAU, R. 1991. Terpenoids, pp. 165–219, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

HOPKINS, R. J., BIRCH, A. N. E., GRIFFITHS, D. W., BAUR, R., and STÄDLER, E., MCKINLAY, R. G. 1997. Leaf surface compounds and oviposition preference of turnip root fly *Delia floralis*: The role of glucosinolate and nonglucosinolate compounds. *J. Chem. Ecol*. 23:629–643.

JEFFREE, C. E. 1986. The cuticle, epicuticular waxes and trichomes of plants, with reference to their structure, functions and evolution, pp. 23–64, *in* B. Juniper and S. R. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

KLINGAUF, F. 1971. Die Wirkung des Glykosids Phlorizin auf das Wirtswahlverhalten von *Rhopalosiphum insertum* (Wald.) und *Aphis pomi* De Geer (Homoptera: Aphididae). *Z. angew. Entomol*. 6:41–55.

KOCH, K. 1992. Die Käfer Mitteleuropas. Ökologie, Goecke & Evers, Krefeld.

KOSIOR, A. 1975. Biology, ecology, and economic importance of cassids (Coleptera, Chrysomelidae, Cassidinae) of the Ojców National Park. *Acta Zool. Cracov*. 20:251–392.

LIN, S., BINDER, B. F., and HART, E. R. 1998. Insect feeding stimulants from the leaf surface of *Populus*. *J. Chem. Ecol*. 24:1781–1790.

MITCHELL, B. K. 1994. The chemosensory basis of host-plant recognition in Chrysomelidae, pp. 141–152, *in* P. H. Jolivet, M. L. Cox, and E. Petitpierre (eds.): Novel Aspects of the Biology of Chrysomelidae. Kluwer Academic Publishers, Dordrecht.

MORRIS, B., FOSTER, S., and HARRIS, M. 2000. 1-Octacosanal and 6-methoxy-2-benzoxazolinone: Compounds from wheat leaf wax that stimulate oviposition by the Hessian fly, *Mayetiola destructor*. *J. Chem. Ecol*. 26:859–873.

MÜLLER, C. 1999. Chemische Ökologie des Phytophagenkomplexes an *Tanacetum vulgare* L. (Asteraceae). PhD dissertation. Free University of Berlin, Germany. Logos-Verlag, Berlin.

MÜLLER, C., and HILKER, M. 2000. The effect of a green leaf volatile on host plant finding by larvae of a herbivorous insect. *Naturwissenschaften* 87:216–219.

NOLDUS, L. P. J. J., POTTING, R. P. J., and BARENDREGT, H. E. 1991. Moth sex pheromone adsorption to leaf surface: Bridge in time for chemical spies. *Physiol. Entomol*. 16:329–344.

PREMACHANDRA, G. S., HAHN, D. T., and JOLY, R. J. 1993. A simple method for determination of abaxial and adaxial epicuticular wax loads in intact leaves of *Sorghum bicolor*. *L. Can. J. Plant Sci*. 73:521–524.

RENWICK, J. A. A., RADKE, C. D., SACHDEV-GUPTA, K., and STÄDLER, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* on cabbage. *Chemoecology* 3:33–38.

SCHOONHOVEN, L. M., JERMY, T., and LOON, J. J. A. V. 1998. Insect–Plant Biology. From Physiology to Evolution. Chapman & Hall, London.

SPENCER, J. L. 1996. Waxes enhance *Plutella xylostella* oviposition in response to sinigrin and cabbage homogenates. *Entomol. Exp. Appl*. 81:165–173.

STÄDLER, E. 1986. Oviposition and feeding stimuli in leaf surface waxes. pp. 105–121, *in* B. Juniper and R. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

STÄDLER, E., and ROESSINGH, P. 1990. Perception of surface chemicals by feeding oviposition insects. *Symp. Biol. Hung*. 39:71–86.

STORK, N. E. 1980. Role of waxblooms in preventing attachment to brassicas by the mustard beetle, *Phaedon cochleariae. Entomol. Exp. Appl*. 28:100–107.

TEUSCHER, E., and LINDEQUIST, U. 1994. Biogene Gifte. Gustav Fischer, Stuttgart.

VARELA, L., and BERNAYS, E. A. 1988. Behavior of newly hatched potato tuber moth larvae, *Phthorimaea operculella* Zell. (Lepidoptera: Gelichiidae), in relation to their host plants. *J. Insect Behav*. 1:261–275.

VINCENT, C., and STEWART, R. K. 1984. Effect of allyl isothiocyanate on field behavior of crucifer-feeding flea beetles (Coleoptera: Chrysomelidae). *J. Chem. Ecol*. 10:33–39.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Ann. Rev. Entomol*. 31:121–144.

WALADDE, S. M., and OCHIENG, S. A. 1990. Tarsi and ovipositor gustatory sensilla of *Maruca testulalis*: Coding properties and behavioral responses. *Symp. Biol. Hung*. 39:551–552.

WHITAKER, J. R., and LEE, C. Y. 1995. Recent advances in chemistry of enzymatic browning. pp. 2–7, *in* C. Y. Lee and J. R. Whitaker (eds.). Enzymatic Browning and Its Prevention. American Chemical Society, Washington, D.C.

WILLMER, P. 1986. Microclimatic effects on insects at the plant surface, pp. 65–80, *in* B. Juniper and S. R. Southwood (eds.). Insects and the Plant Surface. Edward Arnold, London.

YANG, G., ESPELIE, K. E., TODD, J. W., CULBREATH, A. K., PITTMAN, R. N., and DEMSKI, J. W. 1993. Cuticular lipids from wild and cultivated peanut species and the relative resistance of these peanut species to fall armyworm and thrips. *J. Agric. Food Chem*. 41:814–818.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

OLFACTORY RESPONSES OF *Ips duplicatus* FROM INNER MONGOLIA, CHINA TO NONHOST LEAF AND BARK VOLATILES

QING-HE ZHANG,[1,*] GUANG-TIAN LIU,[2] FREDRIK SCHLYTER,[1] GÖRAN BIRGERSSON,[3] PETER ANDERSON,[1] and PETER VALEUR[4]

[1]*Chemical Ecology, Department of Crop Science*
Swedish University of Agricultural Sciences
P. O. Box 44, SE-230 53 Alnarp, Sweden

[2]*Baiyinaobao Natural Preserve*
Chifeng, 025375 Inner Mongolia, P. R. China

[3]*Chemical Ecology, Department of Botany*
Göteborg University
Box 461, SE-405 30 Göteborg, Sweden

[4]*Department of Ecology*
Ecology Building, Lund University
SE-223 62 Lund, Sweden

(Received July 5, 2000; accepted January 6, 2001)

Abstract—Leaf and bark volatiles from nonhost angiosperm trees were tested on *Ips duplicatus* by gas chromatographic–electroantennographic detection (GC-EAD) and by pheromone-baited traps in Sweden and Inner Mongolia, China, respectively. GC-EAD analysis of the headspace volatiles from fresh bark chips of *Betula pubescens* revealed *trans*-conophthorin, two green leaf volatiles (GLVs): 1-hexanol and (*Z*)-3-hexen-1-ol, and two C_8 alcohols: 3-octanol and 1-octen-3-ol, that consistently elicited antennal responses by *I. duplicatus*. The identification of these EAD-active compounds was confirmed in further GC-EAD recordings with synthetic mixtures. Antennal responses were also found to synthetic (*E*)-2-hexen-1-ol and linalool, which have been identifed from the leaves of nonhost birch and aspen species. No antennal responses of *I. duplicatus* were found to hexanal, (*E*)-2-hexenal, and (*Z*)-3-hexyl acetates. In field trapping experiments, blends of EAD-active green leaf alcohols or C_8 alcohols, or *trans*-conophthorin alone resulted in significant reductions (27–60%) in the number of *I. duplicatus* captured compared with pheromone-baited traps. The unsuitable host compound, verbenone (Vn), also significantly reduced trap catches by up to 60% in both experiments. The strongest disruptive effect resulted from the addition of the combination of green leaf alcohols, C_8 alcohols, and verbenone

* To whom correspondence should be addressed. e-mail: qing-he.zhang@vv.slu.se

0098-0331/01/0500-0995$19.50/0

to the pheromone trap, which caused an 84% reduction in trap catch. The blend of two green leaf aldehydes plus the acetate increased the trap catches in 1998 and had no negative or positive effects in 1999. Our results suggest that these nonhost volatiles (NHVs) are important olfactory signals used by *I. duplicatus* in host selection. They may have great significance in developing semiochemical-based management programs for *I. duplicatus* by reducing or stopping attacks on suitable hosts.

Key Words—Coleoptera, Scolytidae, host selection, nonhost volatiles, green leaf volatiles, 1-hexanol, 3-octanol, 1-octen-3-ol, *trans*-conophthorin, verbenone.

INTRODUCTION

The double-spined spruce bark engraver of Eurasia, *Ips duplicatus* (Sahlb.), occurs in the Paleartic from central Scandinavia, across eastern Europe to Siberia and NE China, and in the mountains of central Europe (Postner, 1974; Lekander et al., 1977; Pfeffer, 1995; Zhang, 2001). In Europe, *I. duplicatus* usually occurs on spruce trees that are under attack by *Ips typographus* L., where it occupies the top position of the tree trunk (Schlyter and Anderbrant, 1993). It has so far not been considered as a major forest pest (Postner, 1974), but recent outbreaks in Czech Republic (Knizek and Zahradnik, 1996) and continuous outbreaks since the 1950s in Inner Mongolia, China (Zhang et al., 1995; Liu et al., 1998), as well as its status as a vector of phytopathogenic fungi (Krokene and Solheim, 1996), might alter its economic status. Therefore, the development of efficient and environmentally sound control measures, such as mass trapping with aggregation pheromones or disruption by nonhost volatiles, is needed to control outbreaks of this species.

Suitable hosts of bark beetles are highly scattered throughout mixed species forests and are distributed unevenly in space and time (Atkins, 1966). Therefore, bark beetles commonly utilize specialized and complex semiochemical signals to locate suitable breeding trees (Borden, 1997; Schlyter and Birgersson, 1999). While seeking suitable hosts in flight, bark beetles will less often encounter suitable host trees and their odors, but mostly unsuitable hosts and nonhost trees (Schroeder, 1992; Borden, 1997). Rejection of the latter two types of trees could be based on an imbalance of certain host characteristics and/or a negative response to some nonhost stimuli (Schlyter and Birgersson, 1999).

Recent field studies have shown that the attraction of pheromone/kairomone in over 15 species of conifer-infesting scolytids are disrupted by green leaf volatiles (GLVs: mostly six carbon alcohols, aldehydes, and derivative esters), lately confirmed in Sweden and North America to be produced by the leaves of nonhost taiga angiosperms (Visser, 1986; Whitman and Eller, 1990; Dickens et al., 1991, 1992; Schlyter et al., 1995; Wilson et al., 1996; Borden et al., 1997; Deglow and Borden, 1998a,b; Poland et al., 1998; Byers et al., 1998, 2000; Zhang et al., 1999a,b; de Groot and MacDonald, 1999). Furthermore, the attraction of

I. duplicatus females to its synthetic pheromone in a walking bioassay was strongly inhibited by a combination of verbenone and a mixture of six GLVs (Schlyter et al., 1995).

There is also evidence that volatiles from nonhost bark might play an important role in the host selection of conifer bark beetles (Borden et al., 1998; Byers et al., 1998; Zhang et al., 2000). Guerrero et al. (1997) showed that benzyl alcohol identified in eucalyptus callus elicited electrophysiological responses from the antennae of *Tomicus destruens* (Woll.) and might at high doses (700 mg/day) reduce attacks on host logs. Gas chromatographic–electroantennographic detection (GC-EAD) analysis of the headspace volatiles from fresh bark chips of three nonhost species (*B. pendula* Roth., *B. pubescens* Ehrh. and *P. tremula* L.) revealed five compounds that consistently elicited antennal responses by *I. typographus* (Zhang et al., 2000). Inhibitory effects of these electrophysiologically active nonhost bark volatiles (in combination or alone), including (±)-*trans*-conophthorin (*t*C) and C_8 alcohols, on *I. typographus* have been shown in the field (Zhang and Schlyter, in preparation). In Canada, Borden et al. (1998) and Huber et al. (1999, 2000) found over 10 bark volatile compounds from nonhost angiosperm trees, *Populus* spp., *B. papyrifera* Marsh, and *Acer macrophyllum* Pursh, that elicited antennal responses in five conifer-attacking scolytid species, including three *Dendroctonus*, one *Ips*, and a *Dryocoetes* species. A disruptive effect of these nonhost bark volatiles on the response to a pheromone-kairomone blend by *Dendroctonus* spp. also has been found (Borden et al., 1998; Huber et al., 1999). In addition, single cell responses to unknown compounds from nonhost birch bark have been demonstrated in Scandinavian *Trypodendron lineatum* (Oliver) and *I. typographus* (Tømmerås, 1989; Tømmerås and Mustaparta, 1989).

Our objectives were: (1) to determine whether the nonhost leaf and bark volatiles are detected by *I. duplicatus* antennae, and (2) to test the EAD-active nonhost volatiles (NHVs), alone or combined, for their capability to reduce captures of *I. duplicatus* in pheromone-baited traps in the field.

METHODS AND MATERIALS

GC-EAD Responses to Nonhost Volatiles

Volatiles from fresh bark chips of nonhost birch, *B. pubescens* (one of the most common nonhost species of *I. duplicatus* and *I. typographus* in Europe) were collected by headspace sampling in June 1998, Asa, Småland, southern Sweden, and analyzed by GC-MSD as described by Zhang et al. (2000). The aeration extracts were stored at −20°C before GC-EAD analysis.

Overwintering *I. duplicatus* were collected from the litter around infested spruce trees, in late October at Baiyinaobao, Inner Mongolia, China. They were

immediately sent to Alnarp, Sweden and kept alive at 4°C until used in the electrophysiological experiments.

GC-EAD analysis was carried out with antennae (with the severed head) of both sexes of the overwintering beetles on an HP 6890 gas chromatograph containing a fused silica polar column (HP-Innowax; 30 m × 0.25 mm; 0.25 μm) as described by Zhang et al. (1999b, 2000). The antennal signals were stored and analyzed on a PC equipped with an IDAC-card and the program EAD ver. 2.3 from Syntech, Hilversum, The Netherlands. Two microliters of an aeration extract of fresh *B. pubescens* bark chips was injected. In addition, the EAD activity to two synthetic mixtures (1 μl/injection) was studied to confirm compound identity and obtain replicates of electrophysiological activity. The synthetic mixtures consisted of: (1) GLVs (100 ng/μl for each compound) commonly found from intact leaves of the nonhost birches and aspen (Zhang et al., 1999a) (Figure 1B); (2) nonhost bark compounds (Zhang et al., 2000) and two aggregation pheromone components, (±)-ipsdienol (Id) and (*E*)-myrcenol (EM) (Byers et al., 1990; Schlyter et al., 1992), plus the general inhibitor, verbenone (Vn) (Schlyter et al., 1989) (100 ng/μl for each compound) (Table 1, Figure 2). Each sample (both bark aerations and synthetic mixtures) was tested on four male and two female antennae. In addition, the second synthetic mixture was also analyzed with GC-EAD on a nonpolar column (HP-1; 30 m × 0.25 mm; 0.25 μm, through the cooled on-column injector) on both sexes (two males, one female). Hydrogen was used as the carrier gas. The column temperature was 40°C for the first 2 min, rising to 200°C at 10°C/min, and held for 2 min. Only those compounds that elicited electrophysiological responses (obviously different from background noise) by at least three antennal preparations were considered as antennally active.

Field Response to Nonhost Volatiles

All the field trapping experiments were carried out in an ~2000 ha natural reserve of spruce [*Picea mongolica* (Wu, H.-Q.) Xu, formally *P. koraiensis* Nakai or *P. meyeri* Rehd. or *P. meyeri* var. *mongolica* Wu, as discussed by Xu et al. (1998)] at Baiyinaobao (43°30′–36′N, 117°06′–16′E), Inner Mongolia, China, which is situated in the grassland at the east edge of Hunshandake desert, with a thick layer of sandy soil. Since the 1950s this isolated natural spruce forest has been severely infested by the web-spinning sawflies [*Cephalcia abietis* (L.), *C. alashanica* (Gussakorskij) and *Acantholyda peiyingaopaoa* Hsiao] and *I. duplicatus* (Zhang et al., 1995; Liu et al., 1998). The larger bark beetle competitor, *I. typographus* has not been recorded here.

Experiment 1 (Green Leaf Volatiles, GLVs). This was conducted in early June 1998 in an old clear-cut at the margin of the surrounding stand of dead trees killed by *I. duplicatus* the previous year. Eight black nonsticky, flight barrier

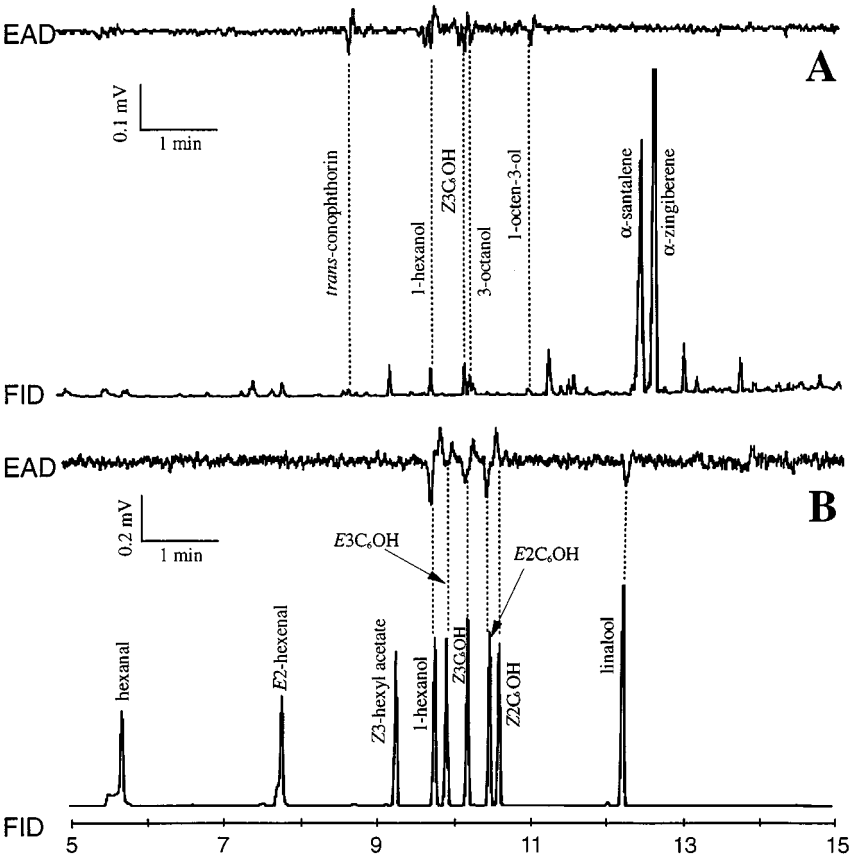


FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD) responses of *Ips duplicatus* antennae to nonhost volatiles: (A) headspace volatiles from fresh bark chips of *Betula pubescens*, (B) a synthetic mixture of eight GLVs and linalool (50 ng/compound), commonly found from leaves of nonhost birch and aspen species.

(slot) traps (Theyson, Germany) were arranged in a curve in order to ensure a similar distance (~20–25 m) to the insect source (the dead trees) for each trap. The distance between traps was ~8 m. The GLVs were chosen and grouped based on their emission from leaves of European birches (*B. pendula* and *B. pubescens*) and aspen (*P. tremula*) (Zhang et al., 1999a), and their electrophysiological activity on *I. duplicatus*.

Experiment 2 (Nonhost Bark Volatiles). Ten flight barrier traps were set up in mid June 1999 in a small glade at the margin of the surrounding stand of dead trees killed by *I. duplicatus* the previous year. The traps were set up in a similar fashion

TABLE 1. CHEMICALS, RELEASE RATES, AND DISPENSERS USED IN FIELD-TRAPPING EXPERIMENTS

Chemical	Source	Purity (%)	Release rate (mg/24 hr)[a]	Dispensers	Exp.
Pheromone			**1.5**		**1, 2**
(±)-Ipsdienol (Id)	W. Francke, Germany	90	1	20 mg Id + 20 mg EM in a wick-dispenser	
(*E*)-Myrcenol (EM)	SciTech, Czech Rep.	95	0.5	disolved in 4 ml octane	
Nonhost volatiles					
Individual GLV alcohols					
1-Hexanol	Aldrich, USA	98	**4**	200 μl in an open # 730 PE-vial[b]	**1**
(*Z*)-3-Hexen-1-ol	Aldrich, USA	98	**6**	200 μl in an open #730 PE-vial	**1**
(*E*)-2-Hexen-1-ol	Aldrich, USA	97	**5**	200 μl in an open #730 PE-vial	**1**
3OH-B			**5**		**1**
(*E*)-3-Hexen-1-ol	Aldrich, USA	98	2.4	200 μl of an 1:1:1 mix	
(*Z*)-2-Hexen-1 ol	Acros, USA	95	2	in an open #730 PE-vial	
(±)-Linalool	Aldrich, USA	97	0.5		
2Ald-Ac			**12.3**		**1, 2**
1-Hexanal	Aldrich, USA	98	5.2	400 μl of an 1:1:2 mix	
(*E*)-2-Hexenal	Aldrich, USA	99	1.6	in #730 PE-vial with 2 mm Ø hole	
(*Z*)-3-Hexenyl acetate	Lancaster, UK	99	5.5	in the lid	
3GLVs			**6**		**2**
1-Hexanol	Aldrich, USA	98	2	200 μl of an 1:1:1 mix	
(*Z*)-3 Hexen-1-ol	Aldrich, USA	98	2	in an open #730	
(*E*)-2-Hexen-1-ol	Aldrich, USA	97	2	PE-vial	
C_8-OHs			**1.8**		**2**
(±)-3-Octanol	Acros, USA	99	1.2	200 μl of an 1:1 mix in an open	
(±)1-Octen-3-ol	Acros, USA	98	1.6	#730 PE-vial	
(±)-***trans*-Conophthorin (*tC*)**	Phero-Tech, CAN	87	**5**	2 Open 250 μl-PE microcentrifuge tubes (RD0249)	**2**
Unsuitable host compound					
(−)-verbenone (Vn)	Bedoukian, USA	99	**0.5**	200 μl in an open #730 PE-vial	**1, 2**

[a]Measured in a mini windtunnel at 20–21°C and 0.7 m/sec for 7–10 days.
[b]PE-vial with 6-mm-diam. opening, 29-mm inner height.

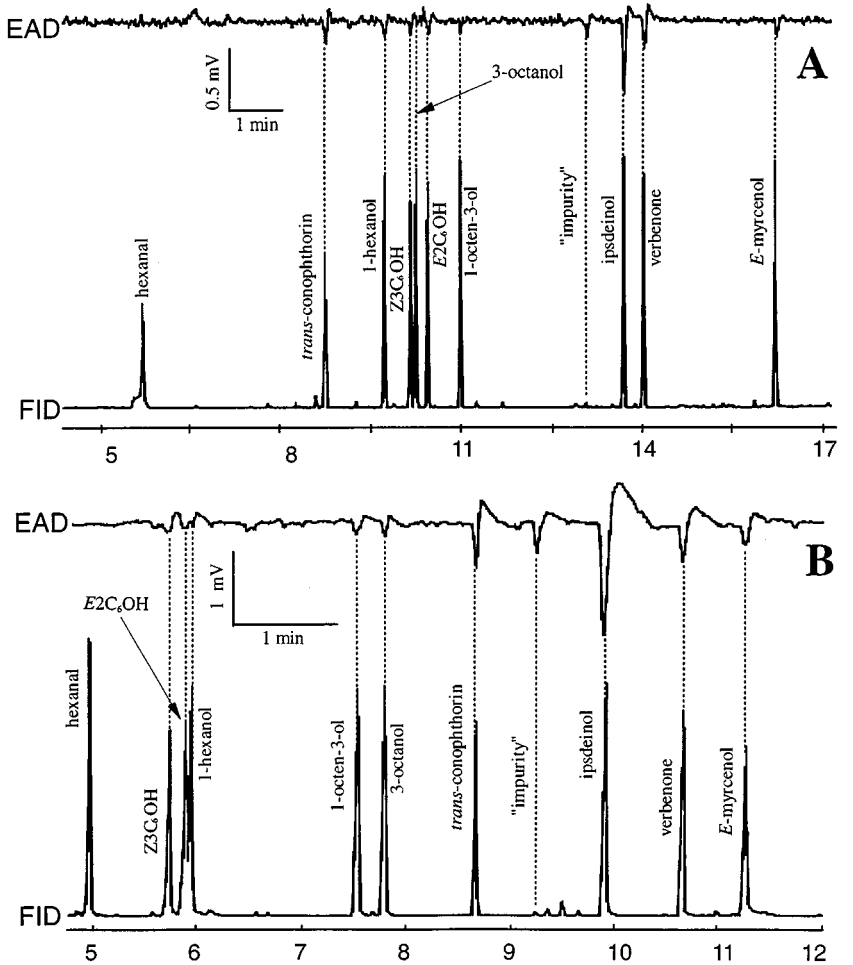


FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD) responses of *Ips duplicatus* antennae to a synthetic mixture of 10 compounds containing nonhost bark volatiles, two pheromone components, and an unsuitable host compound, verbenone (50 ng/compound): (A) on polar GC-column (HP-Innowax), (B) on nonpolar GC-column (HP-1).

to experiment 1 with ~8 m between traps. Nonhost bark volatiles were selected according to their presence in the headspace of fresh bark chips of *B. pendula* and *B. pubescens* and *P. tremula* (Zhang et al., 2000), and their antennal activity on *I. duplicatus*. A blend of nonhost leaf volatiles (2Ald-Ac) tested in 1998 was also repeated in this experiment.

All chemicals, sources, release devices, and release rates are listed in Table 1. In both field experiments, three control treatments were used to assess the effectiveness of candidate NHVs: (1) the positive control, i.e., synthetic pheromone lure (Id + EM, at 1:1, dissolved in octane as 1% solution in a wick-dispenser) (Schlyter et al., 2001); (2) the negative control, consisting of pheromone lure plus the unsuitable host compound, verbenone (Vn); and (3) the blank control, containing only the solvent, octane. The positions of traps together with dispensers were initially randomized and rotated after each replicate (when $\geq$50 beetles were caught in the best trap).

Statistical Analysis

Because of considerable variations in the numbers of beetles captured between replicates and experiments, the counts were converted to proportion (p) of total captured beetles within each replicate. The data were then transformed by arcsin $\sqrt{p}$ in order to fit the assumption of homogeneity of variances for ANOVA. The means were compared by ANOVA followed by Duncan's multiple range test (SPSS 8.0 for Windows) at $\alpha = 0.05$. The sex ratios for the treatments within each experiment were compared with 95% binomial confidence intervals (Byers and Wood, 1980).

RESULTS

GC-EAD Responses. In the GC-EAD analysis of volatiles from *B. pubescens* fresh bark chips, *I. duplicatus* antennae of both sexes consistently responded to *trans*-conophthorin (1.5–2.0 ng), two green leaf alcohols: 1-hexanol (4–5 ng) and (*Z*)-3-hexen-1-ol (6–7 ng), and two C_8 alcohols: 3-octanol (2 ng) and 1-octen-3-ol (1 ng) (Figure 1A). Identification of these EAD-active compounds was done on a combined gas chromatograph and mass selective detector (GC-MSD) by comparison of retention times and mass spectra to those of authentic compounds and was published in detail in Zhang et al. (2000). No repeatable antennal responses were found to any other compounds in the bark aeration extract, including the dominant components, α-santalene (80 ng) and α-zingiberene (100 ng). From the first synthetic mixture mainly containing GLVs (50 ng/compound), all five C_6 alcohols and ($\pm$)-linalool elicited repeatable antennal responses in both sexes (Figure 1B). 1-Hexanol and (*Z*)-3-hexen-1-ol, commonly emitted from both leaves and bark, and (*E*)-2-hexen-1-ol from leaves of nonhost birches and aspen (Zhang et al., 1999a, 2000), seemed to elicit stronger antennal responses than those elicited by (*E*)-3-hexen-1-ol and (*Z*)-2-hexen-1-ol, which are not detected from the nonhost trees. The green leaf aldehydes, hexanal and (*E*)-2-hexenal, and the green leaf acetate, (*Z*)-3-hexyl acetate, did not elicit any GC-EAD responses at the doses

tested. GC-EAD recordings with two different GC-columns (polar and nonpolar) on the second synthetic mixture containing the nonhost bark volatiles and aggregation pheromones showed that both male and female *I. duplicatus* antennae responded strongly to the two pheromone components, (±)-ipsdienol and (*E*)-myrcenol, the nonhost bark volatile, (±)-*trans*-conophthorin, and the unsuitable host compound, Vn (Figure 2A and 2B). Weaker, but repeatable, responses were recorded to 1-hexanol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, (±)-3-octanol, and (±)-1-octen-3-ol. No responses were found to the green leaf aldehyde, hexanal, at the dose tested. The major pheromone component, ipsdienol, elicited the strongest EAD response, which was at least twice as strong as those of (*E*)-myrcenol and the nonhost volatiles. Surprisingly, a strong and repeatable response to a small amount of an unknown impurity (<1 ng) from the synthetic mixture was also found, which is now subject to further identification by GC-MS. There were no differences in antennal responses between males and females.

Field Response to Nonhost Volatiles. In the GLVs experiment (experiment 1), a total of 12,200 beetles were caught during 31 replicates in 1998. The individual green leaf alcohols, 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol, did not significantly reduce any trap catches. The blend (3OH-B) of (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool also did not decrease trap catches. Interestingly, addition of the blend (2Ald-Ac) of two aldehyedes, hexanal and (*E*)-2-hexenal, plus (*Z*)-3-hexenyl acetate to the pheromone trap seemed to significantly increase the trap catches (Table 2). However, the negative control, Vn reduced the trap catches by 40% (Table 2). When considering the sex ratios of beetles caught, the proportion of males was lower in the traps with individual GLV alcohols, 2Ald-Ac, and Vn than that in traps baited with pheromone alone (Table 2). No differences in sex ratio between traps baited with pheromone alone and pheromone plus 3OH-B were found. Thus, the individual GLV alcohols had a inhibitory effect on males (with male catch reduction ranging from 30 to 70%) even though the total number of beetles was not reduced. The addition of 2Ald-Ac to pheromone traps increased the female catches, but not male catches.

In the nonhost bark volatiles experiment conducted in 1999 (experiment 2), a total of 2467 beetles were caught during 18 replicates. Unlike experiment 1, the blend of 2Ald-Ac did not have any significant effect on the trap catches. However, the (±)-*trans*-conophthorin (*t*C), the blend of three GLVs: 1-hexanol, (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol, and the blend of two C_8 alcohols (C_8-OHs): (±)-3-octanol and (±)-1-octen-3-ol, resulted in significant reductions (27%, 33%, and 60%, respectively) in the number of *I. duplicatus* captured compared with the trap baited with pheromone alone (Table 2). Vn, the negative control, also significantly reduced the trap catches by 63%, but its combination with *t*C was not different from the Vn alone. The strongest inhibition of attraction resulted from the addition of the combination of 3GLVs, C_8-OHs, and Vn to the pheromone trap, which caused an 84% reduction in trap catch

TABLE 2. FIELD BEHAVIORAL RESPONSES OF *Ips duplicatus* TO NONHOST LEAF AND BARK VOLATILES AND VERBENONE (VN) ADDED TO ATTRACTANT SOURCE, BAIYINAOBAO, CHIFENG, INNER MONGOLIA, CHINA

Experiment and treatment	% of total beetles captured (mean ± SE)[a]	Males (%)	95% CI	Total catch
1. Nonhost leaf volatiles (GLVs), June 11–13, 1998 (N = 31; Σ = 12110)				
Ph	11.8 ± 1.4b	29.8	26.1–33.9	1520
Ph + 1-Hexanol	13.0 ± 0.9b	19.6	17.0–22.4[b]	1568
Ph + (*Z*)-3-hexen-1-ol	14.4 ± 1.4b	14.2	12.2–16.6[b]	1561
Ph + (*E*)-2-hexen-1-ol	12.3 ± 0.8b	9.9	7.9–12.4[b]	1416
Ph + 2Ald-Ac	25.2 ± 1.4c	16.1	14.3–18.1[b]	3060
Ph + 3OH-B	15.2 ± 1.2b	25.4	22.5–28.5	1890
Ph + Vn	7.3 ± 0.8a	18.2	14.2–22.9[b]	972
Blank	0.9 ± 0.2[c]	42.9	30.0–56.7	123
2. Nonhost bark volatiles, June 7–11, 1999 (N = 18; Σ = 2467)				
Ph	21.1 ± 2.5d	27.3	23.5–31.4	495
Ph + 3 GLVs	14.2 ± 1.5c	27.8	23.2–32.9	331
Ph + 2Ald-Ac[d]	21.1 ± 2.8d	34.1	30.2–38.2	537
Ph + C_8-OHs	8.5 ± 1.2b	30.4	24.7–36.7	224
Ph + Vn	7.7 ± 1.1b	22.6	17.4–28.8	204
Ph + *t*C	15.5 ± 2.1c	31.3	26.8–36.2	371
Ph + Vn + *t*C	5.7 ± 1.2b	22.6	16.4–30.3	137
Ph + 3GLVs + C_8-OHs + Vn	2.7 ± 0.6a	21.1	13.2–32.0	71
Ph + 3GLVs + C_8-OHs + Vn + *t*C	3.2 ± 0.7a	24.1	16.0–34.5	79
Blank	0.8 ± 0.3[c]	38.9	20.3–61.4	18

[a] Numbers in the column with the same letter, within the same experiment, are not significantly different ($P > 0.05$) by ANOVA on arcsin $\sqrt{p}$ followed by Duncan's multiple-range test.
[b] Significantly different from the positive control (pheromone alone; **Ph**), by comparison with 95% binomial confidence intervals.
[c] Blank not included in the ANOVA and the range tests to acheive homogeneity.
[d] One outlier removed for this treatment to acheive homogeneity (mean and SE includes the outlier).

(Table 2). The addition of *t*C to this combination showed no further catch reduction. The active nonhost volatiles seemed to reduce males more than females, but the sex ratios were not significantly different from the traps baited with pheromone alone (Table 2).

DISCUSSION

Electrophysiological Activity. Our results clearly show that *I. duplicatus* antennae are able to consistently detect *trans*-conophthorin, 1-hexanol, (*Z*)-3-hexen-1-ol, 3-octanol, and 1-octen-3-ol, which were commonly found from the headspace samples of bark chips of the nonhost *B. pubescens* (Figure 1A) and other nonhost species such as *B. pendula* and *P. tremula* (Zhang et al., 2000). In similar amounts, *trans*-conophthorin seems to elicit higher antennal responses than the other EAD-active NHVs do. This is the first electrophysiological evidence that *I. duplicatus* antennae not only strongly respond to their aggregation pheromone components, ipsdienol and (*E*)-myrcenol, but also can detect some nonhost leaf and bark volatiles, and the unsuitable host compound, Vn. An identical response pattern has recently been reported for *I. typographus*, the major competitor species of *I. duplicatus* in Europe (Zhang et al., 2000). In addition, a similar antennal activity was found in *Tomicus piniperda* and *T. minor* (Schlyter et al., 2000). However, in this study *trans*-conophthorin was EAD inactive (Schlyter et al., 2000). In a GC-EAD analysis with nonhost bark volatiles, Huber et al. (1999, 2000) found that *trans*-conophthorin elicited antennal responses by five species of North American bark beetles, *D. ponderosae* Hopkins, *D. pseudotsugae*, *D. rufipennis* Kirby, *Ips pini* (Say), and *Dryocoetes confusus* Swaine.

The GC-EAD active green leaf alcohol, (*E*)-2-hexen-1-ol, has not been detected from the nonhost bark, but was found together with 1-hexanol and (*Z*)-3-hexen-1-ol to be abundant in the volatile emission from intact leaves of nonhost birches and aspen (Zhang et al., 1999a). Similar GC-EAD responses to these green leaf alcohols have been reported in *I. typographus* (Zhang et al., 1999a,b), *D. ponderosae* Hopkins (Wilson et al., 1996) and two *Tomicus* species (Schlyter et al., 2000; Poland and Haack, 2000). Surprisingly, hexanal and (*Z*)-3-hexyl acetate, the most common green leaf aldehyde and acetate from many nonhost angiosperm species (Visser, 1986; Zhang et al., 1999a, 2000), did not show any antennal activity in any Eurasian bark beetle species tested (Zhang et al., 1999b, 2000; Schlyter et al., 2000; and this paper).

Field Trapping. In the 1998 test, the blend of the EAD-inactive green leaf aldehydes and acetate increased the trap catches; however, the enchancement was not repeated in the 1999 test. Further study with individual compounds and their combination is needed to clarify these conflicting results. In addition, the EAD-active green leaf alcohols were inactive when tested individually (Table 2).

However, in experiment 2 the combination of 1-hexanol, (*Z*)-3-hexen-1-ol, and (*E*)-2-hexen-1-ol reduced the number of beetles captured. This might be due to either the synergistic effect of different GLV compounds or a density dependent effect of beetle's populations, with higher population density in 1998 than in 1999.

Consistent with our GC-EAD results, field trapping experiments also disclosed the inhibitory effects of (±)-*trans*-conophthorin, a blend of the two EAD-active C_8 alcohols, (±)-3-octanol and (±)-1-octen-3-ol, and the unsuitable host compound, Vn on *I. duplicatus*. Such interruptive activity by these nonhost volatiles has been found in *I. typographus* (Zhang et al., in preparation), and partly in other bark beetle species, such as *T. piniperda, T. minor* (Schlyter et al., 2000), *D. ponderosae*, and *D. pseudotsugae* Hopkins (Huber et al., 1999). When combining GLV-alcohol and C_8 alcohol blends with Vn, a significant reduction of trap catches was achieved in *I. duplicatus*. This may indicate either an additive or a synergistic effect of combined stimuli (Deglow and Borden, 1998a; Poland and Haack, 2000; Schlyter et al., 2000). However, further experiments with controlled overall release rates and dose–response studies are needed to clarify the type of effect.

In the natural habitat of *I. duplicatus* in Inner Mongolia, China, *P. mongolica* is the only dominant and protected tree species of the natural reserve (Zhang et al., 1995). However, there are spruce stands mixed with birch (*B. platyphylla* Suk.) and aspen (*P. davidiana* Dode) as well. Thus, it would be adaptive for *I. duplicatus* to recognize and further avoid the nonhost birch and aspen by the olfactory means when seeking for the host in flight. Attacking a nonhost birch or aspen tree would typically result in death of the attacking beetle. The nonhost volatiles tested in the current study are based on the data and materials of European birches (*B. pubescens* and *B. pendula*) and aspen (*P. tremula*). Recognition and avoidance of the Far East *I. duplicatus* to the leaf and bark volatiles of European angiosperm trees may indicate a similarity in the volatile emissions between the European and Chinese birch and aspen species. However, further study on the volatile emissons of the native *B. platyphylla* and *P. davidiana* from Baiyinaobao Natural Reserve and their potential electrophysiological and behavioral activity on *I. duplicatus* is needed. Particular active volatiles from the nonhost trees in the *I. duplicatus* natural environment in Inner Mongolia, if any, may prove to be even more disruptive than the general GLVs and NHVs found in the European species.

These results show that the nonhost leaf and bark volatiles and the unsuitable host compound, Vn, can significantly reduce the positive responses of *I. duplicatus* to its pheromone sources. This may have potential for the management of *I. duplicatus* by reducing risk of attacks on suitable hosts. In addition, combination of active NHVs and Vn with the pheromone mass-trapping in a "push–pull" fashion may strengthen our combative force against serious outbreaks of *I. duplicatus* in this natural reserve.

Acknowledgments—We thank Dr. F.-M. Yan, Beijing University, for his careful shipment of live beetles and Dr. T. Poland for comments. The permission to "thin" spruce stands at Björnstorp Estate for bark beetle breeding is highly appreciated. This study was supported by grants from the Swedish Council for Forestry and Agricultural Research (SJFR: No.23.0521/96; No.24.0293/98) and an EU-INCO project (CT 98-0151).

REFERENCES

ATKINS, M. D. 1966. Behavioral variation among scolytids in relation to their habitat. *Can. Entomol*. 98:285–288.

BORDEN, J. H. 1997. Disruption of semiochemical-mediated aggregation in bark beetles, pp. 421–438, *in* R. T. Cardé and A. K. Minks (eds.). Pheromone Research: New Directions. Chapman and Hall, New York.

BORDEN, J. H., CHONG, L. J., SAVOIE, A., and WILSON, I. M. 1997. Responses to green leaf volatiles in two biogeoclimatic zones by striped ambrosia beetle, *Trypodendron lineatum. J. Chem. Ecol*. 23:2479–2491.

BORDEN, J. H., WILSON, I. M., GRIES, R., CHONG, L. J., PIERCE, H. D., JR., and GRIES, G. 1998. Volatiles from the bark of trembling aspen, *Populus tremuloides* Michx. (Salicaceae) disrupt secondary attraction by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *Chemoecology* 8:69–75.

BYERS, J. A., and WOOD, D. L. 1980. Interspecific inhibition of the response of the bark beetles, *Dendroctonus brevicomis* and *Ips paraconfusus*, to their pheromones in the field. *J. Chem. Ecol*. 6:149–164.

BYERS, J. A., SCHLYTER, F., BIRGERSSON, G., and FRANCKE, W. 1990. E-myrcenol in *Ips duplicatus*: An aggregation pheromone component new for bark beetles. *Experientia* 45:1209–1211.

BYERS, J. A., ZHANG, Q.-H., SCHLYTER, F., and BIRGERSSON, G. 1998. Volatiles from non-host birch trees inhibit pheromone response in spruce bark beetles. *Naturwissenschaften* 85:557–561.

BYERS, J. A., ZHANG, Q.-H., and BIRGERSSON, G. 2000. Strategies of a bark beetle, *Pityogenes bidentatus*, in an olfactory landscape. *Naturwissenschaften* 87:503–507.

DEGLOW, E. K., and BORDEN, J. H. 1998a. Green leaf volatiles disrupt and enhance response to aggregation pheromones by the ambrosia beetle, *Gnathotrichus sulcatus* (LeConte) (Coleoptera: Scolytidae). *Can. J. For. Res*. 28:1697–1705.

DEGLOW, E. K., and BORDEN, J. H. 1998b. Green leaf volatiles disrupt and enhance response by the ambrosia beetle, *Gnathotrichus retusus* (LeConte) (Coleoptera: Scolytidae) to pheromone-baited traps. *J. Entomol. Soc. Bt. C*. 95:9–15.

DE GROOT, P., and MACDONALD, L. M. 1999. Green leaf volatiles inhibit response of red pine cone beetle *Conophthorus resinosae* (Coleoptera: Scolytidae) to a sex pheromone. *Naturwissenschaften* 86:81–85.

DICKENS, J. C., BILLINGS, R. F., and PAYNE, T. L. 1991. Green leaf volatiles: A ubiquitous chemical signal modifies insect pheromone responses, pp. 277–280, *in* I. Hrdy (ed.). Insect Chemical Ecology. Acadamia Praha, Prague, Czechslovakia.

DICKENS, J. C., BILLINGS, R. F., and PAYNE, T. L. 1992. Green leaf volatiles interrupt aggregation pheromone response in bark beetles infecting pines. *Experientia* 48:523–524.

GUERRERO, A., FEIXAS, J., PAJARES, J., WADHAMS, L. J., PICKETT, J. A., and WOODCOCK, C. M. 1997. Semiochemically induced inhibition of behaviour of *Tomicus destruens* (Woll.) (Coleoptera: Scolytidae). *Naturwissenschaften* 84:155–157.

HUBER, D. P. W., GRIES, R., BORDEN, J. H., and PIERCE, H. D., JR. 1999. Two pheromones of coniferophagous bark beetles found in the bark of nonhost angiosperms. *J. Chem. Ecol*. 25:805–816.

HUBER, D. P. W., GRIES, R., BORDEN, J. H., and PIERCE, H. D., JR. 2000. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. *Chemoecology* 10:103–113.

LEKANDER, B., BEJER-PEDERSEN, B., KANGAS, E., and BAKKE, A. 1977. The distribution of bark beetles in the Nordic countries. *Acta Entomol Fenn*. 32:1–100.

LIU, G.-T., LIU, T., and ZHOU, R. 1998. Prediction of occurrences and population tendency of *Ips duplicatus* Sahlb. by using Markov chain, pp. 258–260, *in* W.-D. Xu and G.-T. Liu (eds.). Study on *Picea mongolica* Forest Ecosystem in Baiyinaobao Natural Preserve, Inner Mongolia. China Forestry Publishing House, Beijing.

KNIZEK, M., and ZAHRADNIK, P. 1996. Mass outbreak of *Ips duplicatus* Sahlberg (Coleoptera: Scolytidae), p. 527, *in* J. Doe (ed.). Proceedings of the XXth International Congress of Entomology, Firenze, Italy, August 25–31, 1996.

KROKENE, P., and SOLHEIM, H. 1996. Fungal associates of five bark beetle species colonizing Norway spruce. *Can. J. For. Res*. 26:2115–2122.

PFEFFER, A. 1995. Zentral- und westpaläarktische Borken- und Kernkäfer (Coleoptera: Scolytidae, Platypodidae). p. 310, Pro Entomologia, Naturhistorsches Museum, Basel.

POLAND, T. M., and HAACK, R. A. 2000. Pine shoot beetle, *Tomicus piniperda* (Coleoptera: Scolytidae), responses to common green leaf volatiles. *J. Appl. Entomol*. 124:63–69.

POLAND, T. M., BORDEN, J. H., STOCK, A. J., and CHONG, L. J. 1998. Green leaf volatiles disrupt responses by the spruce beetle, *Dendroctonus rufipennis*, and the western pine beetle, *Dendroctonus brevicomis* (Coleoptera: Scolytidae) to attractant-baited traps. *J. Entomol. Soc. B. C*. 95:17–24.

POSTNER, M. 1974. *Ips duplicatus*, p. 455, *in* W. Schwencke (ed.). Die Forstschädlinge Europas, 2. Paul Parey, Hamburg.

SCHLYTER, F., and ANDERBRANT, O. 1993. Competition and niche separation between two bark beetles: Existence and mechanisms. *Oikos* 68:437–447.

SCHLYTER, F., and BIRGERSSON, G. 1999. Forest Beetles. pp. 113–148, *in* R. J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford, UK.

SCHLYTER, F., LEUFVÉN, A., and BIRGERSSON, G. 1989. Inhibition of attraction to aggregation pheromone by verbenone and ipsenol: Density regulation mechanisms in bark beetle *Ips typographus*. *J. Chem. Ecol*. 15:2263–2277.

SCHLYTER, F., BIRGERSSON, G., BYERS, J. A., and BAKKE, A. 1992. The aggregation pheromone of *Ips duplicatus* and its role in competitive interactions with *I. typographus* (Coleoptera: Scolytidae). *Chemoecology* 3:103–112.

SCHLYTER, F., LÖFQVIST, J., and JAKUS, R. 1995. Green leaf volatiles and verbenone modify attraction of European *Tomicus*, *Hylurgops*, and *Ips* bark beetles, pp. 29–44, *in* F. P. Hain, S. M. Salom, W. F. Ravlin, T. L. Payne, and K. F. Raffa (eds.). Behavior, Population Dynamics, and Control of Forest Insects, Proceedings of a Joint IUFRO Working Party Conference—February 1994, Ohio State University, OARDC, Wooster.

SCHLYTER, F., ZHANG, Q.-H., ANDERSON, P., BYERS, J. A., WADHAMS, L., LÖFQVIST, J., and BIRGERSSON, G. 2000. Electrophysiological and behavioral responses of pine shoot beetles, *Tomicus piniperda* and *T. minor* (Coleoptera: Scolytidae), to non-host leaf and bark volatiles. *Can. Entomol*. 132:965–981.

SCHLYTER, F., SVENSSON, M., ZHANG, Q.-H., KNIZEK, M., KROKENE, P., IVARSSON, P., and BIRGERSSON, G. 2001. A model for peak and width of signaling windows: *Ips duplicatus* and *Chilo partellus* pheromone component proportions—does response have a wider window than production? *J. Chem. Ecol*. In press.

SCHROEDER, L. M. 1992. Olfactory recognization of nonhosts aspen and birch by conifer bark beetles *Tomicus piniperda* and *Hylurgops palliatus*. *J. Chem. Ecol*. 18:1583–1593.

TØMMERÅS, B. Å. 1989. Host selection by odorous compounds from host and non-host trees in bark beetles. *Fauna Norv. Ser. B* 36:75–79.

TØMMERÅS, B. Å., and MUSTAPARTA, H. 1989. Single cell responses to pheromones, host and non-host volatiles in the ambrosia beetle *Trypodendron lineatum. Entomol. Exp. Appl.* 52:141–148.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol*. 31:121–144.

WHITMAN, D. W., and ELLER, F. J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69–75.

WILSON, I. M., BORDEN, J. H., GRIES, R., and GRIES, G. 1996. Green leaf volatiles as antiaggregants for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *J. Chem. Ecol*. 22:1861–1875.

XU, W.-D., ZOU, C.-J., and LIU, G.-T. 1998. The taxonomy and formation of *Picea mongolica*. pp. 24–33, *in* W.-D. Xu and G.-T. Liu (eds.). Study on *Picea mongolica* forest ecosystem in Baiyinaobao Natural Preserve, Inner Mongolia. China Forestry Publishing House, Beijing.

ZHANG, Q.-H. 2001. *Ips duplicatus*. *In* Y. Baranchikov and M. Montgomery, (eds.). Manual of Main Forest Insect Pests in Northern Asia. USDA Forest Service. in press, (In English and Russian).

ZHANG, Q.-H., SCHLYTER, F., and LIU, G.-T. 1995. Spatial distribution, mortality and sex ratio of overwintering *Ips duplicatus* in a *Picea mongolica* reserve in Inner Mongolia, China with a diffusion model, pp. 109–122, *in* F. P. Hain, S. M. Salom, W. F. Ravlin, T. L. Payne, and K. F. Raffa (eds.). Behavior, Population Dynamics, and Control of Forest Insects, Proceedings of a Joint IUFRO Working Party Conference—February 1994, Ohio State University, OARDC, Wooster.

ZHANG, Q.-H., BIRGERSSON, G., ZHU, J.-W., LÖFSTEDT, C., LÖFQVIST, J. and SCHLYTER, F. 1999a. Leaf volatiles from nonhost deciduous trees: Variation by tree species, season, and temperature and electrophysiological activity in *Ips typographus*. *J. Chem. Ecol*. 25:1923–1943.

ZHANG, Q.-H., SCHLYTER, F., and ANDERSON, P. 1999b. Green leaf volatiles interrupt pheromone response of spruce bark beetle, *Ips typographus. J. Chem. Ecol*. 25:2847–2861.

ZHANG, Q.-H., SCHLYTER, F., and BIRGERSSON, G. 2000. Bark volatiles from non-host angiosperm trees of spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Scolytidae): Chemical and electrophysiological analysis. *Chemoecology* 10(2):69–80.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

FOOD PROCESSING BY ANIMALS: DO BEAVERS LEACH TREE BARK TO IMPROVE PALATABILITY?

DIETLAND MÜLLER-SCHWARZE,[1,*] HEATHER BRASHEAR,[1] ROBIN KINNEL,[2] KATHARINE A. HINTZ,[2] ANASTASIA LIOUBOMIROV,[2] and CATHERINE SKIBO[2]

[1]*SUNY College of Environmental Science and Forestry*
Syracuse, New York

[2]*Department of Chemistry*
Hamilton College
Clinton, New York

(Received October 24, 2000; accepted January 15, 2001)

Abstract—Beavers store and consume tree parts in the bodies of water where they live. We examined whether such soaking renders food more palatable by leaching out undesirable compounds. In experiment 1, saplings of red maple, *Acer rubrum* (RM), were first soaked in a pond for periods of 2, 18, and 36 days, then offered to free-ranging beavers. Soaking for two days rendered RM slightly more acceptable to beavers. To further examine the time window around two days, RM sticks were soaked in distilled water in the laboratory for 1, 2, 4, and 6 days before presenting them to beavers (experiment 2). In experiment 3, twigs of three species were placed on land. Beavers placed RM in the water for 1 to 3 days before consuming the twigs. In experiment 4, sticks were provided in the water at Cranberry Lake Biological Station (CLBS). Most quaking aspen (QA) was consumed during the first night, and most witch hazel, *Hamamelis virginiana* (WH), during the third night. At Allegany State Park (ASP), no such difference was found. Twigs were provided in the water in experiment 5. At ASP, WH was taken after three days in the water, and at CLBS little WH was consumed, and only during the third night. A meta-analysis of all experiments shows that relatively more WH is consumed after two days than any other species. Experiment 6 traced the time beavers left their own harvested branches in the water. Unlike other tree species, WH remained in the water for two to four days before being consumed. Experiment 7 measured the phenolics leached into water from RM twigs and small pieces of bark soaked for 10 and 8 days, respectively. Shredded bark lost 50–60% of leachable phenolics into the water, and twigs 70–80%. We conclude that beavers can use water to leach undesirable compounds from their food. Although this effect was not robust, our study is the first of its kind.

* To whom correspondence should be addressed. e-mail: dmullers@mailbox.syr.edu

0098-0331/01/0500-1011$19.50/1

Key Words—*Acer rubrum*, beaver, *Castor canadensis*, food conditioning, food processing, *Hamamelis virginiana*, leaching, red maple, soaking, witch hazel.

INTRODUCTION

Beavers (*Castor canadensis*) handle, eat, and store food in the bodies of water where they live. In northern climes, they stockpile branches in the water near their lodges as winter food. In addition to these longer-term food caches, beavers also leave harvested branches and logs in the water for one or more days before they consume the bark. Finally, beavers eat bark and leaves while sitting in shallow water. Thus, water is an important medium for storing food and feeding, in addition to its well-known role for floating logs and branches from harvest areas to feeding sites.

We now ask the question: Do beavers use water to process their food? Are they taking advantage of water's ability to leach potentially noxious compounds from pieces of wood to render their food more palatable? If so, beaver might turn otherwise unpalatable tree species into useful food. In turn, such behavior on the part of beavers might force us to reassess what constitutes a suitable food supply and hence, beaver habitat. Many habitat analyses might have to be revised, yielding more available forage than previously thought, after possible food conditioning by beavers.

Humans have long used water for leaching out undesirable, astringent compounds from food. For instance, native North Americans used to soak crushed acorns in streams to render them more palatable by leaching out astringent tannins (Johns, 1990), and to this day South American indigenous farmers soak cassava (manioc, *Manihot esculenta*, Euphorbiaceae) in water (D. M. S; personal observation) to rid it of dangerous cyanogenic compounds, notably linamarin (Johns, 1990).

Food processing by animals has seldom been described. The meadow vole *Microtus pennsylvanicus* cuts winter branches of white and Norway spruce and white and Norway pine and leaves them on the snow for two to three days before eating them. This treatment reduces levels of phenolics by one half, that is, to their summer levels. The high level of protein (12%) and decreased phenolics (1.5%/ dry matter) render the food acceptable. The twigs lose the phenolics possibly by polymerization or oxidation (Roy and Bergeron, 1990).

Pikas, *Ochotona princeps*, of the North American Rocky Mountains are known for the hay piles they accumulate for the winter. In contrast to their phenolics-poor summer diet, they collect phenolics-rich plants, notably alpine avens, *Acomastylis rossii*, for these food caches. As the winter progresses, the phenolic content decreases. When it reaches levels of the summer diet, the pikas consume the stored food (Dearing, 1997).

Mountain beavers (*Aplodontia rufa*) store toxic, alkaloid-containing plants in food caches called haystacks. These plants include tall larkspur (*Delphinium glaucum*), corydalis (*Corydalis caseana*), and corn lily (*Veratrum californicum*) (O'Brien, 1981). We suggest that these alkaloids might be denatured during storage.

The assumption that acorns, buried in the ground by squirrels or jays, lose tannins over the winter has not been confirmed (Dixon et al., 1997).

We noted that beavers in our study area avoid red maple (*Acer rubrum*) (RM). It is often one of the last or even only tree species remaining at the edge of a beaver pond (Müller-Schwarze et al., 1994). Other wildlife also avoid red maple. For instance, porcupines (*Erethizon dorsatum*) reject red maple in both bud and leaf stage, eating it in only one of 812 feeding episodes, while consuming sugar and striped maple more often (Roze, 1989). It is not known what compounds render red maple bark unpalatable. However, red maple leaves contain high levels of phenolics (Shure and Wilson, 1993), including gallic acid and methyl gallate (Abou-Zaid et al., 2000). Red maple also contains small amounts of gramine, an alkaloid (Barbosa et al., 1990). In other studies, beavers consumed RM (e.g., Busher, 1996), even in large amounts, compared to other tree species (Jenkins, 1980), and stored it in their winter food caches roughly in proportion to its abundance at the beaver site (Busher, 1991). However, red maple has less energy content and is less digestible than aspen and alder (Fryxell and Doucet, 1993). Red maple takes almost 30–50 hr to pass through the intestinal tract of a beaver, compared with only 10–20 hr for aspen (Doucet and Fryxell, 1993).

Here, we report results of experiments designed to test whether beavers improve palatability of bark of RM and WH, which are usually less preferred species in our study area, by soaking them in water before eating. In addition, with field observations we tried to establish whether beaver-harvested limbs of different tree species remain in the water for different lengths of time, suggesting leaching of unpalatable species.

METHODS AND MATERIALS

Study Sites

The primary study site was the 250-km^2 forested Allegany State Park (ASP) in Cattaraugus Country in western New York State. It contains about 30 beaver colonies on streams totaling about 70km in length. Additional experiments were performed at Cranberry Lake Biological Station (CLBS), located at the lake of the same name in the mountains of Adirondack Park in northern New York State. There are up to six beaver colonies along the lakeshore and on streams feeding the lake on the 2.6-km^2 forested property owned by the State University of New York.

General Procedures

In experiments 1–3, the sticks or twigs presented on land were stuck in the ground vertically, 30 cm apart, in one row 50 cm above the water's edge, and parallel to it on the evening of day zero. Sticks or twigs were offered in the water in experiments 4 and 5. They were placed in bundles (to mimic bushes and to better find them again) of the same tree species in a shallow part of the pond, and as close as possible to the lodge. The leaves were above water, and the butt ends on the pond bottom. The following morning, and for four mornings thereafter, we recorded the beavers' responses to the samples during the previous night and counted the remaining samples. We found the food samples either left intact and in place, dragged into the water, sampled, removed, or the bark (and leaves, where applicable) fully or partially consumed. Since beavers are active during the night hours, we use "days" in the sense of 24-hr periods in the graphs. In experiment 1, all sticks were replaced every day, and no sticks or twigs were replaced in experiments 2–6.

Experiment 1: Leaching Red Maple Poles in Pond

Experiment 1 duplicated natural behavior of beavers as much as possible. Eighty 2-m-long sections of winter-dormant red maple saplings [mean diameter at thick end 1.97 ± 0.07 (SE) cm; thin end: 1.15 ± 0.076 cm] were harvested at a beaver site on March 2 at 10°C air temperature. They were transported frozen, and the first batch of 20 poles (to be soaked for 36 days) sunk in an ice-covered pond on the following day. A second batch of 20 poles was kept frozen in a snow bank (0°C) and placed in the pond 18 days later. Because the snow bank was receding, a third batch was kept frozen in a walk-in freezer at −10°C, and placed in the pond 34 days after the first. Thus, the batches were soaked in the pond for 2, 18, and 36 days, respectively. Water temperature was +2°C. The pond was ice-covered, with 40–50 cm snow cover. A nonsoaked control sample of 20 poles was kept frozen at −10°C in a walk-in freezer.

For the field tests, the poles were cut into 30-cm-long segments and fed to free-ranging beaver at their colony sites. In addition to the frozen controls, fresh red maple was harvested at the time of provisioning and offered to the beaver as a second control. Finally, sticks of the preferred quaking aspen (*Populus tremuloides*) were also offered as controls. Thus, six treatments in all were used. Consumption of the preferred aspen indicated that beavers were present and attracted to the experimental food.

The sticks were presented in April simultaneously at 10 beaver colonies in a randomized complete block design. The beaver sites represented blocks. (A total of 14 colonies was used because new sites had to be substituted when beavers proved absent at some). For each of the six treatments, two sticks were used per

site per night. The experiment lasted five nights. A total of $6 \times 2 \times 10 \times 5 = 600$ sticks were used, 100 for each treatment. All sticks were replaced on each of the five days of the experiment.

The water temperatures of three streams at beaver sites were +5, +9, and +12°C, respectively. Daily low air temperatures averaged 2.3 ± 1.7°C and daily highs 13.5 ± 1.6°C.

Experiment 2: Leaching Sticks in Containers

To narrow down the effective time window, 30-cm-long RM sticks were collected from saplings at a beaver site not used for preference tests. The sapling pieces were then soaked in tubs with local groundwater and placed on a covered outdoor porch for one, two, four, and six days. To facilitate leaching, the sticks received multiple punctures by rolling them over a nailboard. Half of the water in each tub was replaced each day. Punctured RM sticks, and intact RM sticks. Freshly cut quaking aspen sticks served as controls.

Unlike experiment 1, five trials were run (September 22 to November 27, 1996). The sticks were placed in rows on the shore or the dam, 30 cm apart. Three sticks per treatment (two in trial 1) were placed together, and the sequence of treatment randomized for each site. A total of 770 sticks was offered at eight beaver sites (seven in trial 1), and not replaced for the five days of the experiment.

Cumulative removal and consumption of sticks during each five-day period were recorded by counting intact, sampled, removed, and consumed sticks. The area of bark consumed by beaver on each accepted log was also measured.

Experiment 3: Twigs Provided on Land

In April 19–24, 1999, winter-dormant twigs were provided at five colonies. Altogether 33 QA twigs, 53 RM twigs, and 30 WH twigs were placed. The twigs were not replaced during the five days of the experiment. Minimum air temperatures ranged from −0.5°C to +7.2°C, maximum temperatures from 8.3°C to 14.4°C.

Experiment 4: Sticks Provided in Water

Experiment 4a. From May 3 to 8, 1999, 10 sticks each of RM, QA, and WH were placed at five colonies, for a total of 50 sticks of each species at ASP. Minimum air temperatures ranged from 1.1 to 13.9°C, and maximum temperatures from 21.2 to 27.8°C.

Experiment 4b. A smaller sample of QA, RM, and WH sticks was provided in the water at Cranberry Lake Biological Station (CLBS) in the Adirondack Mountains of New York between July 6 and 16, 1999. The sticks were not replaced during the five days of the experiment.

TABLE 1. TREE SPECIES USED IN EXPERIMENTS

American beech	*Fagus grandifolia*
American hornbeam	*Carpinus caroliniana*
Black cherry	*Prunus serotina*
Black locust	*Robinia pseudoacacia*
Juneberry (serviceberry)	*Amelanchier canadensis*
Quaking aspen	*Populus tremuloides*
Red maple	*Acer rubrum*
Red oak	*Quercus rubra*
Striped maple	*Acer pensylvanicum*
Sugar maple	*Acer saccharum*
White ash	*Fraxinus americana*
Northern wild raisin	*Viburnum cassioides*
Willow	*Salix sp.*
Witch hazel	*Hamamelis virginiana*
Yellow birch	*Betula alleghaniensis*

Experiment 5: Twigs Provided in Water, ASP, and CLBS

Experiment 5a. From May 21 to 27, 1999 a total of 636 twigs (with leaves) were provided in the water at seven sites at ASP. Twelve species of trees and shrubs were used (Fig. 5). The species provided at each site depended on which ones occurred there naturally. Minimum air temperatures ranged from 2.7°C to 10.5°C, maximum temperatures from 11.1 to 23.3°C.

Experiment 5b. A total of 108 [RM, WH, QA, and willow (*Salix sp*.)] were placed at one lake beaver site at CLBS between July 6 and 16, 1999.

Experiments 6a and 6b: Tracing Beaver-Harvested Tree Limbs, Fall and Spring

Limbs and saplings harvested by the beavers themselves were labeled with permanent markers and followed for their fate. On day 0, all branches already in the water were removed. Recording started on day 1 when new branches appeared in the water. The beavers either left branches on land or dragged them into the water. They ate the bark at either location, or built the material into lodge, dam, or (in autumn only) into their food cache. Only a portion was identifiable on subsequent days, since some logs sank to the bottom or were placed on the lodge or into the food cache and covered with other materials such as sticks and/or mud.

Experiment 6a. In fall, at one beaver colony, trees were divided into those that served as immediate food and those that were stored. In the immediate food category we distinguished branches that were eaten ashore from those dragged into the water and then eaten. We recorded the number of days branches and logs remained in the water before consumption after beaver had dragged them into the water.

Experiment 6b. In spring, again all branches found in the water at seven sites were removed on day 0. New branches in the water were marked with permanent markers every meter (because beavers cut them into sections), starting on day 1. We followed the dismemberment of these branches by the beavers. The observations continued for seven days. The water temperatures ranged from 9.5 to 16.5°C at the seven sites over three days.

Experiment 7: Chemical Testing for Amount of Phenolics Leached into Water

The total amount of phenolics available for leaching was tested by extraction of lyophilized bark with methanol–water (8:2). The ground, dry bark was suspended in the solvent and homogenized in a blender. The centrifuged supernatant solution was assayed by the Folin-Ciocalteau method according to Box (1983). This procedure was repeated using distilled deionized water. The average water content was determined by shaving several twigs and lyophilizing the bark. The average proportion of bark to woody material was determined as well.

To test for the loss of phenolic material into water, entire twigs (harvested in the fall at ASP, and stored at −80°c (until use) were soaked in deionized water for 10 days at 4°C and at 25°C, and tested for phenolics after 1, 2, 3, 4, 7, and 10 days, using the Folin-Ciocalteau method.

In a slightly different study, bark from RM twigs of the same origin was cut into small pieces (several mm long) and soaked in deionized water at 4°C for eight days. The concentration of phenols in the water was tested after two, four, six, and eight days, also using the Folin-Ciocalteau method.

RESULTS

Experiment 1. Sticks Soaked in Pond, Presented on Land

We classified the responses as complete consumption, i.e., removal of all bark from a stick; sampling, i.e., leaving bite marks or peeling less than 20% of a stick; and no response. Missing sticks were counted as no response. Eight colonies responded by taking at least one stick; five colonies sampled or ate RM samples. Overall, the beaver responded to only 63 of the 600 logs (10.5%). Of these, 46 (73%) were completely consumed. As expected, the aspen controls were consumed most readily. Thirty-seven percent of these sticks were completely peeled. None was only sampled. The numbers of RM sticks accepted were small (Figure. 1). A Cochran Q test of the overall consumption pattern gave a Q of 56.6 ($P < 0.0001$). However, the acceptance of aspen is responsible for this significance. For RM samples alone, $Q = 6.74$ (not significant). More two-day sticks (10) were accepted

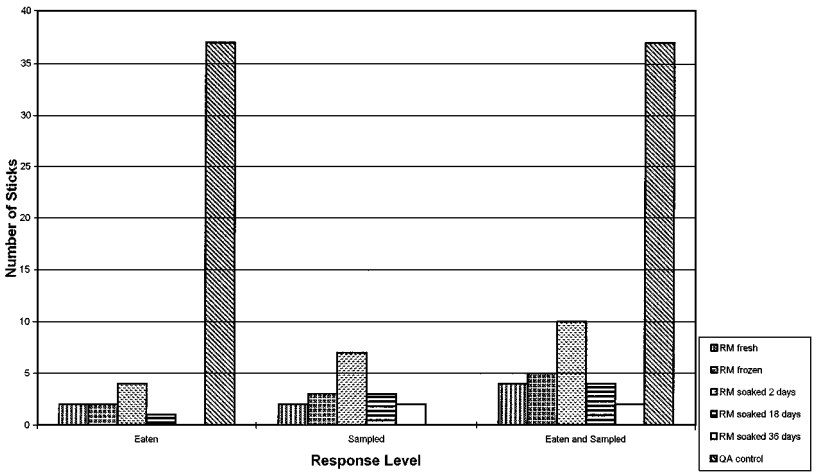


FIG. 1. Responses of beavers to sticks soaked in a pond for 2, 18, or 36 days, and controls (fresh and frozen red maple and fresh quaking aspen). Beavers handled slightly more sticks soaked for two days than other red maple samples. RM = red maple; QA = quaking aspen.

or sampled than the two controls, fresh or frozen RM (4 and 5, respectively). However, the difference is not significant ($\chi^2 = 7.2; df = 4$; NS). The difference between frozen and two-day RM is not significant ($\chi^2 = 2.29$; McNemar test). The difference between 2-day and 36-day samples is significant ($\chi^2 = 6.13$; $P < 0.025$; McNemar test).

Experiment 2. Sticks Soaked in Laboratory, Presented on Land

Of the 770 sticks, 82 (10.7%) were accepted by the beavers. As expected, QA was the most preferred sample, with 29 sticks (35%) peeled totally or partially. Of these, 26 were peeled totally. Beavers handled (consumed and sampled) RM soaked for one day slightly more often (39 sticks) than RM soaked for two, four, or six days (34, 33, and 29, respectively; Figure 2). The differences between consumption of RM soaked for one, two, four, and six days were not significant. However, in trial 2 (of five trials) one-day soaked RM and four-day-soaked RM did differ marginally ($P < 0.10$; Waller-Duncan *k* Ratio *t* test).

Experiment 3. Twigs Provided on Land

The beavers treated the three different species differently: QA was completely removed (consumed?) on the second day. On that day, only 54.7% of the RM and

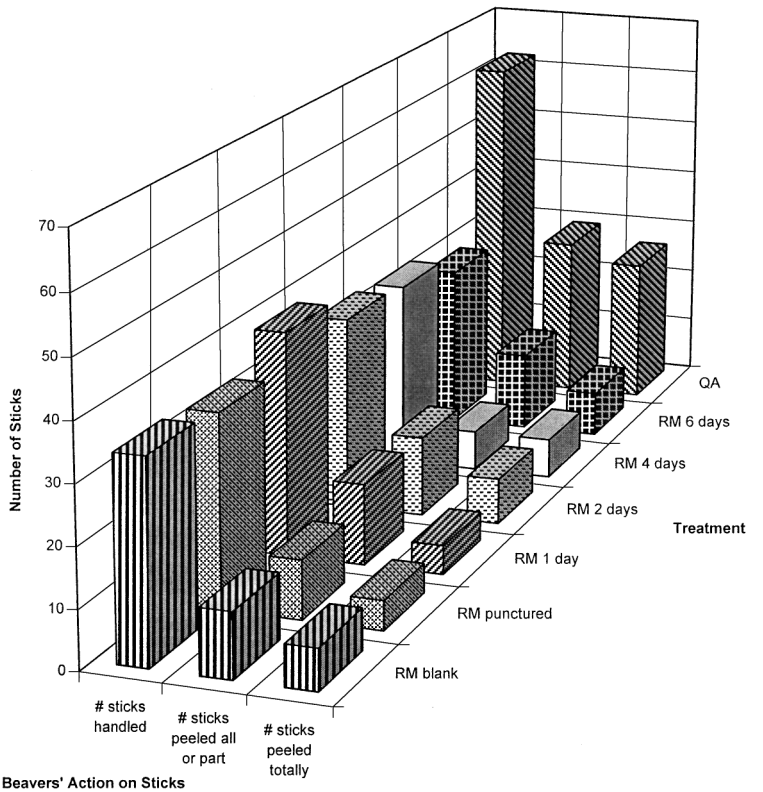


FIG. 2. Numbers of laboratory-leached RM sticks handled or peeled by beavers. Summary of five trials. A total of 770 sticks was offered, 110 sticks per treatment.

43.3% of the WH had been taken. After four days, only 60.4 of the RM, and 63.3% of the WH had been removed. Thirteen of 53 RM twigs were dragged into the water after the first night. After the third night, none was found in the water, but 32 were missing (eaten?) and 21 remained in place (Figure 3). This suggests that the beavers left the RM twigs in the water for one to three nights before further using them, presumably for food.

Experiment 4. Sticks Provided in Water

Experiment 4a. At ASP, beavers removed 33 of 53 aspen sticks presented in water (62.3%), 27 of 54 RM sticks (50%), and 21 of 54 WH sticks (38.9%). In this experiment, there was no indication that some tree species remained longer in the water than others.

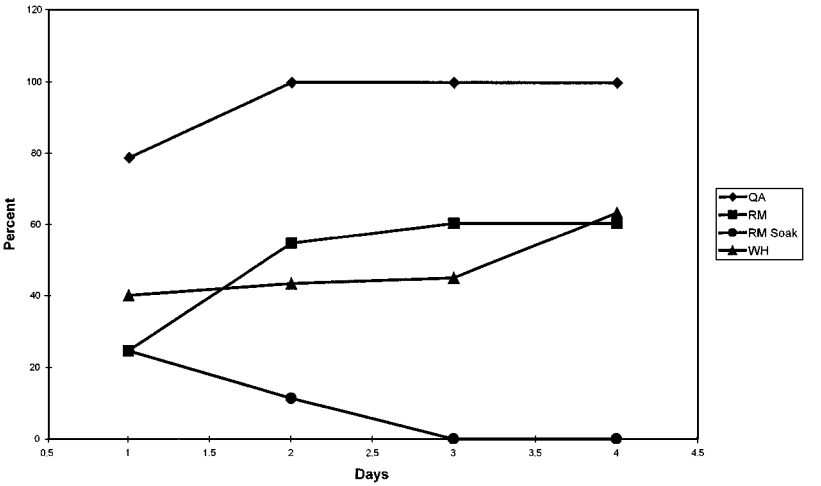


FIG. 3. Consumption and soaking of twigs experimentally provided on land. Abscissa: the four activity periods (nights); ordinate: percent twigs consumed or soaked. QA: quaking aspen ($N = 33$); RM: red maple ($N = 53$); RM Soak: RM pulled into water and not (yet) eaten; WH: witch hazel ($N = 30$).

Experiment 4b. At CLBS, the trend was similar: Of 20 sticks each, 19 aspen sticks, 13 RM sticks, and 12 WH sticks were removed. However, in contrast to ASP, WH was left in the water longer before consumption: On day 1, only one WH stick was taken (=5%), on day 2, five more, and on day 3, six more, for a cumulative total of 12 (60%). This compares with 11 aspen sticks on day 1 (55%), and a cumulative total of 19 on day 3 (95%); and 10 RM logs on day 1 (50%), 3 more on day 2 (65%), and none on day 3, for a cumulative total of 13 on day 3 (65%). After that, very few sticks of any species were taken until day 6. It appears that WH is accepted only after some time in the water (Figure 4). The differences are marginally significant (χ^2test; $P < 0.07$).

Experiment 5. Twigs Provided in Water

Experiment 5a. ASP Site. All willow accepted in this experiment (67%) and most aspen, striped maple, and black cherry were consumed after the first night (day 1) (Figure 5). By contrast, the beavers consumed most of the WH after it had been in the water for three nights. This is similar to the results of experiment 4 (fall). RM was rarely consumed, and hawthorn and black cherry often remained in the water for several nights before being removed.

Experiment 5b. CLBS Site. As usual, most of the quaking aspen twigs (14 of 20) disappeared during the first night. Little RM (6 of 20) and WH (4 of 20) were

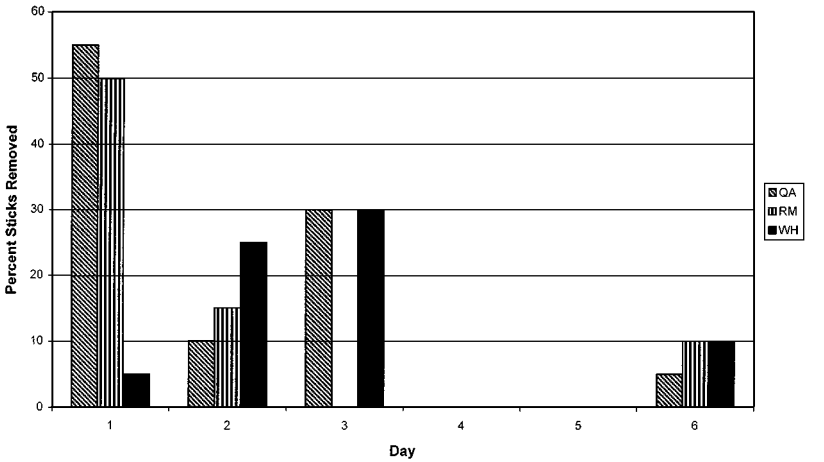


FIG. 4. Removal of experimental sticks at CLBS. Note that most witch hazel sticks were consumed after two or three days in the water.

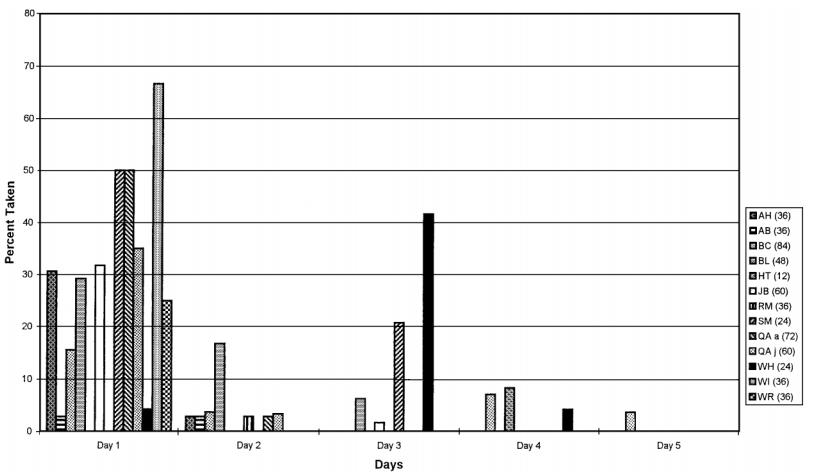


FIG. 5. Time in water of experimental twigs provided in water at ASP. Abscissa: activity periods, ordinate: percent twigs removed by beavers. AH: American hornbeam; QA j: quaking aspen, juvenile growth form; QA a: quaking aspen, adult growth form; AB: American beech; BC: black cherry; BL: black locust; HT: hawthorn; JB: juneberry; RM: red maple; SM: striped maple; WH: witch hazel; WI: willow; WR: wild raisin. Sample sizes in parentheses. Note that witch hazel is eaten after three days, and red maple almost not at all.

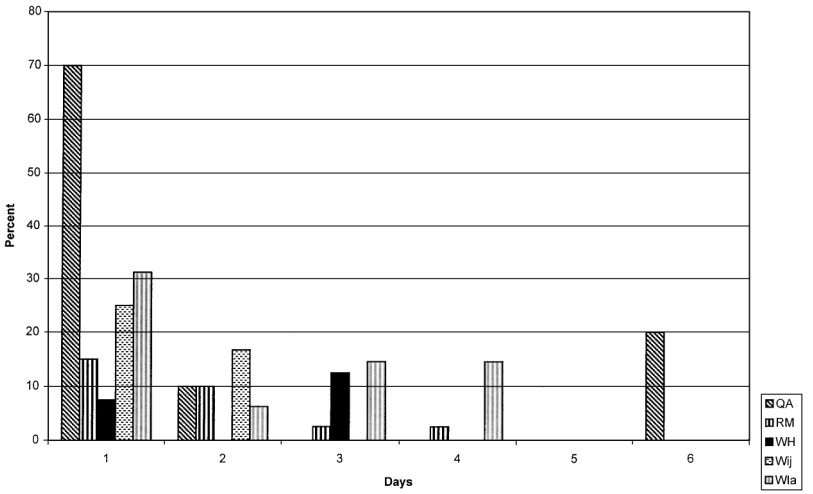


FIG. 6. Consumption of twigs, provided in water at CLBS, over six days.

accepted. Beavers removed WH and also adult willow at high, but not significantly higher levels, during the third night (Figure 6).

A meta-analysis of the numbers of samples taken by beavers in all six cafeteria experiments (food presented in water or land) yields significant ($P < 0.001$) results because the small differences in each experiment point in the same direction. A higher proportion of WH pieces is consumed after three or even four days, than of QA or RM (Figure 7). The pattern for RM is intermediate between QA and WH: About 76% (100 of 131 accepted pieces) of the QA is consumed on the first day, but only 57% (45 of 79) of RM, and 59%(43.5 of 74) of WH. On the second day, the beavers removed a high percentage of RM (26 of 79 = 33% of all RM taken), but only 15% (20 of 131) and 11% (8 of 74) of QA and WH, respectively. During the third night, 31% (23 0f 74) of the WH was removed, but only 7% (9 of 131) of the QA and 8% (6.5 of 79) of the RM. Similarly, on day 4 the relative level of accepted WH was higher (8.5 of 74 = 11.5%) than those for QA (2 of 131 = 1.5%) and RM (1.5 of 79 = 1.9%). It should be pointed out that the animals did not deplete one species before proceeding to the next, less palatable species.

Experiment 6: Beavers' Own Harvest

During the fall, a total of 23 trees were debarked on shore. These belonged to five species: white ash, American beech, American hornbeam, quaking aspen, and sugar maple. Twenty-one trees representing nine species were peeled in the water: witch hazel, American hornbeam, American beech, white ash, yellow birch, sugar maple, quaking aspen, red maple, and red oak. Four trees representing three

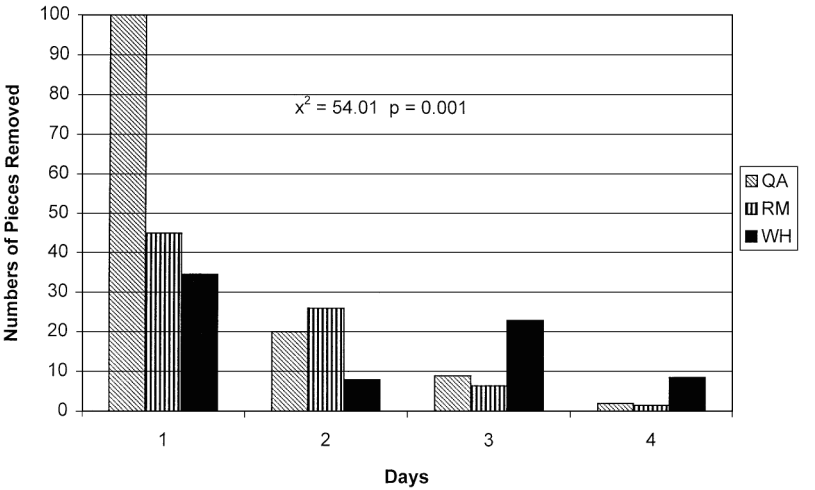


FIG. 7. Meta-analysis of all six cafeteria experiments that measured responses to sticks or twigs presented in water or on land. Abscissa: activity period, ordinate: number of pieces (sticks or twigs) accepted by beavers. QA: quaking aspen; RM: red maple; WH: witch hazel. Relatively more witch hazel is consumed after three days.

species were debarked partially on shore and partially in the water: black cherry, red maple, and white ash. All seven witch hazel limbs were eaten in the water, while most of the white ash and American beech were eaten on land (Figure 8).

As for the time limbs were left in the water before eating, witch hazel stands out. Most WH logs (4) were left in the water for three days, with one for two days and two for four days. Red maple (only two) and yellow birch (also only two) limbs spent two days in the water before consumption. Other tree species, such as striped maple, remained in the water for varying amounts of time, ranging from 0 to 15 days (Figure 9).

In the spring, most (60%) of 10 aspen branches harvested and brought into the water by beavers at four sites were eaten after one day. Of six branches present for two days, two (33%) were consumed, and one of three present for three days (33%).

Experiment 7. Amount of Phenolics Leached into Water

Assays of extracts of the freeze-dried bark consistently revealed the presence of 1.4–1.6% of leachable phenolic material in the dry bark. The mass of twigs consists of an average of 26.7%bark. The bark contains an average of 43.6% water. Thus, twigs contain a total of approximately 22.6 mg of leachable phenolics per 10 g of twig, the amount extracted by the methanol–water mix. Use of distilled, deionized water resulted in only 11.5 mg of leachable tannins per 10 g of twig. This is half the amount that was leached into the methanol–water mix.

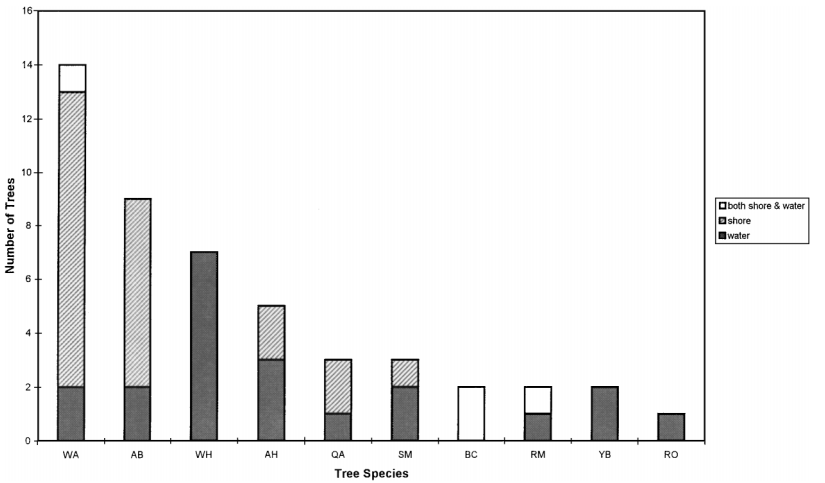


FIG. 8. Tree species selected and cut by beavers and then consumed on land, in water, or both. WA: white ash; AB: American beech; WH: witch hazel; AH: American hornbeam; QA: quaking aspen; SM: sugar maple; BC: black cherry; RM: red maple; YB: yellow birch; RO: red oak.

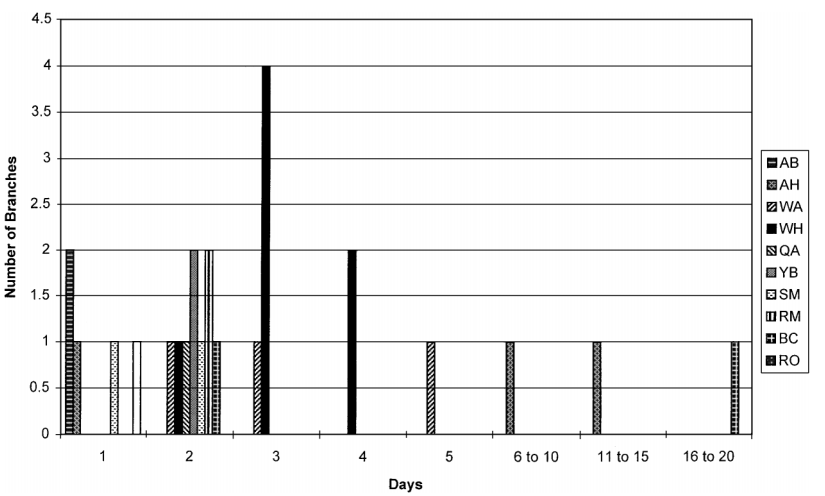


FIG. 9. Numbers of days after harvesting that beavers left trees in the water before they consumed the bark. Tree species, as in Fig. 8. One beaver colony at ASP, fall.

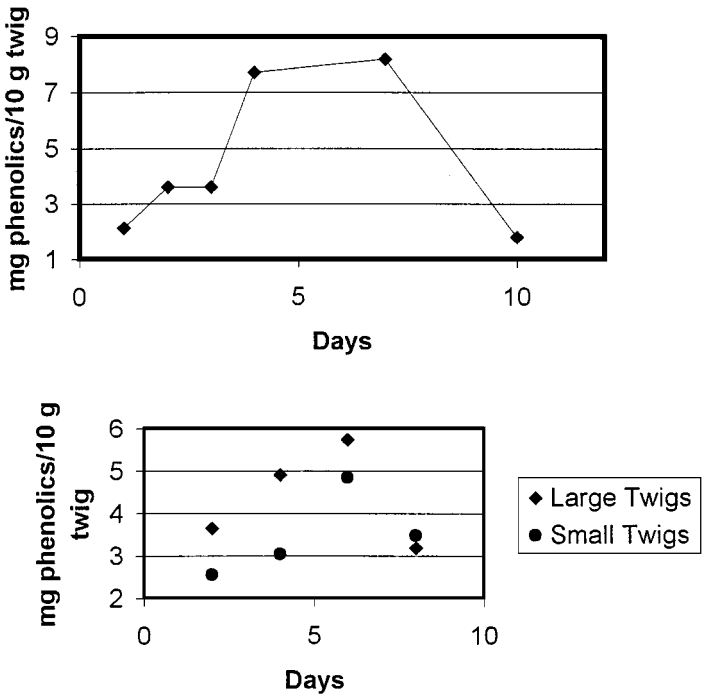


FIG. 10. Top: cumulative amount of phenolics leached into deionized water from RM twigs after 1, 2, 3, 4, 7 and 10 days. Bottom: cumulative amounts of phenolics leached into dionized water from large and small RM twigs. Note the decrease of accumulated phenolics after several days.

Soaking *whole twigs* shows a linear increase in the amount of phenolics during the first six days, followed by a drop during the last two days of the experiment (Figure 10). During the first six days, the concentration of phenolics in the water increased to 70–80% of the leachable phenolics. Essentially no difference was observed when the experiment was carried out at 25°C.

The soaking of *bark pieces* in water produced similar results. Again, phenolic concentration dropped during the latter part of the experiment (Figure 10). In this study, 50–60% of the material leachable in water was removed from the woody material.

DISCUSSION

Our experiments repeatedly revealed that beavers leave certain tree species, especially witch hazel, in the water one to three days before eating them. Although

the effect is not robust, several experiments showed the same tendency. Therefore, pooling of the data of several experiments yielded highly significant differences among the tree species' time periods in the water before being eaten. Busher (1996) found that beavers in Massachusetts store a higher percentage of witch hazel than of other tree species in the water near their lodge as part of their fall food cache. They consumed very little witch hazel immediately after harvesting. Witch hazel may be more palatable after some time in the water.

We have to distinguish the physical process of leaching from the biological process of degradation of phenolics by fungi. A compromise between leaching out of undesirable compounds and loss of nutrients also, has to be reached. Therefore, soaking may be effective during a "window of opportunity" of about two to three days. This is indicated by two lines of evidence: Leaching in the pond for longer than two days did not improve palatability (experiment 1). In the laboratory (experiment 7), chemical leaching of phenolics reached a peak after two days of soaking.

In the published literature, two other mammals appear to process food in an analogus fashion, even though water is not involved. Voles cut conifer twigs and leave them on snow for two to three days (while the levels of phenolics drop) before they eat them. (Roy and Bergeron, 1990). Pikas reduce phenolics in their haypiles by long-term storage. In this case, the phenolics also serve as food preservatives (Dearing, 1997).

Water-soluble compounds from food leach better at higher water temperatures, with longer extraction times, and at smaller particle sizes. Running water possibly enhances extraction, too. The beavers have running water and long extraction times for winter-stored branches. Beavers in more southern climates do not store food for the winter. Whether they utilize the warmer water for leaching in their day-to-day feeding remains to be investigated.

Except for experiment 1, our tests were designed without replacement of sticks during the five days of the experiment. Therefore, the density of the offered sticks decreased over the five days. This may have affected the choice by the beavers, although, in contrast to laboratory experiments with forced choices, they had vast amounts of natural food available. Replacement of consumed food would most likely have led the beavers to consume more aspen and reject more of the undesirable samples. Experiment 5 indicates how the cafeteria of a variety of available food can influence choice: red maple twigs were hardly eaten at all when several other, more palatable species were also offered.

Familiarity with certain tree species at each particular site may also play a role. Experiment 5a tried to address this aspect of food choice by offering tree species that occur at a particular site.

It is clear from the chemical study that phenolic material is gradually released into water when RM twigs or bark are soaked. This provides a viable way for beavers to render the RM (and possibly other species) twigs more palatable.

The chemistry suggests that soaking for a period of five days leaches out more than half of the phenolics capable of being leached, and this agrees with the field observations that beavers will take RM logs after two to three days of soaking.

Less clear is the reason for the drop in the concentration of the phenolics in the water during the last days of the study. While the tannins may be subject to enzymatic or chemical degradation, it is not likely that the phenolic material would be removed from the solution. More likely is polymerization, catalyzed by oxygen or traces of acid, of the released phenols to begin removing them effectively from the solution.

If beavers indeed use leaching of less palatable tree species regularly, management implications arise: As preferred tree species become depleted, the animals can switch to processing less palatable species. Witch hazel does not occur at many sites and is not present in large numbers. Use of RM, however, may indicate depletion and current quality of food resources. For instance, Shadle and Austin (1939) found that the first beavers to colonize Allegany State Park in recent times used mostly aspen (44.1% of their diet) and very few maples, birches, black cherry trees, beeches, and ash trees (combined 19.6%), while today these latter species are a substantial part of the beavers' diet (combined 57.4%) in the same study area (Müller-Schwarze and Schulte, 1999).

Acknowledgments—We thank the managers of Allegany State Park for providing facilities and continued support. We are grateful to the staff of the Cranberry Lake Biological Station of the College of Environmental Science and Forestry, State University of New York, for supporting our work. Christine Müller-Schwarze assisted in the winter collection of red maple.

REFERENCES

ABOU-ZAID, M. M., GRANT, G. G., HELSON, B. V., BENNINGER, C. W., and DE GROOT, P. 2000. Phenolics from deciduous leaves and coniferous needles as sources of novel pest control agents for lepidopteran forest pests, pp. 398–416, *in* F. Shahidi and C.-T. Ho (eds.). Phytochemicals and Phytopharmaceuticals. AOCS Press, Champaign, Illinois.

BARBOSA, P., GROSS, P. PROVAN, G. J., PACHECO, D. Y., and STERMITZ F. R. 1990. Allelochemicals in foliage of unfavored trees of the gypsy moth. *J. Chem. Ecol*. 16:1719–1730.

BOX, J. D. 1983. Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Water Res*. 17:511–525.

BUSHER, P. E. 1991. Food caching behaviour by the North American beaver, *Castor canadensis*, in western Massachusetts, pp. 111–114 *in* B. Bobek, K. Perzanowski, and W.L Regelin (eds.). Transactions Eighteenth IUGB Congress, Krakow 1987, Vol. 1. Swiat Press, Krakow.

BUSHER, P. E. 1996. Food caching behavior of beavers (*Castor canadensis*): Selection and use of woody species. *Am. Midl. Nat*. 135:343–348.

DEARING, M. D. 1997. The manipulation of plant toxins by a food-hoarding herbivore, *Ochotona princeps*. *Ecology* 78:774–781.

DIXON, M. D., JOHNSON, W. C., and ADKISSON, C. S. 1997. Effects of caching on acorn tannin levels and Blue Jay dietary performance. *Condor* 99:756–764.

DOUCET, C. M., and FRYXELL, J. M. 1993. The effect of nutritional quality on forage preference by beavers. *Oikos* 67:201–208.

FRYXELL, J. M., and DOUCET, C. M. 1993. Diet choice and the functional response of beavers. *Ecology* 74:1297–1306.

JENKINS, S. H. 1980. A size-distance relation in food selection by beavers. *Ecology* 61:740–746.

JOHNS, T. 1990. With Bitter Herbs They Shall Eat It: Chemical Ecology and Origins of Human Diet and Medicine. University of Arizona Press, Tucson.

MÜLLER-SCHWARZE, D., and SCHULTE, B. A. 1999. Behavioral and ecological characteristics of a "climax" population of beaver (*Castor canadensis*), pp. 161–177, *in Beaver Protection, Management and Utilization in Europe and North America*. P. Busher and R. Dzieciolowski (eds.). Kluwer/Plenum, New York.

MÜLLER-SCHWARZE, D., SCHULTE, B. A., SUN, L., MÜLLER-SCHWARZE, C., and MÜLLER-SCHWARZE, A. 1994. Red maple (*Acer rubrum*) inhibits feeding by beaver (*Castor canadensis*). *J. Chem. Ecol*. 20:2021–2034.

O'BRIEN, J. P. 1981. Summer food habits of the mountain beaver in northeastern California. *Murrelet* 62:86–87.

ROY, J., and BERGERON, J. 1990. Branch-cutting behavior by the vole *Microtus pennsylvanicus*: A mechanism to decrease toxicity of secondary metabolites in conifers. *J. Chem. Ecol*. 16:735–741.

ROZE, U. 1989. The North American Porcupine. Smithsonian Institution Press, Washington, D. C.

SHADLE, A. R., and AUSTIN, T. S. 1939. Fifteen months of beaver work at Allegany State Park. *J. Mammal*. 20:299–303.

SHURE, D. J., and WILSON, L. A. 1993. Patch-size effects on plant phenolics in successional openings of the southern Appalachians. *Ecology* 74:55–67.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

LABORATORY AND FIELD EVALUATION OF PREDATOR ODORS AS REPELLENTS FOR KIORE (*Rattus exulans*) AND SHIP RATS (*R. rattus*)

GARY N. BRAMLEY[1,*] and JOSEPH R. WAAS

Department of Biological Sciences
University of Waikato
Private Bag 3105, Hamilton, New Zealand

(Received February 22, 2000; accepted January 6, 2001)

Abstract—Predator odors may serve to stop rats from entering conservation areas or to decrease predation, food consumption, and other damage by rats in areas tainted with predator odor. We compared the efficacy of real predator odors and synthetic odors (derived from the urine and feces of carnivores) as rat repellents with real herbivore odors as controls in a Y maze. We tested six predator odors: cat (*Felis catus*) urine and feces, mongoose (*Herpestes auropunctatus*) feces, *n*-propylthietane, *S*-methyl, methyl butanol, and isopentyl-methyl sulphide. The herbivore odors we used were: red deer (*Cervus elaphus*) urine, guinea pig (*Cavia porcellus*) feces, and white rabbit (*Oryctolagus cuniculus*) urine. Ship rats (*Rattus rattus*) and kiore or Polynesian rats (*R. exulans*) showed no aversion to any of the six predator odors when compared with herbivore odors. Ship rats, however, may have avoided synthesized odors more than real ones. We applied two odors (*S*-methyl, methyl butanol and *n*-propylthietane) to purpose-built feeders in native forest but recorded no change in either visitation rate or duration of visits for rodents [rats and mice (*Mus musculus*)] or possums (*Trichosurus vulpecula*). The consumption of maize at feeders was correlated with the number and duration of possum visits, but only weakly correlated with the number of visits by rodents. Consumption of maize was unaffected by the odor associated with the feeder. It is unlikely that the odors we tested will be useful in deterring rodents or possums from areas where they have been removed for economic, public health or conservation reasons.

Key Words—*Rattus rattus*, ship rat, semiochemicals, odor avoidance, predator odors, Polynesian rat, *R. exulans*, rat repellents, brushtail possums, pest management.

* To whom correspondence should be addressed.
[1] Current address: P.O Box 785, Kerikeri, New Zealand.

INTRODUCTION

Predator-based repellents have been used experimentally to reduce damage to crops caused by wildlife (Sullivan et al., 1985a,b; Boag and Mlotkiewicz, 1994) and food consumption by domestic herbivores (Arnould et al., 1993). Mason et al. (1994) believed that volatile sulfurous compounds in the feces and urine of carnivores, derived from the digestion of meat, mediated the responses of prey animals, and this is supported by data (e.g., Nolte et al., 1994). Mason et al. (1994) used the term semiochemical to describe these sulfurous chemicals that provide biologically important information to other animals. They hypothesized that omnivores such as rats (genus *Rattus*) should be attracted to semiochemicals because they may provide them with reliable information on sources of food, while herbivores should be repelled from areas contaminated with semiochemicals to avoid encountering a predator. However, many authors have reported that rats avoid predator odors or show reduced activity in their presence (reviewed by Kats and Dill, 1998). The behavioral and physiological responses of prey species to repellents include the avoidance of treated food and traps, freezing, hiding, analgesia, and physiological arousal (Kats and Dill, 1998).

Burwash et al. (1998) tested eight artificially synthesized semiochemicals (and one real one) and, despite no statistically significant results, concluded from trends in their data that ship rats (*Rattus rattus*) avoid odors derived from carnivores. They went on to suggest that the application of such chemicals might reduce rat damage to the economically important macadamia nut (*Macadamia integrifolia*) crop in Hawaii, by causing rats to reduce their consumption.

Published accounts of mammals' ability to discriminate and avoid predator odors have been contradictory, in part because different authors have interpreted the same behavior in different ways (Burwash et al., 1998 and references therein). Interpretations may have been further clouded because the efficacy of predator-based repellents varies with cover (Merkens et al., 1991), different test arenas can produce different results (Garbe et al., 1993), and both these factors have varied in different trials. Rodent responses to semiochemicals appear to be dependent on the context of the experiment and might also vary with experience (e.g., Wuensch, 1992), although this remains untested. Furthermore, responses to semiochemicals appear to differ in different congeneric species, subspecies, strains, and populations. For example, Bramley et al. (2000) showed that rats from a predator-naïve population of Norway rat (*Rattus norvegicus*) avoided semiochemicals, but rats from another (predator experienced) population did not. Differences in laboratory mouse strains in response to predator odors have also been recorded (Dell'Omo et al., 1994).

In addition to the variability found in different populations, subspecies and strains of rodent, some authors have used "no odor" treatments as a control for predator odors and in so doing have failed to provide adequate experimental

controls. This may have produced erroneous results in some cases (see Wolff and Davis-Born, 1997 and Mappes et al., 1998, for a critique).

In New Zealand, there are four species of rodent [roof or ship rat, Norway rat, Polynesian rat or kiore (*R. exulans*) and the house mouse[2]]. All four species were introduced with human voyagers (Atkinson and Moller, 1990; Holdaway, 1996). Although the four species do not occur in the same places (Atkinson and Moller, 1990) or habitats (King et al., 1996; King and Moller, 1997; Bramley, 1999), all are regarded as pests because of their adverse effects on native wildlife and vegetation (Atkinson, 1985; Towns, 1991). Ship rats are most common on the two main islands of New Zealand (Innes, 1990), while kiore are limited to smaller offshore islands, having been replaced, first by Norway rats and then ship rats in most habitats on the two main islands (Atkinson and Moller, 1990; King and Moller, 1997).

Control of rodent populations has relied on extensive poisoning using second-generation anticoagulants (MacDonald, 1984). In New Zealand, rat populations have been eradicated from offshore islands and greatly reduced in large areas of forest on the main islands using these poisons (Taylor and Thomas, 1993; Innes et al., 1995). Reinvasion to mainland management areas is rapid (Innes et al., 1995), and the threat of reinvasion to often-visited island reserves is always present (Wace, 1986). Thus, if rats could be shown to reliably avoid predator odors, then this response could be exploited by conservation managers as part of an arsenal of techniques to protect native species from rats in New Zealand. It could also be economically useful since ship and Norway rats are two of the most serious world-wide pests of corps and stored foods and they harbor diseases such as bubonic plague and typhoid (MacDonald, 1984). Repelling rats from areas such as zoological gardens and wildlife parks or sanctuaries may also prove beneficial to the animals living there.

We determined whether two species of rat (kiore and ship rat) would discriminate and avoid odors derived from the urine and feces of predators in a Y-shaped maze. Garbe et al. (1993) showed that mouse responses to odor varied with the kind of arena used. Moreover, when animals are housed in captivity, there is always the possibility that their behavior will change as a result of being held captive (Ward et al., 1996). Because of these important considerations, we also examined the responses of ship rats in the wild to two of the odors we used in our laboratory trials. The two semiochemicals we tested in our field trials (*S*-methyl, methyl butanol and *n*-propylthietane) had both proved effective in deterring Norway rats from Kapiti Island in laboratory trials (Bramley et al., 2000). Both the artificial semiochemicals we used are components of predator urine or feces [cats (*Felis catus*) and mustelids, respectively]. Both these predators are found on North Island,

[2]There are two species of house mouse recognised *M. musculus* and *M. domesticus*. Mice in New Zealand share morphometric characteristics with both species and await genetic determination (Murphy and Pickard, 1990)

New Zealand. Thus, it is likely that free-living rats on North Island had encountered them or their odors before. All visits to feeders were recorded on videotape, and thus we were also able to collect incidental observations on the avoidance behavior of brushtail possums (*Trichosurus vulpecula*).

METHODS AND MATERIALS

Animals. Thirty animals were used in the laboratory trials: 15 kiore and 15 ship rats. All animals were adults, and 10 ship rats and nine kiore were females. The ship rats were caught in cage traps baited with apple and peanut butter along the banks of the Waikato River at Tamahere, south of Hamilton City, North Island, New Zealand. Kiore were captured using traditional Maori pitfall traps on islands in the Hen and Chickens group near Whangarei, northern North Island, New Zealand, and supplied to us by the Ngati Wai tribal resource management group. Both groups of rats were collected between January and April 1997.

At the University of Waikato facilities, the ship rats were housed individually in plastic bottomed, wire cages (46 × 24 × 20 cm high) and kiore were housed individually in plastic tubs with wire tops (40 × 27 × 15 cm deep). All animals had *ad libitum* access to water and Sharpes Diet 86 rat chow (Sharpes Grains and Seeds, Lower Hutt, New Zealand), occasional seed mixes, and pieces of apple; kiore were also given fresh grass seed heads. Cages were provided with a tin can and a cardboard tube to act as retreats. We used wood shavings as bedding and changed it weekly. The rats were housed in two separate rooms and maintained on a constant 12L:12D photoperiod, with lights on at 22:00 hr. Temperatures in the rooms were between 16 and 24°C. Ship rats were tested in May 1997 and kiore in June 1997.

Odors. We examined the subjects' responses to nine different odors. Six of the odors were natural, from the feces or urine of live animals, and three odors were the commercially synthesized volatile ingredients of urine or feces (provided by New Zealand Industrial Chemists, Gracefield, Lower Hutt, New Zealand). The natural odors were: cat urine (supplied by Five Cross Roads Veterinary Clinic, Hamilton, New Zealand), mongoose feces (collected in Hawaii by A. Woolhouse of New Zealand Industrial Chemists), guinea pig feces, New Zealand white laboratory rabbit urine, and red deer urine. The cat and rabbit urine was collected via catheterization of laboratory animals held at the Veterinary Clinic or Waikato Polytechnic (Hamilton, New Zealand). Guinea pig feces were collected from the guinea pig cages at the Polytechnic. The deer urine was collected from animals kept at the deer unit of AgResearch Ruakura (Hamilton, New Zealand). Artificially synthesized chemicals were provided as 1% solutions in paraffin oil. Chemicals used were isopentyl methyl sulfide [IPMS, found in red fox, (*Vulpes vulpes*) urine], *n*-propylthietane [PT, found in stoat (*Mustela erminea*) and ferret (*Mustela furo*)

anal sac secretions, and hence probably also in their feces], and *S*-methyl, methyl butanol (SMMBO, found in cat urine). Thus, there were three synthesized predator smells, three natural herbivore smells, and three natural predator smells. Of the natural odors, one of the herbivore and two of the predator smells were from feces. Urine and feces was always collected from more than one donor animal, but only one donor was used at a time (i.e., samples were not pooled) except in the case of the deer urine. All natural odors were stored frozen.

Islands in the Hen and Chickens group are free of mammalian predators of kiore. Kiore are the only rat known to live there (Atkinson and Moller, 1990), so kiore collected there should be naïve to all the smells we tested. Ship rats collected on the North Island should be familiar with felids, canids, and mustelids, which have long-established populations on North Island, New Zealand. There were also cows, sheep, deer, goats, and rabbits on farms near where the ship rats were caught. There are no feral populations of guinea pigs in New Zealand (King, 1990), and it is unlikely any of the rats we used had ever come into contact with guinea pig odor.

In the field trials, odors were supplied as 5% w/v impregnated in casein squares measuring approximately 30 × 30 × 5 mm high and weighing 4.8–5.2 g (New Zealand Industrial Chemists, Wellington, New Zealand). Only two odors were used in the field trials (SMMBO and PT). We chose to use 5% w/v concentrations because we wanted the odors to be detectable at some distance in forest, and for them to persist for at least two weeks. The chemistry of herbivore urine and feces has not been well studied (A. Woolhouse, New Zealand Industrial Chemists, Wellington, New Zealand, personal communication), and it was not possible to use synthetic herbivore derived odors as a control. Instead, we used two other controls: odor-free casein squares applied to the feeder and no casein squares. The casein squares had a slight odor of their own, and thus these controls represent an odor (casein) and a no odor (no square) treatment.

Maze Experiments. Animals were collected one at a time from the captive colony and transported in their cages, in darkness, to a separate trial room. They were then transferred into an experimental arena lit with red light. When the animals were in the arena, the red light was turned off and all observations were completed using a 20-cm-diam. spotlight with an infrared filter. A Hitachi 12 V, 7 Ah, sealed lead–acid battery, powered the spotlight. Tests were videoed using a Videotronic Tri-Q CCD camera and a Panasonic AG5260 video recorder. After being tested, animals were returned to their cage and then to the captive colony.

We used a Y maze made of PVC piping (10 cm in diameter) to examine the responses of both groups of rats to odors (Figure 1). The odor stimulus was presented inside an opaque glass container as either 0.1 ml of solution pipetted onto a 1-cm^2 piece of Whatman filter paper or as 0.1 g of thawed feces. Odors were presented in the maze with distilled water controls, presented in the same way, in the other choice arm. The position of the odor (left or right) and the type of odor (numbered 1–9) were recorded on the video tape after the trial, but were unknown

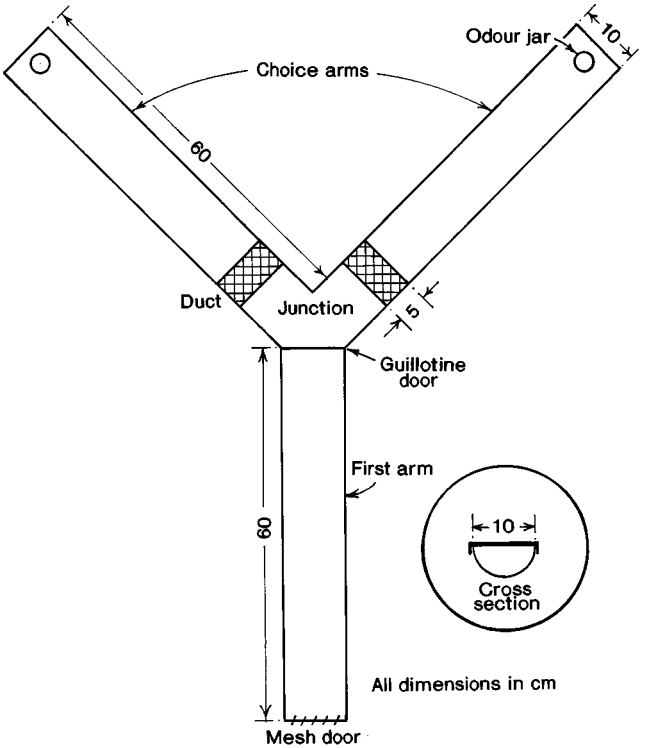


FIG. 1. The Y maze apparatus used to test the responses of rats to the odor of predators and herbivores.

to us when we scored the rats' behavior. Three identical mazes were used and after each test the maze was washed using hot water and Vircon soap powder and then towel dried.

Ship rats and kiore were tested separately according to an incomplete block design using 18 blocks. Each block consisted of five rats. One block (five rats and five odors) was tested per day. Each rat was randomly allocated a position in six blocks (i.e., received each of the six odors once over the 18 days of the trial). Tests began 1 hr after the lights in the colony had gone off and continued sequentially until five animals were tested. Each test was usually conducted within 40 min of the previous one. Prior to the start of each test, rats were placed in the first arm of the maze, with the guillotine door shut (Figure 1). The video recorder was then started and the observer left the room. After 5 min, the guillotine door was opened (from outside the room) and the rats were free to explore the maze for 15 min. We recorded: (1) the time until the rat first visited each of the arms (a visit was

defined as occurring when the rat's head was more than 5 cm down the arm); (2) the number of visits to the odor and control arm; (3) the time spent in each arm; and (4) a total activity score for each rat, which was the number of visits to each arm summed together.

The day before being tested, each rat was introduced to the maze as outlined above, but in the absence of any odors. We recorded the same measures for each rat on videotape to detect any left or right bias and to establish that the rats would explore the maze. In the middle of the series of tests and at the end of the series, the rats were again introduced to clean mazes and their movements recorded. This was to check that each rat would still explore the maze in the absence of smells and that the rats had not developed a left or right preference during the trials. Recordings of rats in a clean maze were done after the day's tests (on other rats) had concluded.

Field Experiments. Four feeding stations (Figure 2) were set up in each of seven different forests or forest remnants on North Island. The feeding stations

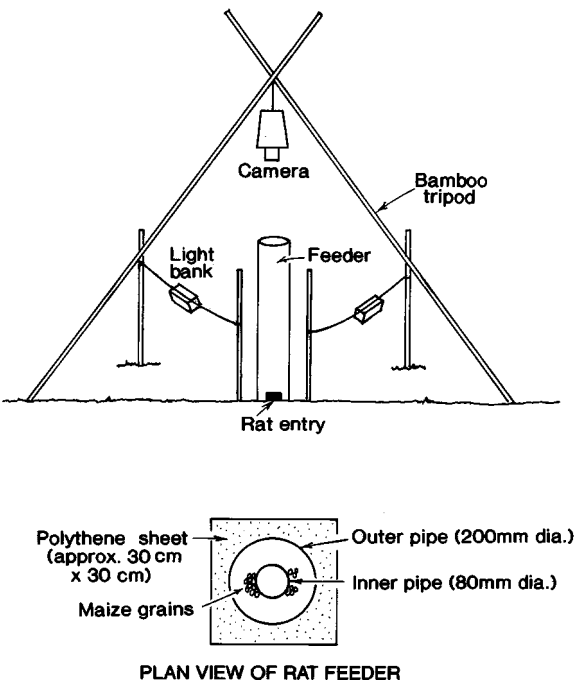


FIG. 2. A rat feeding station and camera used to record visits by rodents (rats and mice) and possums (*Trichosurus vulpecula*). Each feeder consisted of two PVC pipes, and odor sources were attached to the outside of the larger pipe, near the rat entrance. Four stations were positioned in each of seven forested areas. All four cameras were connected to a central time-lapse video recorder.

were positioned approximately 20 m from a central point, one each in a north, south, east, and west direction. The seven forest sites were: Tahae Block (Pureora Forest Park), Pouakani Scenic Reserve (near Pureora), Yarndley's Bush (north of Te Awamutu), Walter Scott Reserve (29 km west of Te Awamutu), Whewell's Bush (Matangi), Te Kauri Scenic Reserve (Kawhia), and Te Tapui Scenic Reserve (13 km east of Matamata). The seven study areas were widely separated geographically (Figure 3). They also differed in topography, climate, altitude, aspect, geology, history, dominant vegetation, and probably in rodent density and species composition too. We expected that each site would have ship rats and mice, but the relative densities of each species were unknown and we did not know whether or not Norway rats were present at any site.

Each feeding station consisted of a feeder (made up of an inner PVC pipe 8 cm diam., 1 m high and an outer PVC pipe 20 cm diam., 1 m high). The inner pipe was filled with approximately 550 g of maize grains and had two small holes cut at the bottom to allow the corn to flow out. The larger pipe had three squares (10×10 cm) removed from the bottom to allow rodents access to the inner pipe (Figure 2). Each feeder was positioned on top of a 30×30-cm piece of black polythene to protect the corn from ground moisture and was held in place by guy ropes attached to surrounding vegetation or support poles. Each feeder was monitored by a Videotronic Tri-Q CCD camera positioned approximately 1.65 m above the ground, suspended on a tripod of three bamboo poles tied together at the top.

The feeder and camera units were numbered according to their position with feeder and camera 1 being the northernmost one. Each camera was secured inside an inverted plastic bucket to protect it from the elements. On either side of the feeder (approximately 55 cm above the ground), a bank of 16 infrared-light-emitting diodes provided illumination for the cameras. These two light banks lit up an area of approximately 50×50 cm around the base of the feeder. The four cameras were connected via approximately 20 m of cable to a Panasonic WJ-420 four-input screen splitter. The splitter was connected to a GYYR VHS timelapse recorder (model TLC 1800X) set to record 48 hr on a 3-hr tape. The splitter, video recorder, and camera control boxes were protected inside plastic boxes and covered with polythene to exclude water. The splitter and video recorder were connected to a Motormate PS-200-2 DC-AC power inverter. The power inverter, lights, and cameras were connected to three 63 Ah Sonnenschein 12 V lead–acid batteries connected in parallel via a Grasslin 12 V electronic timer.

Three casein squares impregnated with the odors were attached to the outer pipe of each feeder by using plastic electrical insulation tape. The casein squares were attached between the entrances shown in Figure 2. A Latin square design was used to assign the four treatments (SMMBO, PT, casein, or no odor) to the four feeders. Study areas at each forest patch were located on the flattest ground we could find. On day 1 of a test, cameras and feeders were assembled and feeders were filled with a known amount of maize. The timer and video recorder were set

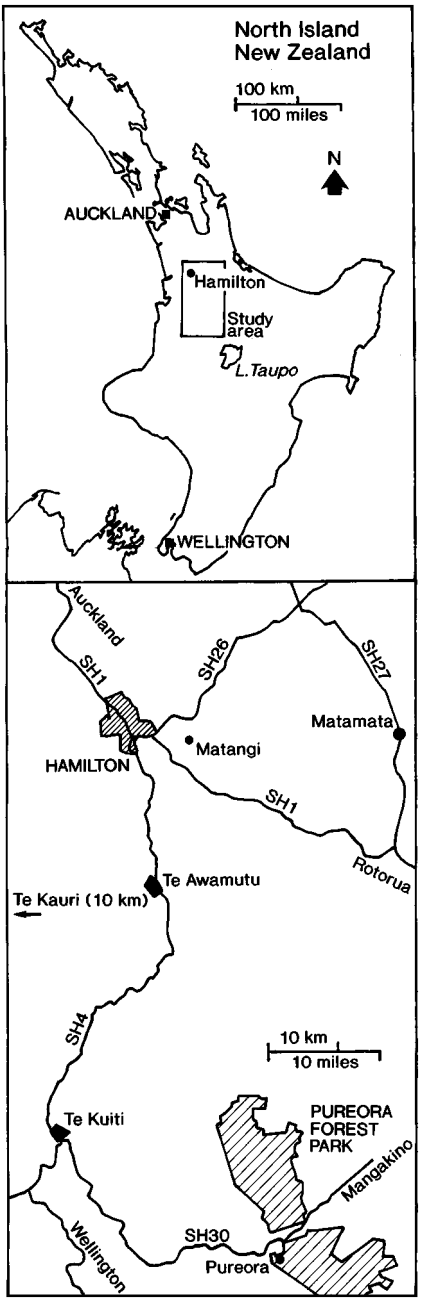


FIG. 3. The location of North Island towns near forest remnants where the field evaluation of ship rat response to predator odors was conducted.

to begin recording after dark and to stop recording before dawn. The exact length of recordings varied slightly with season. Once the system was in place, activity at each feeder was automatically recorded on videotape for the next three nights. After three nights, we returned, weighed the remaining corn, added fresh corn if required, removed the videotape, and left the area for 10 nights. After 10 nights, we returned and removed the casein squares impregnated with odor and weighed any remaining corn. We then wiped the outside of each plastic feeder with methyl alcohol to remove any remaining smell and allowed it to air dry. We refilled the feeders, reset the video recorder, and placed new casein squares impregnated with different odors on each feeding station. We video taped activity for a further three nights, and then returned and weighed the corn again (replenishing it if necessary). We allowed the feeders to remain *in situ* for 10 more days. After this, we reweighed any remaining corn, then moved the feeders to the next site. Thus, from each site we had six nights of recording from two sessions separated by 10 days. Each of the feeders had a different smell associated with it (according to our Latin square design) in each test. We began these experiments on December 20, 1997, and concluded them on June 21, 1998. We watched each video four times (focusing on one feeder during each viewing). We recorded the number of visits made by rodents (rats or mice) and brushtail possums (*Trichosurus vulpecula*) to each feeder and the duration of the visits. We then summed the number and duration of visits for: (1) the first night and (2) the three nights of each session. We separated our data in this way because subjects might habituate to the odors over the three days of a test. We also recorded consumption of maize on nights 1–3 and nights 4–10 of each session for each odor.

Data Analysis. In the *maze experiments*, we used the Generalized Linear Model (GLM) function of MINITAB (version 12.1) to investigate the responses of each species of rat in a Y maze. For each species and each variable, we created a model that included the rat (numbered 1–15) and the block (numbered 1–18) as random effects and the odor as a fixed effect. We then grouped the odors into herbivore, real carnivore, and synthesized carnivore and created models for each measure using odor type as a fixed predictor and rat and block as random predictors. We also created a model investigating the effect of sex on activity for each species. We used Tukey's tests to discriminate pairwise differences where GLM results were significant.

In the *field experiments*, to determine whether rats visited feeders differentially according to the treatments attached to them, we again used the GLM procedure. We created different models for each measure and specified the smell as a fixed predictor and location, test (1 or 2), and feeder (1–4) as random effects. To check that most maize consumption was by the rodent and possum visitors we recorded (and not by, for example, diurnal birds), we correlated the consumption on nights 1–3 with the number of visits by rats and possums for those three nights in both sessions.

RESULTS

Maze Experiments. Exploratory data analysis showed that the number of visits to the arm containing the distilled water control were similar to the number of visits to the odor arm for both groups of rats. Thus, we chose not to use the distilled water arm as a control because it was likely that the odor arm affected the subject's behavior throughout the whole maze. Instead we used the data collected when rats were exposed to herbivore odors as the control.

In our experiments with kiore, neither the odor nor the odor type significantly affected the variables we measured (Table 1). In no case was the block significant either, which indicates that rat behavior did not vary significantly on different days. There was a high level of individual variation in the sample, and the individual rat was a significant predictor for all of our measures except time in the odor arm (Table 1). Male kiore were more active [9.89 $\pm$ 1.56 (mean $\pm$ SE) visits] than female kiore (5.00 $\pm$ 0.87 visits; $F_{1,89} = 8.66$, $P = 0.004$). The effect of sex is implicit in the designation "rat" in our model, and thus is already partially accounted for.

One of the female ship rats escaped during her first test and could not be recaptured alive. Another female rat showed an abnormally high level of activity (approximately 230 visits to the left arm in a 15-min trial) and so was excluded from the analysis. This reduced our sample size to 13 animals. Of the variables we measured, only the number of visits to the odor may have been affected by the odor type ($F_{2,77} = 3.1$, $P = 0.054$) with herbivore and real predator odors being visited more often (14.32 $\pm$ 2.38 visits, and 13.96 $\pm$ 1.78, respectively) than artificially synthesized predator odors (10.33, $\pm$ 1.89; $T = 2.26$, $P = 0.07$). All other variables were unaffected by odor or odor type (Table 2), but some behaviors

TABLE 1. *F* VALUES FROM GENERALIZED LINEAR MODELS DESIGNED TO DETERMINE EFFECT OF SEMIOCHEMICALS ON KIORE BEHAVIOR[a]

		Visits to		Time in		Time to approach	
Factor	Activity	Odor	Control	Odor	Control	Odor	Control
Fixed effects							
Odor	0.72	0.75	0.54	0.69	1.23	0.95	0.65
Odor type	0.17	0.25	0.16	0.53	0.22	1.44	0.51
Random effects							
Rat	2.29*	2.21*	2.15*	1.01	1.22	2.37*	2.55**
Block	1.20	1.01	1.21	1.09	1.08	1.11	1.03

[a] *F* values marked with one asteriks are significant at $P = 0.05$, those marked with two asterisks are significant at $P = 0.01$. Nine odors were tested in all: three real predator odors, three synthesized predator odors, and three herbivore odors. The odors were each tested individually in one model "odor" and then assigned into groups (real predator, synthesized predator, and herbivore) for as "odor type" in a second model to see if kiore differed in responses according to odor source.

TABLE 2. *F* VALUES FROM GENERALIZED LINEAR MODELS DESIGNED TO DETERMINE EFFECT OF SEMIOCHEMICALS ON SHIP RAT BEHAVIOR[a]

		Visits to		Time in		Time to approach	
Factor	Activity	Odor	Control	Odor	Control	Odor	Control
Fixed effects							
Odor	1.14	1.78	1.05	0.73	1.23	1.44	1.13
Odor type	1.92	3.1	0.3	0.24	0.43	1.67	0.12
Random effects							
Rat	9.06**	6.80**	5.67**	1.96	1.77	1.6	3.44**
Block	1.89*	1.44	1.91*	0.84	0.71	1.42	2.05*

[a] *F* values marked with one asterisk are significant at $P = 0.05$, those marked with two asterisks are significant at $P = 0.01$. Nine odors were tested in all: three real predator odors, three synthesized predator odors, and three herbivore odors. The odors were each tested individually in one model as "odor" and then assigned into groups (real predator, synthesized predator, and herbivore) for "odor type" in a second model to see if ship rats differed in responses according to odor source.

(activity, number of visits to the control, time to approach the control; Table 2) were affected by the block number, indicating that rats behaved differently on different days. There was no trend in the behavioral changes through the period of the trial, for example, rats did not become predictably more or less active as the trial progressed, rather they were more active on some days than they were on others. The individual rat significantly affected all measures except the time spent in the odor and control arms, and the time to approach the smell, indicating high individual variability among rats. Ship rat activity was also affected by sex ($F_{1,77} = 8.58$, $P = 0.005$). Female ship rats were more active than males (males 17.4 ± 3.06 visits; females 30.5 ± 3.00 visits). The effect of the sex of the rat is again accounted for in our models.

Rats Use of a Clean Maze. Each rat explored an empty maze on three occasions. Kiore made a total of 104 visits to the left choice arm and 109 visits to the right choice arm ($\chi^2 = 0.12$, $df = 1$, NS). Seven of the rats remained in the first arm during their first experience in the maze. A further three rats remained in the first arm on two occasions, thus 10 of the kiore (66%) chose not to explore the maze on at least one occasion.

Ship rats made a total of 339 visits to the left choice arm and 361 to the right choice arm ($\chi^2 = 0.69$, $df = 1$, NS). Only two of the ship rats remained in the first arm on one occasion, and one of those two remained in the first arm on a second occasion. We concluded that both species of rat readily explored the maze in the absence of odors, although kiore were more reluctant to explore the maze when they first experienced it and were less active overall. Neither species of rat showed a preference for one side of the maze.

Field Experiments. On some occasions, either one of the cameras or the time-lapse video recorder failed to work. Two of the cameras stopped recording

after one night at Yarndley's Bush. During the second test at Yarndley's Bush, the video recorder stopped working after one night, thus reducing the number of feeding stations for which we had data for all three nights from 56 to 46. On some occasions, maize weights were not recorded because the remaining maize became wet and heavy due to rain. These losses reduced our sample size for maize consumption on nights 1–3 from 56 to 50, and on nights 4–13 from 56 to 52.

Average maize consumption on days 1–3 varied between 120 g (at feeders with SMMBO) and 159 g (at feeders with odor-free casein squares on them). The amount of maize consumed on days 1–3 of a test did not vary according to odor ($F_{3,49} = 0.31$, $P = 0.82$), but more than five times as much corn was eaten during the second test (219 $\pm$ 30 g) as there was during the first test (48 $\pm$ 10 g, $F_{1,49} = 22.38$, $P = 0.0001$). Maize consumption for days 4–13 of each test did not vary with smell either ($F_{3,51} = 0.36$, $P = 0.78$), but did vary with the test (again more maize was eaten in the second test, $F_{3,51} = 7.48$, $P = 0.009$) and with the location ($F_{6,51} = 9.78$, $P = 0.0001$). The amount of maize consumed on days 4–13 varied from an average of 120 g at Pouakani Scenic Reserve to 519 g at Walter Scott Scenic Reserve.

The number of rodents visiting feeders on night 1 of a test did not vary according to the semiochemical that was attached to the feeder (means ranged from 4.0 to 7.1 visits per feeder, $F_{3,55} = 0.81$, $P = 0.49$), but at some locations rat activity was generally higher (means ranged from 0.6 to 19.3 visits per location; $F_{6,55} = 7.64$, $P = 0.0001$). The number of brushtail possums visiting on the first night of a test did not vary according to odor either (range of means 0.6–2.1 visits per feeders, $F_{3,55} = 1.72$, $P = 0.18$), but there was also more possum activity at some locations (means ranged from 0.1 to 2.4 visits per location, $F_{6,55} = 2.49$, $P = 0.03$). More possum visits were recorded during the first night of the second test (mean = 2.0 visits) than on the first night of the first test (mean = 0.6 visits; $F_{1.55} = 7.35$, $P = 0.01$).

The number of rat and mouse visits over the three-day test did not vary according to odor (range of mean values = 20.1–25.0; $F_{3,45} = 0.21$, $P = 0.89$), but did vary according to location (range of mean values = 1.0–60.9 visits; $F_{6,45} = 9.64$, $P = 0.0001$). The same was true for possums (range of mean values = 4.1–5.8; $F_{3,45} = 0.32$, $P = 0.81$ for odor, and range of mean values = 0.4–12.3 visits; $F_{6,45} = 7.49$, $P = 0.0001$ for location). The amount of time spent at each feeder by rats and mice on night one was not dependent on the odor attached to the feeder (means ranged between 948 sec and 1578 sec; $F_{3,55} = 0.17$, $P = 0.92$), although it did vary with location (range of means = 146–4030 sec, $F_{6,55} = 3.46$, $P = 0.007$). Possums spent more time at feeders on the first night of the second test than they did on the first night of the first test (mean = 110 sec for test 1, mean = 1130 sec for test 2; $F_{1,55} = 10.61$, $P = 0.002$). The amount of time spent at feeders by possums on the first night of the second test did not vary according to smell ($F_{3,55} = 2.12$, $P = 0.11$).

When we considered the total amount of time spent at a feeder during the three nights of video recording, location was the only significant predictor for rodents (mean values between 69 sec and 10279 sec; $F_{6,45} = 3.72$, $P = 0.006$) and for possums (mean values between 77 sec and 8468 sec; $F_{6,45} = 3.59$, $P = 0.008$).

The total maize consumption at each feeder was correlated with the number of possums that we recorded visiting the feeders ($r = 0.57$, $P = 0.0001$) but only weakly correlated with the number of rodents ($r = 0.20$, $P = 0.17$). The maize consumption on days 1–3 was weakly correlated with the amount of time rats and mice spent at a feeder ($r = 0.27$, $P = 0.08$), but moderately correlated with the amount of time possums spent there ($r = 0.57$, $P = 0.0001$). The number of rodent visits and possum visits was unrelated ($r = 0.03$, $P = 0.83$), but the length of time spent at feeders by rodents was correlated with the length of time spent there by possums ($r = 0.332$, $P = 0.02$).

DISCUSSION

It might be expected that rats would be more likely to avoid the odors of predators that they encounter most frequently (Dickman, 1992) or those that pose the most significant threat (Jedrzejewski et al., 1993). We included both familiar and unfamiliar predators of ship rats in our laboratory experiment, yet neither species showed avoidance of any of the odors at the concentrations we used. It appears from both the field and laboratory experiments that semiochemicals would not be effective rat repellents in the sense that we envisaged. Not only do ship rats and kiore visit areas tainted with carnivore odors as often as they visit control areas (tainted with herbivore odors) in laboratory trials, they also spent similar amounts of time and consumed similar amounts of food in treatment and control areas in field trials.

Interestingly, the two species of rat we studied did not appear to be attracted to the predator odors, as predicted by Mason et al. (1994), either. This may be because they cannot tell the odors apart. However, ship rats appeared to be able to discriminate at least some of the odors, since they spent more time in arms containing odors from real predators or herbivores than they did in arms containing synthesized semiochemicals. Since the synthesized chemicals smelled stronger to the human nose than the real ones, it may be that this difference in behavior is due to strength or pungency of the odor stimulus rather than the odor itself. Kemble and Bolwahnn (1997) reported that pungency alone could explain mouse aversion of novel chemicals, although this has not been tested for the species of rat we studied. The laboratory tests conducted by Burwash et al. (1998) used only one (familiar) real odor; the other eight were synthesized. Burwash et al. (1998) do not report any dilution of their chemicals, and although they used small amounts (0.01 ml), the avoidance behavior they report may be a response to strong novel odors, rather

than avoidance of predator odors *per se*. The odors we used in our field trials were stronger than those we used in the laboratory trial, although they were familiar, and there was no effect on rat behavior. Thus, pungency alone cannot explain the different results reported by Burwash et al. (1998) and this study. Kiore showed no differences in their responses to odors, and it remains unknown whether they can discriminate among the odors we tested or not.

Discrimination tests for both species of rat would be useful because they could help to interpret their lack of avoidance behavior. It seems likely that ship rats and kiore can distinguish the odors, because rats are generally well endowed with olfactory neurons (Nef, 1998). Furthermore, olfaction is considered to be one of the most important means of communication (Brown, 1985) and orientation (Lavenex and Schenk, 1998) for rats. Olfaction has been well-documented as a way of detecting predators for laboratory rodents (Kats and Dill, 1998). In order to observe odor discrimination in the absence of an avoidance response, it may be necessary to determine whether rats can be trained to recognize the odor as significant by operant conditioning (e.g., Terry and Johanson, 1996). If the rats are able to discriminate predator odors from others, then it remains to be explained why, in the context of our experiments, they ignore the information these semiochemicals can potentially convey, and what methods they employ to detect and avoid predators in the wild.

Cocke and Thiessen (1986) and Ward et al. (1996) both reported changes in the level of physiological arousal of animals [gerbils (*Meriones unguiculatus*) and hedgehogs (*Erinaceous europaeus*)] in laboratory trials that were not manifested in behavioral changes. It is possible that the rats we studied were showing changes to their metabolism that we could not measure.

Both the ship rats and the kiore we tested in laboratory trials showed a high degree of individual variation. Burwash et al. (1998) suggested that it is the high level of individual variability that has allowed ship rats to become such a globally successful species. We have previously found (Bramley et al., 2000) that, despite high individual variation, individual Norway rats from a predator-naïve population avoided predator odors, while individuals that had presumably come in contact with predators showed no such aversion. We suggested that avoidance might be an innate behavior for Norway rats but that it might be modified by experience, with experienced rats being more accurate at assessing risk. This may also be true for ship rats, since the ship rats we studied had presumably encountered predators or their odors in the past. Because we used odors from only one donor animal at a time, differences in quality of the individual odors may partially explain the high individual variation in our results.

The kiore that we studied were from predator-free islands. They showed no innate aversion to odors. In order to make sense of these observations and understand the development of odor-mediated avoidance, experiments are necessary that manipulate the individual experience of the rats being tested. Only by knowing

the experience of each individual being tested will experimenters be able to draw sensible conclusions about how responses to predator odors might vary with age or experience. The role individual experience plays in discriminating odors and responding to them has usually been studied by changing the background odor in the weaning period (e.g., Terry and Johanson, 1996) or by cross-fostering (e.g., Wuensch, 1992). Neither approach is suitable for studying predator avoidance. In order to mimic the experience of encountering a predator in the wild and to allow meaningful ecological interpretations, it will be necessary to pair the presentation of a semiochemical with a negative stimulus. Measuring the number of presentations necessary to engender avoidance and the length of time individuals maintain avoidance responses after experience would be useful in helping workers determine whether chemicals provide meaningful ecological information or not.

Laboratory studies have shown, usually with laboratory strains of Norway rat or house mouse, that odors are important for individual recognition (Gheusi et al., 1997), social interactions (Hurst et al., 1994; Hurst and Barnard, 1995), orientation in the home range (Lavenex and Schenk, 1998), feeding (Galef, 1993), and mate selection (Kavaliers and Colwell, 1995). Laboratory studies have also shown that laboratory rats and mice recognize and avoid predator odors (see Kats and Dill, 1998, for a review). This reductionist laboratory approach has allowed significant progress in our understanding of rodent social systems. However, little is known about how wild populations of rat respond to either conspecific or heterospecific odors, and whether wild rats and mice use odors when exploring their home range. The few studies that have been conducted in the wild (Dickman, 1992; Stapp and Van Horne, 1996; Bramley, 1999) have produced contradictory results. Large enclosures represent a kind of middle ground between these two approaches and have allowed successful manipulation of the presence, density, and encounters of wild animals (e.g., Drickamer, 1997).

We detected no preference for either side of the maze by ship rats or kiore, but the behavior of ship rats appeared to change according to the experimental block they were assigned to. This change was unexpected but should have had only a minimal effect on our results since smells were positioned in blocks throughout the trial and rats were randomly assigned to blocks. Thus, five randomly chosen rats received one randomly chosen smell each on any given day, and any smell tested on a day when the rats were inactive would also have been presented to other rats on other days.

In the field trials we conducted, the one consistently significant predictor of maize consumption and visitation rate was the location of the test. This is probably because rodent and possum density varied at the different sites, and feeders in areas with more animals received more visits. It appears from our field trials that possums were unaffected by predator odors. This result contrasts with data reported by Woolhouse and Morgan (1995), who applied semiochemicals (one of which

was PT) to pine (*Pinus radiata*) seedlings and recorded a decrease in consumption by possums. It may be that propylthietane has an aversive taste.

The strong correlation between maize consumption and possum visits implies that possums were eating most of our maize. Nonetheless, rodents also visited the feeders often, and since rodent and possum visits were not correlated (either positively or negatively), it appears possums did not exclude rats and mice from visiting. The positive correlation we observed between the amount of time spent at feeders for rats and possums is further support that neither species adversely affects the behavior of the other.

Rats and possums consumed more maize during the second test in an area than they had in the first test. This result is to be expected since resident animals have had longer to locate feeders, overcome neophobia, and assess food palatability by the time the second trial began.

It is apparent from this study and that of Bramley et al. (2000) that all rodents, even congeneric species, do not respond to predator odors in the same way. Studies of the type outlined above looking at the development of aversion and manipulating experimental conditions such as cover and risk are needed to help clarify how heterospecific odors are used by different species, and under what conditions avoidance evolves and is maintained by a population. Future research should also focus on the effect of age on avoidance responses and what other antipredator strategies might operate under different conditions.

Acknowledgments—This work was supported by a grant in aid from the New Zealand Lottery Grants Board (grant AP:52697) to the authors. We wish to thank John Innes, who helped with the grant application and commented on earlier versions of the manuscript; Joanne Baltus, who translated Arnould et al. (1993) from French; and C.M. King, Graham Hickling, and C.R. Dickman, whose comments improved an earlier version of the manuscript. Hori Parata, Moya Armstrong, and Mere Roberts helped me obtain the kiore. Carey Bramley, Gavin Reynolds, and Jackie Todd helped with the field experiments.

REFERENCES

ARNOULD, C., ORGEUR, P., SEMPERE, A., SIGNORET, J. P., and VERDIER, M. 1993. Repulsion alimentaire chez trois especes d'ongules en situation de paturage: effet des excrements de chien. *Rev. Ecol. (Terre Vie*) 48:121–131.

ATKINSON, I. A. E. 1985. The spread of commensal species of *Rattus* to oceanic islands and their effects on island avifaunas, pp. 35–81, *in* P. J. Moors (ed.). Conservation of Island Birds: Case Studies for the Management of Threatened Island Species. International Council for Bird Preservation Technical Publication No. 3.

ATKINSON, I. A. E., and MOLLER, H. 1990. KIORE, pp. 175–192, *in* C. M. King (ed.). The Handbook of New Zealand Mammals. Oxford University Press, Auckland, New Zealand.

BOAG, B., and MLOTKIEWICZ, J. A. 1994. Effect of odor derived from lion feces on behavior of wild rabbits. *J. Chem. Ecol*. 20:631–637.

BRAMLEY, G. N. 1999. Habitat use and responses to odors by rodents in New Zealand. PhD thesis. Department of Biological Sciences, University of Waikato, Hamilton, New Zealand, 260 pp.

BRAMLEY, G. N., WAAS, J. R., and HENDERSON, H. V. 2000. The responses to predator odours by two populations of wild Norway rats (*Rattus norvegicus*). *J. Chem. Ecol*. 26:705–719.

BROWN, R. E. 1985. The Rodents II: Suborder Myomorpha, pp. 345–457, *in* R. E. Brown and D. W. MacDonald (eds.). Social Odors in Mammals, Volume 1. Oxford University Press, Oxford.

BURWASH, M. D., TOBIN, M. E., WOOLHOUSE, A. D., and SULLIVAN, T. P. 1998. Laboratory evaluation of predator odors for eliciting an avoidance response in roof rats (*Rattus rattus*). *J. Chem. Ecol*. 24:49–66.

COCKE, R., and THIESSEN, D. D. 1986. Chemocommunication among prey and predator species. *Anim. Learn. Behav*. 14:90–92.

DELL'OMO, G., FIORE, M., and ALLEVA, A. 1994. Strain differences in mouse response to odours and predators. *Behav. Proc*. 32:105–116.

DICKMAN, C. R. 1992. Predation and habitat shifts in the house mouse, *Mus domesticus*. *Ecology* 73:313–322.

DRICKAMER, L. C. 1997. Responses to odors of dominant and subordinate house mice (*Mus domesticus*) in live traps and responses to odors in live traps by dominant and subordinate males. *J. Chem. Ecol*. 23:2493–2506.

GALEF, B. G. 1993. Functions of social learning about food: A causal analysis of effects of diet novelty on preference transmission. *Anim. Behav*. 46:257–265.

GARBE, C. M., KEMBLE, E. D., and RAWLEIGH, J. M. 1993. Novel odors evoke risk assessment and suppress appetitive behaviors in mice. *Aggress. Behav*. 19:447–454.

GHEUSI, G., GOODALL, G., and DANTZER, R. 1997. Individually distinctive odors represent individual conspecfics in rats. *Anim. Behav*. 53:935–944.

HOLDAWAY, R. N. 1996. Arrival of rats in New Zealand. *Nature* 384:225–226.

HURST, J. L., and BARNARD, C. J. 1995. Kinship and social tolerance among female and juvenile wild house mice: Kin bias but not kin discrimination. *Behav. Ecol. Sociobiol*. 36:333–342.

HURST, J. L., FANG, J., and BARNARD, C. J. 1994. The role of substrate odors in maintaining social tolerance between male house mice (*Mus musculus domesticus*): Relatedness, incidental kinship and the establishment of social status. *Anim. Behav*. 48:157–167.

INNES, J. G. 1990. Ship rat, pp. 206–225, *in* C. M. King (ed.). The Handbook of New Zealand Mammals. Oxford University Press, Auckland, New Zealand.

INNES, J. G., WARBURTON, B., WILLIAMS, D., SPEED, H., and BRADFIELD, P. 1995. Large-scale poisoning of ship rats (*Rattus rattus*) in indigenous forests of the North Island, New Zealand. *N.Z. J. Ecol*. 19:5–17.

JEDRZEJEWSKI, W., RYCHLIK, L., and JEDRZEJEWSKA, B. 1993. Responses of bank voles to odors of seven species of predators: Experimental data and their relevance to natural predator-vole relationships. *Oikos* 68:251–257.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Ecoscience* 5:361–394.

KAVALIERS, M., and COLWELL, D. D. 1995. Odours of parasitized males induce aversive responses in female mice. *Anim. Behav*. 50:1161–1169.

KEMBLE, E. D., and BOLWAHNN, B. L. 1997. Immediate and long-term effects of novel odors on risk assessment in mice. *Physiol. Behav*. 61:543–549.

KING, C. M. (ed.) 1990. The Handbook of New Zealand Mammals. Oxford University Press, Auckland, New Zealand, 600 pp.

KING, C. M., and MOLLER, H. 1997. Distribution and response of rats, *Rattus rattus* and *R. exulans*, to seedfall in New Zealand beech forests. *Pac. Conserv. Biol*. 3:143–155.

KING, C. M., INNES, J. G., FLUX, M., KIMBERLEY, M. O., LEATHWICK, J. R., and WILLIAMS, D. S. 1996. Distribution and abundance of small mammals in relation to habitat in Pureora Forest Park. *N.Z. J. Ecol*. 20:215–240.

LAVENEX, P., and SCHENK, F. 1998. Olfactory traces and spatial learning in rats. *Anim. Behav*. 56:1129–1136.

MACDONALD, D. W. (ed.). 1984. The Encyclopedia of Mammals (2). George Allen and Unwin, London.

MAPPES, T., KOSKELA, E., and YLONEN, H. 1998. Breeding suppression in voles under predation risk of small mustelids: laboratory or methodological artifact? *Oikos* 82:365–369.

MASON, J. R., EPPLE, G., and NOLTE, D. L. 1994. Semiochemicals and improvements in rodent control, pp. 327–346, *in* B. G. Galef, M. Mainardi, and P. Valsecchi (eds.). Behavioral Aspects of Feeding: Basic and Applied Research in Mammals. Harwood Academic Publishers, Chur, Switzerland.

MERKENS, M., HARESTAD, A. S., and SULLIVAN, T. P. 1991. Cover and efficacy of predator-based repellents for Townsend's vole (*Microtus townsendii*). *J. Chem. Ecol*. 17:401–412.

MURPHY, E. C., and PICKARD, C. R. 1990. House mouse, pp. 225–242, *in* C. M. King (ed.). The Handbook of New Zealand Mammals. Oxford University Press, Auckland, New Zealand.

NEF, P. 1998. How we smell: The molecular and cellular bases of olfaction. *News Physiol. Sci*. 13:1–5.

NOLTE, D. L., MASON, J. R., EPPLE, G., ARANOV, E., and CAMPBELL, D. L. 1994. Why are predator urines aversive to prey? *J. Chem. Ecol*. 20:1505–1516.

STAPP, P., and VAN HORNE, B. 1996. Do olfactory cues mediate interactions between rodents on northern shortgrass prairie? *Can. J. Zool*. 74:226–232.

SULLIVAN, T. P., NORDSTROM, L. O., and SULLIVAN, D. S. 1985a. Use of predator odors as repellents to reduce feeding damage by herbivores I. Snowshoe hares (*Lepus americanus*). *J. Chem. Ecol*. 11:903–919.

SULLIVAN, T. P., NORDSTROM, L. O., and SULLIVAN, D. S. 1985b. Use of predator odors as repellents to reduce feeding damage by herbivores II. Black-tailed deer (*Odocoileus hemionus columbianus*). *J. Chem. Ecol*. 11:921–935.

TAYLOR, R. H., and THOMAS, B. W. 1993. Rats eradicated from rugged Breaksea Island (170 ha), Fiordland, New Zealand. *Biol. Conserv*. 65:191–198.

TERRY, L. M., and JOHANSON, I. B. 1996. Effects of altered olfactory experiences on the development of infant rats' responses to odors. *Dev. Psychobiol*. 29:353–377.

TOWNS, D. R. 1991. Response of lizard assemblages in the Mercury Islands, New Zealand, to removal of an introduced rodent : The kiore (*Rattus exulans*). *J. R. Soc. N.Z.* 21:119–136.

WACE, N. M. 1986. The rat problem on oceanic islands—research is needed. *Oryx* 20:79–86.

WARD, J. F., MACDONALD, D. W., DONCASTER, C. P., and MAUGET, C. 1996. Physiological response of the European Hedgehog to predator and nonpredator odor. *Physiol. Behav*. 60:1469–1472.

WOLFF, J. O., and DAVIS-BORN, R. 1997. Response of grey-tailed voles to odours of a mustelid predator: A field test. *Oikos* 79:543–548.

WOOLHOUSE, A. D., and MORGAN, D. R. 1995. An evaluation of repellents to suppress browsing by possums. *J. Chem. Ecol*. 21:1571–1583.

WUENSCH, K. L. 1992. Fostering house mice onto rats and deer mice: Effects on response to species odors. *Anim. Learn. Behav*. 20:253–258.

Journal of Chemical Ecology, Vol. 27, No. 5, 2001

EFFECTS OF LONG-TERM OPEN-FIELD OZONE EXPOSURE ON LEAF PHENOLICS OF EUROPEAN SILVER BIRCH (*Betula pendula* ROTH)

AMMAR SALEEM,[1,*] JYRKI LOPONEN,[1] KALEVI PIHLAJA,[1] and ELINA OKSANEN[2]

[1]*Laboratory of Environmental and Physical Chemistry*
Department of Chemistry, University of Turku
FIN-20014 Turku, Finland

[2]*Department of Ecology and Environmental Science*
University of Kuopio
P.O.B 1627, 70211 Kuopio, Finland

(Received June 26, 2000; accepted January 3, 2001)

Abstract—The response of phenolic compounds as a result of long-term low open-field ozone exposure was studied in ozone-sensitive and ozone-tolerant clones of European silver birch (*Betula pendula* Roth). The saplings were exposed to 1.5–1.6 times the ambient (elevated) ozone and ambient air (as control) over three growing seasons from May 1996 until August 1998. Quantification by modified Folin-Ciocalteau assay showed a 16.2% increase in total phenolics in elevated ozone plants as compared to that in controls and a corresponding 9.9% increase of 10 phenolic compounds quantified by HPLC. Five nonflavonoids and five flavonoids showed 8.4% and 11.4% increases, respectively. The phenolic results indicated slightly higher ozone sensitivity of clone 5 as compared to clone 2. The most ozone-responsive phenolic compounds in clone 2 and clone 5 were (+)-catechin (CT), chlorogenic acid (CGA), 5-*p*-coumaroylquinic acid (5CQA), 3-*p*-coumaroylquinic acid (3CQA), myricetin galactopyranoside (MG), quercetin-3-*O*-glucuronopyranoside (QGR), and quercetin-3-*O*-arabinofuranoside (QA). Increased phenolic content in ozone-exposed plants was related to impaired growth and accelerated leaf senescence, indicated by enhanced autumn leaf yellowing and lower chlorophyll and Mg content. The change in carbon allocation towards defensive phenolics at the expense of growth was greater in the ozone-sensitive clone as compared to tolerant clone.

Key Words—*Betula pendula* Roth, silver birch, ozone, phenolics.

* To whom correspondence should be addressed. e-mail: amsale@utu.fi

0098-0331/01/0500-1049$19.50/0

INTRODUCTION

Increasing tropospheric ozone levels impose one of the greatest risk factors to the stability of European forest trees and forest ecosystems in the 21st century (Matyssek and Innes, 1999). Although vast literature is available on the effects of ozone on plants, knowledge about the long-term mechanisms of ozone impact on forest trees under natural environmental conditions is still insufficient. This open-field ozone experiment was established in 1996 aiming to highlight current knowledge caps in empirical data (such as carryover mechanisms of ozone) and to provide realistic information about the impact of slightly elevated ozone exposure on European silver birch (*Betula pendula* Roth), the most important deciduous pulpwood species in Finland. In this first step, exposed saplings were harvested and analyzed for chemical and biochemical changes in relation to different growth responses determined for tolerant and sensitive clones. The emphasis was on the variation of relative contents of phenolics in ozone-tolerant and -sensitive birch clones grown over three growing seasons under low open-field ozone exposure and under ambient air (serving as control). Another part of plants in this extensive multiyear experiment will be used in actual quantitative ozone risk assessment after long lasting chronic exposure.

Plants contain a wide variety of phenolic compounds, such as simple phenols, flavonoids, stilbenes, tannins, lignans, and lignin. These substances function as cell-wall constituents providing mechanical stability and forming water and gas impermeable cell wall layers. Phenolics also function as natural pesticides, fungicides, antiherbivore factor, pigments, antibiotics, signal substances, attractants for pollinators, protective agents against UV radiation, excessive light, and air pollutants; as well as taking part in senescence, regeneration, and degradation processes due to cell damage. Pollutant-stress-induced changes in phenolics have been proposed to be connected with higher energy demand and consequent increase in respiration of stressed plants, with oxidation of these substances to quinones, with lignin production separating healthy and damaged (necrotic) leaf tissues, and with accelerated senescence (e.g., Bennett and Wallsgrove, 1994; Giertych et al., 1999). Ozone-caused increases have been reported especially in phenolic acid, tannin and terpenoid concentrations in several tree species (Rosemann et al., 1991; Jordan et al., 1991; Lavola et al., 1994; Wellburn and Wellburn, 1996), providing protection against oxidative damage (Langenheim et al., 1994; Foy et al., 1995; Ormrod et al., 1995). It has been suggested that ozone-induced accumulation of phenolics specifically concerns those compounds that are needed for defense (Richter and Wild, 1988; Foy et al., 1995; Lavola, 1998).

The phenolics of birch species, especially those of European silver birch leaves, have been well documented by Ossipov et al. (1995, 1996) and Keinänen and Julkunen-Tiitto (1998). In our previous study, (Oksanen and Saleem, 1999), hydroxycinnamic acid derivatives, e.g., neochlorogenic acid (NCGA); caffeoylquinic

acid derivatives (CQA); chlorogenic acid (CGA); hydroxybenzoic acid derivatives, e.g., (GPS); 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-*D*-glucopyranose and flavonol glycosides, e.g., myricetin glycosides (MG), quercetin glycosides (QG), and kaempferol glycosides (KG) were tentatively identified and quantified on the basis of study by Ossipov et al. (1996). In the present study, more efficient extraction and chromatographic conditions were used to improve separation, identification and quantification of phenolic compounds.

METHODS AND MATERIALS

Plant Material. Forty saplings of ozone-sensitive *Betula pendula* clone 2 and ozone-tolerant clone 5, determined previously in Pääkkönen et al. (1993), were randomly placed in two replicate open-air elevated-ozone exposure fields and two replicate ambient-ozone control fields in May 1996. These fast-growing, commercially used clones originated from southern (Valkeakoski, clone 2) and central (Ristiina, clone 5) Finland. The plants were grown in 20-dm^3 pots, filled with a mixture of prefertilized *Sphagnum* peat and quartz sand (2 : 1). The saplings were irrigated as needed and fertilized with 0.2% Superex-9 solution (19 : 5 : 20, N-P-K) once a week until July. During the growing seasons, the positions of plants were randomized by rotation within each field throughout the experiment. Each year in October, the pots were protected with excised conifer branches and plants were overwintered at the experimental site covered with snow.

Open-Field Ozone Exposure. The experiment was carried out by using the open-field exposure system at Kuopio University experimental area in central Finland, described in detail by Wulff et al. (1992). Ozone was produced from pure oxygen with a Fisher OZ 500 generator, and dispensed from the upwind vertical tubes. Ozone concentrations were continuously monitored at the center of each exposure field with a Dasibi 1008-RS ozone analyzer. Ozone was administered during the day and night throughout the growing seasons according to the 1.5- to 1.6-Fold ambient ozone profile. During inappropriate weather conditions (rain, fog, dew, and strong wind) ozone fumigation was automatically stopped. The cumulative ozone exposures AOT0, AOT30, and AOT40 (accumulated over threshold 0, 30, and 40 ppb, respectively), and the 7-hr (11:00–18:00) and 24-hr mean values for ozone concentrations are given in Table 1.

Sampling and Extraction. Ten green, mature sun-leaves from five plants per clone and treatment from the middle of the canopy were frozen in liquid nitrogen on August 27, 1998. Freeze-dried leaf material (500 mg) was homogenized for 40 sec with Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechink, Germany) in 20 ml of 80% methanol at room temperature. The homogenate was subjected to continuous stirring for 1 hr followed by centrifugation at 1500 *g* for 10 min. The pellet was extracted twice, and the combined extract was evaporated to dryness

TABLE 1. OZONE DATA FROM MAY 20, 1996 TO AUGUST 31, 1998.

Ozone	1996		1997		1998	
	A	E	A	E	A	E
AOT0 (ppm hr)	71.2	107.0	67.6	107.1	70.3	104.9
AOT30 (ppm hr)	5.5	33.4	4.7	30.1	3.1	24.7
AOT40 (ppm hr)	1.0	17.3	0.8	13.9	0.3	10.2
7-h mean (ppb)	30.2	45.8	27.5	42.0	26.1	34.0
24-h mean (ppb)	24.1	36.3	22.4	35.5	21.8	31.0

[a] AOT exposures have been calculated for each growing season (May–September) and for each 24 hr/day. Values are means for the two ambient-ozone (control, **A**) and / elevated-ozone blocks (**E**).

under low pressure. Dried extracts were redissolved in HPLC water to a final weight:volume ratio of 0.04 g/ml and stored at -18°C. All chemicals and eluents for HPLC analysis as well as Folin reagent for total phenolics concentration assay were purchased from Fluka BioChemica (Buchi, Switzerland).

Total Phenolics Concentration (TPC). The Folin-Ciocalteau method for quantification of total phenolics, was chosen on the basis of its high sensitivity, lower interference, and speed as compared to other competitive assays (Waterman and Mole, 1994). The assay (Waterman and Mole, 1994) was slightly modified for this study by homogenizing the contents at 1500 *g* for 10 min and measuring the absorbance at 730 nm instead of usual 765 nm. Extract (0.1 ml) was mixed with 5.9 ml water and of the diluted extract 1.0 ml was mixed with 1.0 ml of Folin reagent. The mixture was allowed to stand for 2–5 min and 2 ml of 20% Na_2CO_3 was added. The mixture was stirred and incubated for 10 min at room temperature, homogenized at 1500 *g* for 8 min, and its absorbance was measured at 730 nm on a Perkin-Elmer Spectrophotometer 550. A standard curve was drawn with known concentrations of gallic acid. The results were calculated according to the mathematical procedure of Segal (1975).

HPLC Analysis. Each extract was filtered through a Millex-HV_{13} filter, 0.45 mm (Millipore, Bedford, Massachusetts) before HPLC injection. Separations were performed on a Spherisorb 5 ODS-2 column (250 $\times$ 4.6 mm ID, 5 μm, Phase Separations Ltd.). The HPLC system consisted of an Hitachi L-6200 Intelligent Pump connected to a L-4250 UV-VIS Detector (Merck, Tokyo, Japan) and Chrompac C-R6A Integrator (Shimadzu, Kyoto, Japan). Two solvents were used for elution: A, acetonitrile; B, 5% formic acid. The elution profile was 0–5 min., 100% B (isocratic); 5–60 min, 0–30% A in B (linear gradient); 60–70 min, 30–70% A in B (linear gradient). The flow rate was maintained at 1.0 ml/min and the column back pressure at 70–136 bar. The detection wavelength was fixed at 280 nm. The injection volume of the sample was 20 μl.

HPLC-ESI-MS Analysis. HPLC-ESI-MS analysis was performed with a Perkin Elmer Sciex API-365 triple quadrupole mass spectrometer (Sciex, Toronto,

Canada). The instrument was equipped with a pneumatically assisted ion spray interface and Macintosh data system. The HPLC system consisted of Perkin-Elmer Series 200 HPLC system with a UV-VIS detector. Samples were introduced into the system by a Perkin-Elmer Series 200 Autosampler (Perkin-Elmer, Norwalk, Connecticut). All chromatographic conditions were kept similar to the HPLC-DAD analyses except the use of 0.4% instead of 5% formic acid as eluent B. Before MS, the main part of the flow was split off and only 20% was introduced into the mass spectrometer. The mass spectrometer was operated in negative ion mode with a scan range of *m/z* 100–1100 in 0.30 amu steps. The needle voltage was set at −4000 V, the orifice voltage at −35 V and the ring voltage at −220 V. The nebulizer gas flow was set at 9 and for N_2 curtain gas at 12. The heated nitrogen gas temperature was set at 300°C and the gas flow rate was 7000 cm^3/min.

Growth Measurements. On May 28, 1998, 10 plants per clone per treatment were measured for dry mass of shoot (stem + leaves) and roots. Leaf area growth for another 10 plants per clone per treatment was followed from May 8 to June 5, 1998. On August 31, 1998, 10 plants per clone per treatment were measured for stem height, base diameter, and proportion of yellowed leaves (Percent of all leaves).

Quantitative Analyses. Leaf samples for Rubisco, soluble protein, chlorophyll, and carotenoid concentration determination were collected on August 31, 1998, from mature sun-leaves from 10 saplings per clone per treatment at 10:00–13:00 hr. Frozen leaf disks (2.05 cm^2) were weighed, and a crude extract was prepared for Rubisco concentration using 2 ml of the extraction buffer (Gezelius and Hallen, 1980). The amount of total Rubisco protein was assayed by PAGE as described by Rintamäki et al. (1988), using purified Rubisco protein (Sigma Chemical Co.) as a standard. Rubisco was quantified on a dry weight basis by scanning the areas and intensities of bands in Coomassie-stained gels (Adobe Photoshop Program, version 5.0). An aliquot for soluble proteins was quantified by using Bradford reagent with BSA as standard. Samples for chlorophyll and total carotenoid quantification were taken from the crude extract before centrifugation. Chlorophyll concentrations were determined with a UV-1201 Shimadzu spectrophotometer using buffered 80% acetone as solvent and calculated with corrected equations (wavelengths 646.6 and 663.6 nm) as described by Porra et al. (1989). Total leaf carotenoids were analyzed spectrophotometrically according to Wellburn and Lichtenthaler (1984), measuring absorbance at 470 nm. Bulk samples for starch determination, collected from 10 plants per treatment per clone, were freeze-dried, milled, and analyzed by standard enzymatic techniques (Boehringer kit for food analysis).

Nutrient Analysis. Nutrients were analyzed from dried and ground bulk samples collected from green middle canopy leaves on August 31, 1998. Atomic absorption spectrophotometry (AAS) was used for Ca, K, Mg, and P quantification, and nitrogen concentration was determined by the Kjeldahl method.

TABLE 2. HPLC-DAD (T_R and HPLC-ESI-MS λ_{max}) and (M/Z) data of phenolics of *B. pendula Roth* leaf extract

Peak	T_R(min)	$\lambda^a{}_{max}$ (nm)	*m/z*	Compound[b]
1	17.1	280	305(M-H), 611(2M-H)	(+)-Catechin(CT)
2	21.0	251, 300 sh, 330	353(M-H), 707(2M-H)	Chlorogenic acid (CGA)
3	21.9	315	337(M-H)	5-*p*-Coumaroylquinic acid (5CQA)
4	22.5	285	327(M-H), 655(2M-H)	(GPS)
5	23.3	315	337(M-H), 675(2M-H)	3-*p*-Coumaroylquinic acid(3CQA)
6	25.5	250, 300 sh, 335	353(M-H), 707(2M-H)	Neochlorogenic acid (NCGA)
7	35.8	260, 300 sh, 361	479(M-H), 959(2M-H)	Myricetin galactopyranoside(MG)
8	40.5	261, 302 sh, 360	463(M-H), 927(2M-H)	Quercetin-3-*O*-galactopyranoside (QGL)
9	41.0	265, 299 sh, 350	477(M-H), 955(2M-H), 301(M-glucuronic acid)	Quercetin-3-*O*-glucuronopyranoside (QGR)
10	43.5	260, 300 sh, 361	433(M-H), 867(2M-H)	Quercetin-3-*O*-arabinofuranoside (QA)

[a]sh = shoulder; [b]GPS = 1-(4″-hydroxyphenyl)-3′-oxopropyl-β-*D*-glucopyranose.

Statistical Analysis. Due to the randomized location and rotation of plants throughout the experiments, no significant field effects were revealed in any parameter by ANOVA with a SPSS/PC package (version 8.0). Two-way ANOVA using plot (field) means (±standard errors) was used to test differences between treatments. Differences were considered significant at $P < 0.05$.

RESULTS

Phenolic Determination. Extraction was carried out with 50%, 60%, 70%, and 80% acetone, methanol, and ethanol to get higher yields of phenolics. Results on the basis of peak areas from HPLC analyses indicated that 80% methanol was the most efficient solvent for extracting phenolics. The Folin-Cioaclteau assay was slightly modified for the determination of total phenolics. The homogenization of the contents immediately before measuring the absorption noticeably reduced the turbidity, and 730 nm proved to be a more suitable absorbance wavelength and gave higher total phenolic concentration than 765 nm. These minor changes provided better reproducibility in triplicate measurements under our test conditions. HPLC-DAD was supported with HPLC-ESI-MS for quick recognition of phenolic compounds of interest in birch leaf extracts. HPLC identification with diode array detection at 280 nm was used to get characteristic UV spectra of individual compounds. The shapes of spectra and UV maximum maxima of each compound were then compared with standard compounds. Ten phenolic compounds were identified and quantified on the basis of results from HPLC-DAD and HPLC-ESI-MS. The *m/z* values (Table 2) from HPLC-ESI-MS confirmed the identification of each compound corresponding to a particular peak (Figure 1).

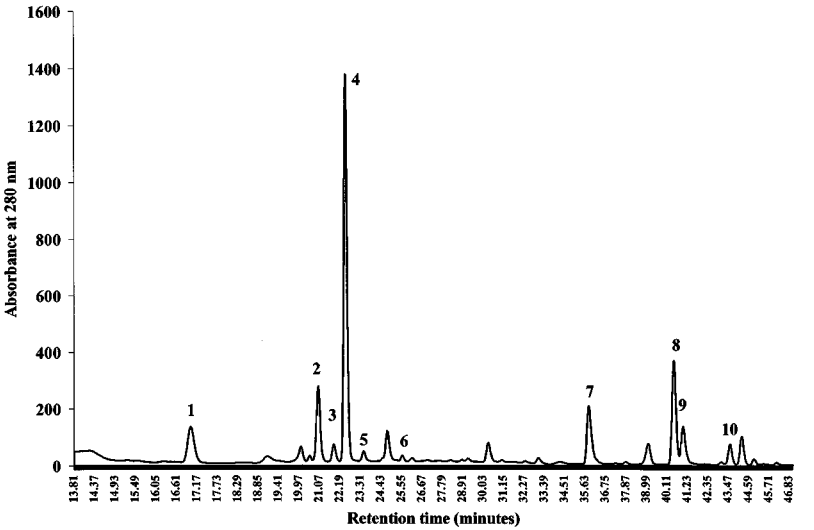


FIG. 1. HPLC chromatogram of *Betula pendula* Roth leaf extract. Numbers in chromatogram correspond to compounds in Table 2.

Phenolics in Different Treatments. The ozone-induced increase in total phenolic contents was determined for pooled data (two clones together) in order to compare TPC and HPLC methods (Figure 2). TPC results showed a 16.2% increase in total phenolics in ozone treatment, while 10 identified phenolics (HPLC) showed a 9.9% increase (Figure 2). The latter rise in 10 selected compounds provided more compact information of the trend due to the nonspecificity of TPC assay (Figure 2). In addition, there was a 2.9% higher ozone response of nonflavonoids as compared to flavonoids in pooled data (Figure 2). Based on 10 identified phenolics, the results showed an 11.4% increase in phenolic contents in clone 5 as compared to clone 2, indicating a greater responsiveness of the sensitive clone 5 (Figure 2 and 3a). The ozone-induced increase in the concentration of 10 phenolics for clone 2 and clone 5 is shown in Figure 3. The highest increases in clone 2 were found in 3CQA (31.5%), CGA (31.2%), MG (30.6%), QA (26%), and QGR (25.3%) and in clone in 5, 5CQA (53.3%), QGR (38.4%), CT (33.7%), CGA (31%), and 3CGA (28.2%) (Figure 3).

Growth Responses and Leaf Yellowing. Mean leaf size in emerging new foliage was significantly smaller in both clones from May 8 to June 5, 1998, in elevated-ozone plants (Figure 4). The difference between treatments was greater in ozone sensitive clone 5 (Figure 4). Stem and root dry weight was unaffected in the tolerant clone 2, whereas in clone 5 lower dry weights in ozone plants were observed on May 31, 1998, accompanied by increased shoot/root ratio (Table 3). Stem height and base diameter growth were unaffected in both clones, when determined on August 31, 1998 (Table 3), indicating ozone-induced

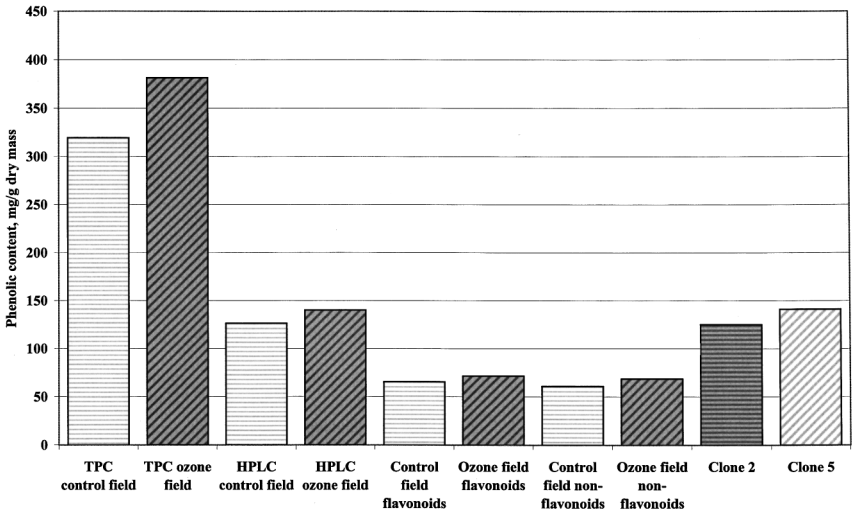


FIG. 2. Overall increase of phenolic concentration in *B. pendula* leaf extracts in elevated ozone plants as compared with controls (data were pooled for two clones). Results are average values of three measurements.

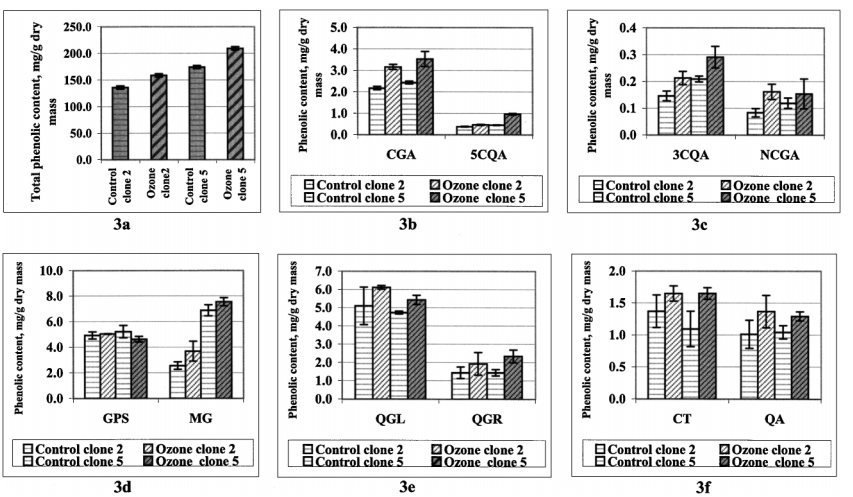


FIG. 3. Response of (a) total phenolic content and 10 phenolics (b–f) of *B. pendula* leaf extracts in elevated ozone plants as compared with control plants in clone 2 and clone 5. Values are means of ± SE, $N = 3$. See Table 2 for names of compounds.

TABLE 3. LONG-TERM GROWTH RESPONSES OF *B. pendula* CLONES 2 (TOLERANT) AND 5 (SENSITIVE), DETERMINED DURING THE THIRD GROWING SEASON AFTER START OF OZONE EXPOSURE[a]

Response	Clone	Control	Elevated ozone	*p*
May 31, 1998.				
DWT of shoot (g)	2	44.3 ± 16.2	46.0 ± 21. 9	0.961
	5	60.7 ± 5.6	42.5 ± 3.5	**0.045**
DWT of roots (g)	2	129.2 ± 18.9	86.0 ± 16.5	0.826
	5	189.0 ± 5.6	49.0 ± 2.0	**0.018**
Shoot/root ratio	2	0.34 ± 0.08	0.53 ± 0.08	0.549
	5	0.32 ± 0.05	0.87 ± 0.03	**0. 041**
August 31, 1998.				
Stem height (cm)	2	214.8 ± 18.5	201.0 ± 6.9	0.889
	5	184.7 ± 7.6	185.0 ± 12.1	0.976
Base diameter (mm)	2	19.8 ± 1.3	20.1 ± 0.7	0.872
	5	18.5 ± 0.8	18.5 ± 12.1	0.999
Yellowed leaves(%	2	7.8 ± 1.1	12.9 ± 2.3	0.564
of all leaves)	5	9.5 ± 1.7	23.8 ± 3.4	**0.038**

[a] Values are means ± SE. Two-way ANOVA (P-values are bold, when ≤ 0.05), $N = 2$.

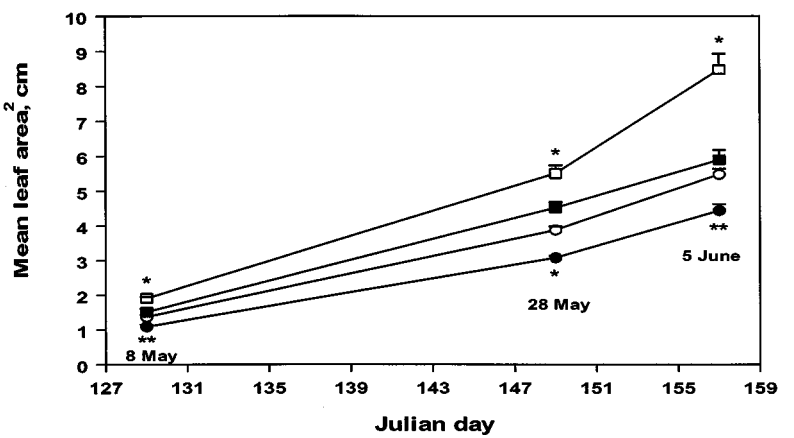


FIG. 4. Effects of elevated ozone on leaf size of new emerging foliage in *B. pendula*, determined between May 8 and June 5, 1998. Symbols: (■) control, clone 2; (□) control, clone 5; (●) elevated ozone, clone 2; (○) elevated ozone, clone 5. Two-way ANOVA (*p* values for clone 2 are 0.023 on May 8, 0.027 on May 28, and 0.039 on June 5. Corresponding *P* values for clone 5 are 0.06, 0.048, and 0.012, respectively). $N = 2$.

TABLE 4. EFFECTS OF LONG-TERM OZONE EXPOSURE ON STARCH, RUBISCO, SOLUBLE PROTEIN, CHLOROPHYLL AND CAROTENOID CONCENTRATIONS IN *B. Pendula* CLONES 2 AND 5[a]

Response	Clone	Control	Elevated ozone	*P*
Total Rubisco concentration	2	77.02 ± 1.99	78.88 ± 6.43	0.808
(mg/g DWT)	5	85.72 ± 3.94	89.12 ± 4.91	0.643
Total soluble protein conc.	2	161.44 ± 38.67	157.97 ± 11.50	0.939
(mg/g DWT)	5	155.61 ± 14.31	174.70 ± 1.78	0.317
Total chlorophyll (a + b),	2	2.19 ± 0.04	1.45 ± 0.75	0.433
(mg/g DWT)	5	2.30 ± 0.16	1.03 ± 0.02	0.016
Total carotenoids,	2	268.31 ± 32.10	163.61 ± 34.51	0.157
(μg/g DWT)	5	261.88 ± 42.03	229.32 ± 6.62	0.524
Starch	2	81.18 ± 5.33	78.01 ± 9.5	0.605
(mg/g DWT)	5	99.41 ± 9.59	79.59 ± 4.15	0.245
Nutrient concentrations, (mg/g DWT)				
N	2	10.98 ± 0.31	11.06 ± 0.56	0.591
	5	13.68 ± 1.51	12.84 ± 0.50	0.699
P	2	3.07 ± 0.24	3.24 ± 0.19	0.622
	5	3.40 ± 0.41	3.18 ± 0.23	0.713
Ca	2	6.10 ± 0.57	7.09 ± 0.41	0.403
	5	7.91 ± 1.30	7.97 ± 0.78	0.834
Mg	2	10.66 ± 5.22	2.23 ± 0.08	0.052
	5	16.65 ± 9.15	2.34 ± 0.15	**0.031**
K	2	7.45 ± 0.49	7.49 ± 0.43	0.763
	5	5.58 ± 0.58	6.27 ± 0.86	0.725

[a]Leaf samples collected on August 31, 1998. Two-way (P-values are bold, when ≤ 0.05), N = 2 Values are means ± SE.

reduction in resource allocation to leaves rather than to stem growth. In addition, yellowing of leaves was (14.3%) enhanced in ozone plants of clone 5 (Table 3).

Quantitative Analysis. Rubisco and soluble protein concentration were not affected by elevated ozone in either clone (Table 4). Total chlorophyll concentration was lower in clone 5 ozone-exposed plants, accompanied by reduced content of Mg. In clone 2, the reduction in chlorophyll and Mg was not significant. Total carotenoid and starch tended to decrease in elevated-ozone plants of both clones but were not significant (Table 4). Foliar N, P, Ca, and K concentrations were also unaffected by ozone (Table 4).

DISCUSSION

The results provide good evidence that elevated atmospheric ozone levels have detrimental effects on the well being of European silver birch (evidenced by elevated phenolic levels) leading to reduced growth and increased leaf senescence.

Changes in Shoot–Root Balance. High ozone sensitivity of clone 5 was confirmed by significantly lower shoot and root growth, a higher proportion of yellowed leaves, as well as lower chlorophyll and Mg concentrations in ozone-exposed plants. In both clones, increased shoot–root ratio in ozone plants indicated shifts in assimilate transport at the expense of roots, leading to altered crown and root architecture, followed by predisposition to other stress factors, such as water and nutrient deficiency and pathogens, and impaired competitiveness in the long term. In recent reviews, changes in resource allocation and disturbed shoot and root balance have been considered as crucial factors underlying the deleterious ozone impact on European forest trees, long-term stand development, and forest ecosystem structure (Skärby et al., 1998; Matyssek and Innes, 1999).

Ozone-Induced Increase in Phenolics. The results of this experiment were consistent with earlier findings, although ozone-induced increases in phenolics were generally higher in our previous study (Oksanen and Saleem, 1999). In our previous work, quantitative comparisons focused on GPS and CGA, which are known to be one among the most abundant nonflavonoid phenolic compounds in *Betula pendula* and *Betula pubescens* (Keinänen and Julkunen-Tiitto, 1998). The ozone-induced increase in phenolics was over two times higher in the sensitive clone 5 as compared to tolerant clone 2 (Oksanen and Saleem, 1999). GPS was less important in the present experiment. The reasons for different responses may be explained by annual variation in ozone concentrations and by seasonal changes in phenolics, relating to phenology of trees, i.e., the samples in the first study were collected in June during a high ozone episode and fast growth period of leaves, whereas in the present study the samples were collected at the end of growing season. Previously, ozone-induced increases in contents of papyriferic acid and flavonoids were also reported in stem samples after low, short-term field ozone exposure in these same silver birch clones (Lavola et al., 1994).

Role of Phenolics in Ozone Stress. Changes in phenolic compounds can be regarded as a phytochemical adaptation to the biotic and abiotic environment (Dixon and Paiva, 1995; Lavola, 1998). Our results support the earlier findings in deciduous trees (Bryant et al., 1987; Julkunen-Tiitto and Meier, 1992; Lavola, 1998) that in birch, a high degree of variation in the production of phenolic compounds is due to phenotypic plasticity, enabling the adaptation to spatial and temporal variation in environmental stress factors like ozone. The accumulation of cell wall phenolics and flavonoids has been shown to prevent water loss and increase the general stress tolerance of the plant (Elstner et al., 1994; Dixon and Paiva, 1995). Induction of antioxidative phenolics such as quercetin and chlorogenic acid may further increase resistance against ozone damage by scavenging free oxygen and hydroxyl radicals and hydrogen peroxide (Appel, 1993; Foy et al., 1995). The increase of chlorogenic acid in ozone-exposed plants in this experiment may indicate an increase in antioxidant activity (e.g., Larson,

1988; Elstner et al., 1994; Rice-Evans et al., 1997), as reported in *Mahonia repens*, where chlorogenic acid was found to be a highly efficient scavenger of free oxygen radicals ameliorating the oxidative stress responses in plants (Grace et al., 1998). In addition, accumulation of triterpenoids on the plant surfaces may contribute ozone resistance by reflectance and ozonolysis of terpenoids (Langenheim, 1994). Recently, in *Pinus nigra* needles collected in a polluted area in Poland, an increase of phenolic levels was concluded to indicate an activation of senescence processes, correlating negatively with the main nutrients (Giertych et al., 1999). Similar relations were observed also in our study with lower Mg and chlorophyll concentrations in the sensitive clone, indicating accelerated leaf senescence.

Growth Versus Phenolic Allocation. The present results are in accord with various carbon nutrient balance (CNB) models (Bryant et al., 1983; reviewed in Herms and Mattson, 1992), which explain that, due to metabolic costs, increased allocation of carbon resources to chemical defense and repair processes under stressful situations results in lower biomass production and growth. The increase in total phenolics and concomitant reduction in growth is also in agreement with the protein competition model (PCM) of plant phenolic allocation of Jones and Hartley (1998). This model assumes that impaired growth and reduced demand for protein synthesis is accompanied by increased investment in carbon-based phenolics in fast-growing species because both processes use the same amino acid precursor—phenylalanine (Jones and Hartley, 1998). The present results indicate that a change in carbon allocation towards defensive phenolics at the expense of growth was greater in the ozone-sensitive clone 5 as compared to tolerant clone 2. Previously, a higher content of flavonol glycoside was detected in ozone-tolerant cultivars of soybean than in sensitive ones (Foy et al., 1995).

Ecological Implications and Conclusions. Because several phenolic biosynthesis-affecting environmental stress factors, such as increased ozone concentration, oxides of nitrogen, UV-B radiation, and elevated CO_2 levels together with increasing temperature and changing precipitation, affect forest trees simultaneously, the amounts of carbon-based phenolic compounds have been predicted to change in forest ecosystems (Jones and Hartley, 1998). Integrating the highly variable effects of single environmental factors to estimate the net effect of global change is a complex task. However, the present results suggest that increasing ozone levels tend to increase phenolic concentrations in birch trees, as proposed by Jones and Hartley (1998) and Lavola (1998). The results indicate an overall ozone-induced increase in foliar CT, CGA, 5CQA, 3CQA, MG, QGR, and QA. This was regarded as a source of concern, because altered resource allocation towards phenolics was accompanied by growth reductions and disturbed shoot/root balance, especially in the sensitive birch clone.

Acknowledgments—The study was funded by the Academy of Finland and Finnish Cultural Foundation grants.

REFERENCES

APPEL, H. M. 1993. Phenolics in ecological interactions: The importance of oxidation. *J. Chem. Ecol*. 19:1521–1552.

BENNETT, R. N., and WALLSGROVE, R. M. 1994. Secondary metabolites in plant defence mechanisms. *New Phytol*. 127:617–633.

BRYANT, J. P., CHAPIN, F. S., III, and KLEIN, D. R. 1983. Carbon–nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

BRYANT, J. P., CHAPIN, F. S., III, REICHARDT, P. B., and CLAUSEN, T. P. 1987. Response of winter chemical defence in Alaska paper birch and green alder to manipulation of plant carbon/nutrient balance. *Oecologia* 72:510–514.

DIXON, R. A., and PAIVA, N. L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7: 1085–1097.

ELSTNER, E. F., OSSWALD, W., VOLPERT, R., and SCHEMPP, H. 1994. Phenolic antioxidants. *Acta Holticl*. 381:304–335.

FOY, C. D., LEE, E. H., ROWLAND R., DEVINE, T. E., and BUZZELL, R. I. 1995. Ozone tolerance related to flavonol glycoside genes in soybean. *J. Plant Nutr*. 18:637–647.

GEZELIUS, K., and HALLEN, M. 1980. Seasonal variation in ribulose bisphosphate carboxylase activity in *Pinus sylvestris*. *Physiol. Plant*. 48:88–98.

GIERTYCH, M. J., KAROLEWSKI, P., and DE TEMMERMAN, L. O. 1999. Foliage age and pollution alter content of phenolic compounds and chemical elements in *Pinus nigra* needles. *Water Air Soil Pollut*. 110:363–377.

GRACE, S. C., LOGAN, B. A., and ADAMS, W. W., III. 1998. Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. *Plant Cell Environ*. 21: 513–521.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: to grow or defend. *Q. Rev. Biol*. 67:283–335.

JONES, C. G., and HARTLEY, S. E. 1998. Global change and plant phenolic concentrations: species level predictions using the protein competition model, pp. 23–50, L. J. De Kok and I. Stulen (eds.). *in Responses of Plant Metabolism to Air Pollution and Global Change*. Backhuys Publishers, Leiden, The Netherlands.

JORDAN, D. N., GREEN, T. H., CHAPPELKA, A. H., LOCKABY, B. G., MEHLDAHL, R. S., and GJERSTAD, D. H. 1991. Response of total tannins and phenolics in loblolly pine foliage exposed to ozone and acid rain. *J. Chem. Ecol*. 17:505–513.

JULKUNEN-TIITTO, R., and MEIER, B. 1992. Variation in growth and secondary phenolics among field-cultivated clones of *Salix myrsinifolia*. *Planta Med*. 58:77–80.

KEINÄNEN, M., and JULKUNEN-TIITTO, R. 1998. High-performance liquid chromatographic determination of flavonoids in *Betula pendula* and *Betula pubescens* leaves. *J. Chromatogr. A* 793:370–377.

LANGENHEIN, J. H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol*. 20:1223–1280.

LARSON, R. A. 1988. The antioxidants in higher plants. *Phytochemistry* 27:969–978.

LAVOLA, A. 1998. Phytochemicals of deciduous trees in relation to environmental changes. PhD thesis. University of Joensuu, Publications in Sciences No. 46, 39 pp.

LAVOLA, A., JULKUNEN-TIITTO, R., and PÄÄKKÖNEN, E. 1994. Does ozone stress change the primary or secondary metabolites of birch (*Betula pendula* Roth)? *New Phytol*. 126:637–642.

MATYSSEK, R., and INNES, J. L. 1999. Ozone—a risk factor for forest trees and forests in Europe? *Water Air Soil Pollut.* 116:199–226.

OKSANEN, E., and SALEEM, A. 1999. Ozone exposure results in various carry-over effects and prolonged reduction in biomass in birch (*Betula pendula* Roth). *Plant Cell Environ.* 22:1401–1411.

ORMROD, D. P., LANDRY, L. G., and CONKLIN, P. L. 1995. Short-term UV-B radiation and ozone exposure effects on aromatic secondary metabolite accumulation and shoot growth of flavonoid-deficient *Arabidopsis* mutants. *Physiol. Plant*. 93:602–610.

OSSIPOV, V., NURMI, K., LOPONEN, J., PROKOPJIEV, N., HAUKIOJA, E., and PIHLAJA, K. 1995. HPLC isolation and identification of flavonoids from white birch *Betula pubescens* leaves. *Biochem. Syst. Ecol*. 23:213–222.

OSSIPOV, V., NURMI, K., LOPONEN, J., HAUKIOJA, E., and PIHLAJA, K. 1996. High performance liquid chromatographic separation and identification of *Betula pubescens* and *Betula pendula*. *J. Chromatogr A* 721:59–68.

PÄÄKKÖNEN, E., PAASISALO, S., HOLOPAINEN, T., and KÄRENLAMPI, L. 1993. Growth and stomatal responses of birch (*Betula pendula* Roth.) clones to ozone in open-air and chamber fumigations. *New Phytol*. 125:615–623.

PORRA, R. J., THOMPSON, W. A., and KREIDEMANN, P. E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochem. Biophys. Acta* 975:384–394.

RICE-EVANS, C. A., MILLER, N. J., and PAGANGA, G. 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci*. 2:152–159.

RICHTER, C. A., and WILD, A. 1988. Phenolic compounds in needles of Norway spruce trees in relation to novel forest decline. I. Studies on trees from a site in the northern Black Forest. *Biochem. Physiol. Pflanzen* 188:305–320.

RINTAMÄKI, E., KEYS, A. J., and PARRY, M. A. J. 1988. Comparison of the specific activity of ribulose-1,5-bisphosphate carboxylase–oxygenase from some C_3 and C_4 plants. *Physiol. Plant*. 74: 326–331.

ROSEMANN, D., HELLER, W., and SANDERMANN, H. JR., 1991. Biochemical plant responses to ozone. II. Induction of stilbene biosynthesis in Scots pine (*Pinus sylvestris* L.) seedlings. *Plant Physiol*. 97:1280–1286.

SEGAL, I. H. 1975. Biochemical Calculations. John Wiley & Sons, New York.

SKÄRBY, L., RO-POULSEN H., WELLBURN, F. A., and SHEPPARD, L. 1998. Impact of ozone on forests: a European perspective. *New Phytol*. 139:109–122.

WATERMAN, P. G., and MOLE, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, London, pp. 83–84.

WELLBURN, A. R., and LICHTENTHALER, H. 1984. Formulae and program to determine total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. pp. 9–12, *in* C. Sybesma (ed.). *Advances in Photosynthesis Research*, Vol II.

WELLBURN, F. A. M., and WELLBURN, A. R. 1996. Variable patterns of antioxidant protection but similar ethene emission differences in several ozone-sensitive and ozone tolerant plant sections. *Plant Cell Environ*. 19:754–760.

WULFF, A., HÄNNINEN, O., TUOMAINEN, A., and KÄRENLAMPI, L. 1992. A method for open-air exposure of plants to ozone. *Ann. Bot. Fenn.* 29:253–262.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

VARIABLE DIETS AND CHANGING TASTE IN PLANT–INSECT RELATIONSHIPS

J. ALAN A. RENWICK[1,*]

Boyce Thompson Institute
Ithaca, New York 14853

(Received January 1, 2001; accepted January 19, 2001)

Abstract—The host ranges of phytophagous insects are determined to a large degree by plant chemistry. Specialist insects are often closely associated with plants that produce characteristic chemicals, which may act as attractants or stimulants to aid in finding or recognizing a host. Generalist insects are generally believed to rely on the presence of repellents or deterrents to ensure avoidance of unsuitable plants. However, the chemistry of any plant can be highly variable, as a result of growth characteristics, genetic variation, or environmental factors. Such variable chemistry may provide windows of opportunity for nonadapted insects to utilize a plant or for a plant to become resistant to a normally adapted herbivore. Differences in insect responses to plant constituents may also result from genetic variation or environmental factors. In particular, dietary experience has been found to influence the ability of insects to taste plant chemicals that may serve as signals of suitability or unsuitability. Certain dietary constituents appear to suppress the development of taste sensitivity to deterrents in an insect, whereas the presence of specific stimulants in the diet may result in the development of dependence on these compounds. These findings further emphasize the fact that the dynamics of plant biochemistry along with plasticity in the sensory system of insects might be expected to play a major role in the evolution of new plant–insect relationships.

Key Words—Host selection, taste, feeding stimulants, feeding deterrents, diet, *Manduca sexta*, *Pieris rapae*, sensory plasticity.

INTRODUCTION

The vast majority of phytophagous insects are known to be specialists on a limited number of closely related plants. The evolution of such plant–insect associations

*e-mail: jar14@cornell.edu
[1]ISCE Silverstein-Simeone Award 1999.

0098-0331/01/0600-1063$19.50/0

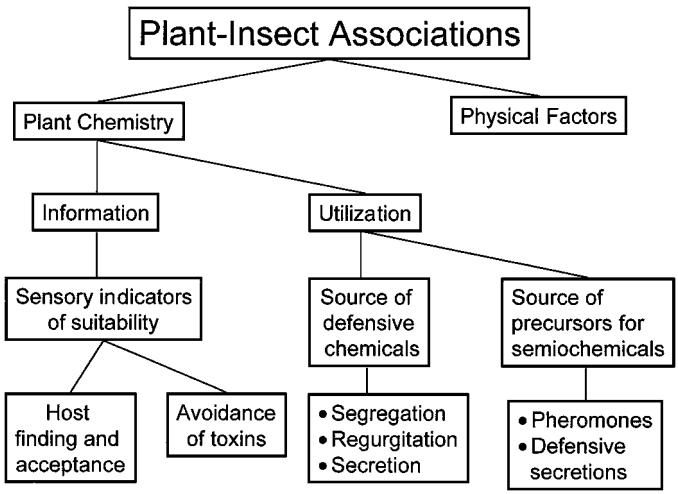


FIG. 1. Schematic representation of the value of plant chemistry to adapted insects.

is believed to be driven in part by the insects' need to avoid competition and to escape from enemies (Bernays and Chapman, 1994; Schoonhoven et al., 1998). This process has involved adaptation to the physical and chemical defenses that plants have developed against generalist herbivores and pathogens. By specializing, these insects may take advantage of unique architecture or characteristic chemistry of their host plants in a variety of ways (Figure 1).

Plant chemicals may be useful to the insect as a basic source of information about the plant, or they may be utilized more directly for defense or communication purposes. Chemicals for defense might be ingested and sequestered in various body parts to discourage potential predators (Brower, 1984). Alternatively, they are often regurgitated directly from the crop, or they may be stored in special glands for secretion when threatened by a potential enemy (Rowell-Rahier and Pasteels, 1993). Some host plant chemicals also are used as precursors for semiochemicals, including pheromones (Hughes, 1974).

The information afforded by plant chemistry is critical in guiding the process of host selection by specialist insects. Chemicals emanating from a plant may have a negative or positive effect on an approaching insect. The presence or absence of specific volatiles can promote landing, and key compounds at the surface may then signal the suitability of a plant, resulting in acceptance for oviposition or feeding (Renwick and Huang, 1994). The presence of repellents or deterrents usually signals unsuitability of a plant, and the resulting rejection behavior allows the insect to avoid contact with, or ingestion of, toxins that are likely to be encountered in the plant. For the Lepidoptera, host finding and acceptance is largely the responsibility

of the female adult, which must select a site for oviposition that is optimal for the hatching larvae. However, larvae must also be capable of recognizing new host plants after completely defoliating a plant or after falling from their first host plant (del Campo, 1999). It is clear, therefore, that discrimination among plants and recognition of specific hosts by adults and larvae are highly dependent on the insect's ability to perceive and respond to specific cues. Although many plant–insect systems have now been studied to elucidate the chemical basis for specialization, the significance of variability in plant chemistry and variability in the insect responses to plant constituents has seldom been addressed. Here we examine the results of experiments with two model systems to illustrate how such variations might play a role in the evolution of host range expansion and/or plant escape from herbivory.

Pieris–CRUCIFER MODEL SYSTEM

The specialization of many insects on members of the Cruciferae (=Brassicaceae) has long been linked to the presence of glucosinolates in these plants (Feltwell, 1982). This chemical association was first demonstrated by Verschaffelt (1910) for *Pieris* species, and the observation has served as a basis for several classical studies on the role of these compounds in host selection. The involvement of glucosinolates in stimulating feeding by larvae and oviposition by adults has since been confirmed for *P. brassicae* and *P. rapae* (Thorsteinson, 1953; David and Gardiner, 1966; Renwick et al., 1992). More recent studies have shown that ovipositing *P. rapae* adults respond most strongly to indole glucosinolates, such as glucobrassicin, and less strongly to aliphatic glucosinolates, especially those with sulfur in the side chain, such as glucocheirolin (Huang and Renwick, 1994). Larval preferences for different glucosinolates have received less attention, but feeding stimulation clearly occurs in response to any one of many aromatic or aliphatic glucosinolates (Renwick and Lopez, 1999).

P. rapae recently has served as a model insect for studying the chemical basis for restricted host ranges. Despite the strong association of this insect with glucosinolate-containing plants (including members of the Tropaeolaceae, Capparaceae, and Resedaceae, as well as most of the Cruciferae), some crucifers are avoided. Work on two of the unacceptable crucifers has shown that these plants contain strong oviposition deterrents. The deterrents in *Erysimum cheiranthoides* have been identified as cardenolides (Sachdev-Gupta et al., 1990), and deterrents that explain rejection of *Iberis amara* were identified as cucurbitacin glycosides (Huang et al., 1993). The fact that these unacceptable plants contain reasonably high concentrations of stimulatory glucosinolates indicates that the deterrent effect must outweigh the effects of the stimulants. These results have reinforced the idea that acceptance or rejection of a plant may depend to a large extent on a balance

of stimulants and deterrents perceived by the insect (Miller and Strickler, 1984; Renwick and Huang, 1994; Renwick, 1996). This idea has been tested by growing the normally unacceptable *E. cheiranthoides* in nutrient solutions containing increasing concentrations of nitrogen. At higher levels of nitrogen, the glucosinolate/cardenolide ratio in foliage increased substantially, and the plants became acceptable for oviposition (Hugentobler and Renwick, 1995).

Comparative studies with *Pieris napi oleracea* and *P. rapae* have shown that these related species have different thresholds for response to the various stimulants and deterrents. Oviposition by *P. napi* was more strongly stimulated by the aliphatic, sulfinyl, and sulfonyl glucosinolates than by the indole representatives of the group (Huang and Renwick, 1994). Furthermore, this species was less deterred by concentrations of cardenolides and cucurbitacin glycosides that effectively blocked oviposition by *P. rapae*. Similar differences were found in the responses of larvae to feeding deterrents in *E. cheiranthoides* and *I. amara*. Both cardenolides and cucurbitacin glucosides deterred feeding by *P. rapae*, but had little or no effect on *P. napi* (Chew and Renwick, 1995). The cardenolides that were most effective as oviposition deterrents were not the most active feeding deterrents. In general, the strophanthidin-based cardenolides were the most active oviposition deterrents, whereas the digitoxigenin glycosides were more effective against larval feeding (Renwick, 1996).

SENSORY PERCEPTION OF CONTACT STIMULI

Recognition of host plants by phytophagous insects clearly depends on a series of behavioral responses to visual, olfactory, and/or gustatory cues. In the case of *Pieris rapae* butterflies, visual cues are important for locating potential hosts, and, after landing, contact with nonvolatile cues at the leaf surface results in acceptance or rejection of the plant (Chew and Renwick, 1995). Taste receptors on the tarsi are responsible for perception of the glucosinolates and possible deterrents such as cardenolides at the leaf surface. Electrophysiological recordings from these receptors have been used to explain observed variation in behavioral responses to individual compounds. In particular, comparisons between *P. napi* and *P. rapae* have shown that differences in responses of the tarsal receptors to compounds could be related to differences in behavioral responses of the butterflies (Du et al., 1995; Städler et al., 1995).

In the case of larvae, host recognition depends almost entirely on the use of taste receptors that are present on the mouthparts. The most important chemoreceptors appear to be the medial and lateral sensilla styloconica located on the maxillary galea of the caterpillars. These sensilla are innervated by neurons that respond to glucosinolates and others that respond to deterrents (Schoonhoven, 1987). Recent studies have shown that *Pieris* caterpillars have specialist deterrent receptors that are particularly sensitive to cardenolides, as well as more general deterrent

receptors that respond to a wide range of compounds (van Loon and Schoonhoven, 1999). As in the case of adults, larvae of the different *Pieris* species are likely to have different sensitivities to both stimulants and deterrents, and electrophysiological responses can be correlated with the observed behavioral responses.

EFFECTS OF DIETARY EXPERIENCE

The responses of insect larvae to chemical cues from potential host plants may often be influenced by dietary experience. Feeding behavior may be affected by various forms of learning, including habituation, sensitization, and imprinting (induction of preference) (Szentesi and Jermy, 1989). Early work on *Pieris brassicae* showed that when late-instar larvae were given a choice of food plants, they generally preferred the plant on which they were reared (Johansson, 1951). Larvae reared to fifth instars on *Tropaeolum majus* also developed a preference for this plant. Thus, induced preference was clearly indicated, although some feeding also occurred on the less preferred plants. However, Ma (1972) found that *P. brassicae* that had fed on *Brassica oleracea* refused to feed on *T. majus*, and the mechanism of this rejection behavior was never explained. Subsequent work with *Pieris rapae* has shown that cabbage-reared larvae of this species also will starve to death rather than feed on nasturtium (*Tropaeolum majus*) (Renwick and Huang, 1995). However, adults from the same colony readily oviposited on nasturtium, and the hatching larvae fed and developed normally. Likewise, when neonate larvae were transferred from a Parafilm substrate to nasturtium, they fed normally. In experiments with different instars, the development of refusal behavior was found to occur in first instars, within 24 hr of inititiation of feeding on cabbage.

Subsequent experiments with other host plants, including noncrucifers, showed that larvae transferred from cabbage to *Brassica juncea* (Cruciferae), *Sinapis alba* (Cruciferae), *Cleome spinosa* (Capparidaceae), and *Reseda luteola* (Resedaceae) all fed readily on their new hosts. These results suggested that nasturtium has some unique deterrent property that is absent in other hosts. The possibility of imprinting as a mechanism for rejection of a new host was tested by making the reverse switch. However, those larvae transferred from nasturtium to cabbage fed almost immediately on the new plants (Renwick and Huang, 1995).

The presence of a feeding deterrent in nasturtium has subsequently been demonstrated, and the major active compound has been identified as chlorogenic acid (Huang and Renwick, 1995a). Additional experiments were performed with larvae reared on a variety of host plants to determine whether some unique factor in cabbage was responsible for triggering sensitivity to the deterrents in nasturtium. However, larvae reared from egg hatch to third instar on *Brassica juncea*, *Sinapis alba*, *Cleome spinosa*, and *Reseda luteola* all refused to feed on nasturtium. These results suggested that many host plants have the qualities necessary for *P. rapae*

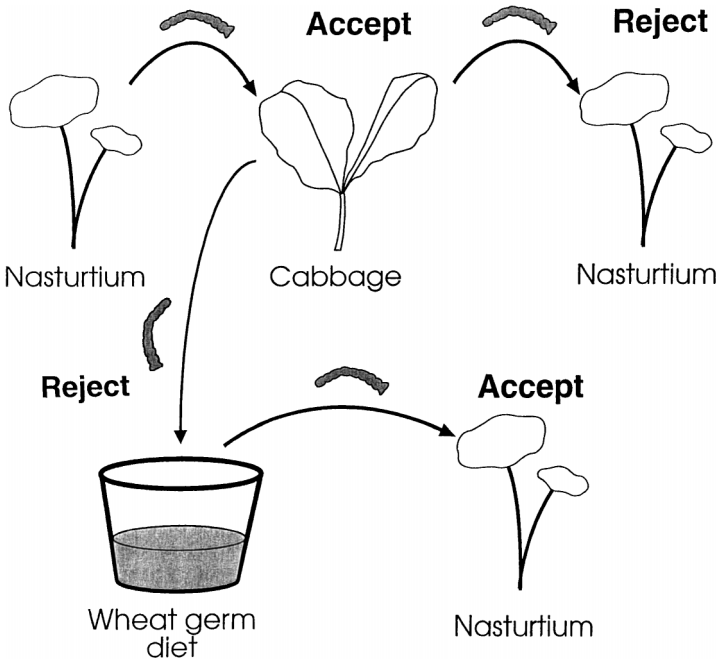


FIG. 2. Effect of dietary experience on acceptance or rejection of new diets by larvae of *Pieris rapae*.

larvae to develop sensitivity to the deterrents. However, when larvae were reared on wheat germ diet, and third instars were transferred to nasturtium, they accepted the plant and fed normally (Figure 2). This means that the wheat germ diet either lacks an inducer or contains chemicals that suppress development of larval sensitivity to the deterrent in nasturtium. Larvae reared on nasturtium from the time of hatching had limited sensitivity, whereas larvae that were reared exclusively on the wheat germ diet remained almost completely insensitive to the deterrents (Renwick and Huang, 1995).

We have suggested that the development of sensitivity to deterrents in plants is suppressed by continuous exposure of *P. rapae* larvae to these compounds. However, it was not clear whether exposure to one deterrent would suppress sensitivity to other unrelated deterrents. Experiments to answer this question were performed by rearing larvae on cabbage leaves that were treated with individual deterrent compounds and then testing the response of these larvae to other deterrents. The results showed that dietary experience can, in fact, affect sensitivity to compounds that are not present in the diet. Thus, a type of cross-habituation exists (Huang and Renwick, 1995b).

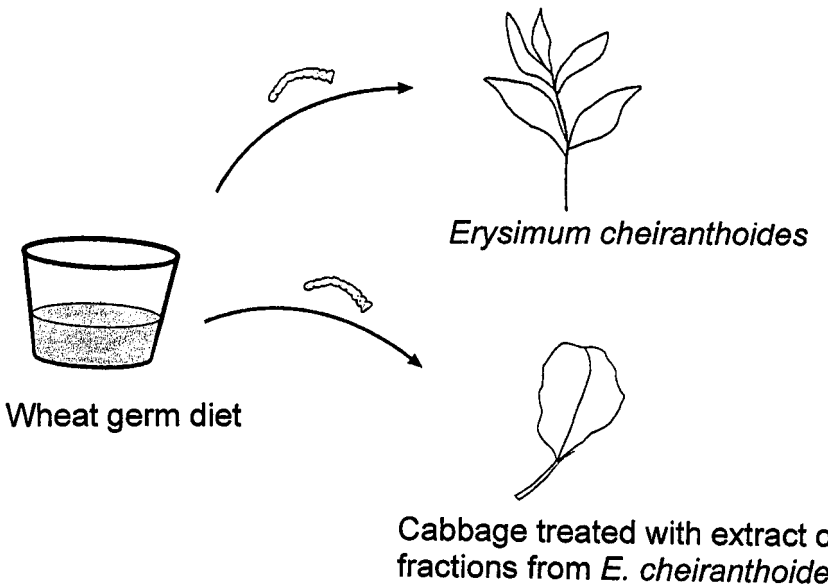


FIG. 3. Bioassay for toxicity of an unacceptable plant to *Pieris rapae* and for monitoring isolation of toxin from plant extracts.

Detailed studies on the development of larval sensitivity to feeding deterrents suggested that the sensory system of second instars is most plastic, but a gradual increase in sensitivity of larvae at any stage occurs after their transfer from artificial diet to cabbage. The evidence suggests that *P. rapae* could serve as an excellent model for analysis of the physiological and biochemical processes involved in sensory development. In addition, suppression of taste sensitivity allows us to test the effects of feeding on a plant that would not normally be eaten. When *P. rapae* larvae are reared on wheat germ diet and then transferred to a normally unacceptable plant, some feeding is likely to occur. This approach has been used to examine possible effects of *P. rapae* feeding on *Erysimum cheiranthoides*. Diet-reared fifth instars consumed considerable amounts of *E. cheiranthoides* foliage but became sick within 48 hr and died soon after (Renwick et al., unpublished results). This provided a clear indication that *E. cheiranthoides* contains compounds that are toxic to *P. rapae* (Figure 3). Solvent extracts of *E. cheiranthoides* applied to cabbage foliage were also toxic to the diet-reared larvae, and preliminary HPLC has resulted in the isolation of several cardenolides that could explain the toxic activity (Renwick, unpublished results).

Recent work has shown that the source of suspected suppressing compounds in artificial diet is the wheat germ itself. Exhaustive extraction and partitioning of

wheat germ, using feeding deterrent assays to monitor for activity, resulted in the isolation of a highly deterrent methanol-soluble fraction. Subsequent separation by HPLC yielded active compounds that have been characterized as apigenin-based flavones (Huang and Renwick, 1997).

Manduca sexta AS A COMPARATIVE MODEL

The discovery of diet-dependent sensitivity to deterrents in *Pieris rapae* prompted additional studies to determine whether other insects exhibit the same phenomenon. *Manduca sexta* appeared to be an ideal candidate, since Städler and Hanson (1978) had already found that plant-reared larvae of this species were deterred by extracts of artificial diet. These authors were the first to suggest that diet-induced changes in food selection behavior of *M. sexta* could be related to changes in sensory responses. Experiments in our laboratory were designed to determine whether diet-dependent acceptance of a nonhost plant (cowpea) by this species could be the result of differences in sensitivity to deterrents. The results clearly indicated that dietary modification of food acceptance was due, not to differences in response to a deterrent, but to the development of dependence on host plant constituents (del Campo, 1999; del Campo and Renwick, 1999). Larvae reared on

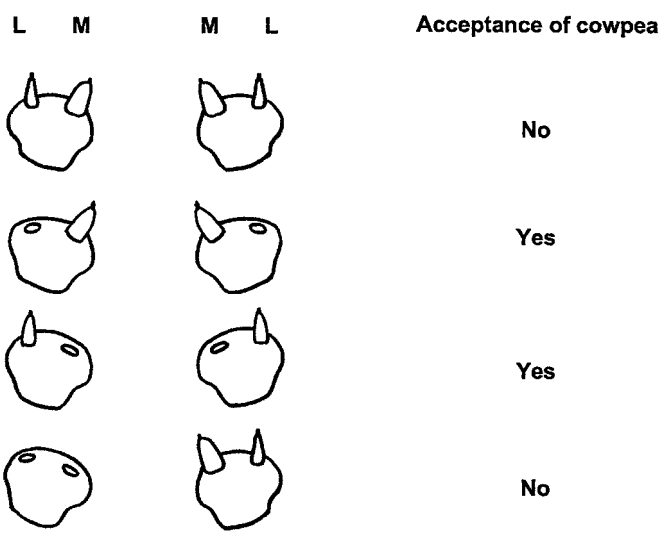


FIG. 4. Effects of selective removal of lateral (L) and medial (M) sensilla styloconica from *Manduca sexta* larvae on acceptance of cowpea leaf discs as food. Test larvae were reared on potato foliage to become dependent on the presence of a specific stimulant for feeding.

a wheat germ diet will feed on the nonhost cowpea, whereas potato-reared larvae require a specific feeding stimulant for acceptance of cowpea as food. A single compound was isolated from potato foliage to explain this effect (del Campo and Renwick, 2000), and the development of dependence on this compound could be related to changes in the responses of a particular chemoreceptor on the mouthparts of the larvae (del Campo, 1999). Since feeding discrimination in caterpillars has been linked to the responses of two maxillary hairs, the medial and lateral sensilla styloconica, to stimulants and deterrents (Schoonhoven, 1987), ablation experiments were performed to determine which of these receptors are involved in the development of this chemical dependence. Discrimination was measured by testing the acceptance of cowpea leaf disks by potato-reared *M. sexta* larvae. The intact larvae refused to feed on the cowpea disks, but after bilateral removal of either the medial or lateral sensilla, the larvae fed on the disks. However, after unilateral removal of both medial and lateral sensilla, the larvae still refused to feed (del Campo, 1999) (Figure 4). Thus, discrimination appears to require input from both sensilla. However, electrophysiological recordings indicated that dietary experience affected the responses of only the lateral sensilla to the potato compound. As a result of these studies, del Campo et al. (2001) have concluded that specialization by this insect involves a peripheral neural mechanism.

DEPENDENCE OF *Pieris rapae* ON A HOST STIMULANT

The possible development of *P. rapae* dependence on specific host compounds was examined by using the same approach as for *M. sexta*. Cowpea disks were generally accepted as food by neonate larvae, but larvae that had fed on cabbage refused the cowpea. The development of this refusal behavior was related to the length of time spent feeding on cabbage. However, when extracts of various host plants were applied to cowpea leaf disks, crucifer-fed larvae were stimulated to feed at rates that were little affected by the plant on which they had been reared. A general addiction to glucosinolates was suggested, and a single glucosinolate was found to be sufficient to elicit feeding by cabbage-reared larvae. Since bioassays of cowpea extracts failed to show any deterrent activity, the conclusion was reached that addiction to glucosinolates is responsible for the fixation of *P. rapae* larvae on their host plants (Renwick and Lopez, 1999).

DEPENDENCY AND SENSITIVITY OF *P. napi oleracea*

Comparative studies on *P. n. oleracea* have revealed the existence of a somewhat different dependence mechanism. Neonate larvae of this species will not feed

on untreated cowpea leaf disks. However, when glucosinolates are applied to the disks, feeding occurs (W. Q. Zhang, unpublished results). Since the naïve neonates require glucosinolates for initiation of feeding, prior experience is not necessary for the development of dependence. Instead, it appears that *P. napi* larvae have an innate craving for glucosinolates upon hatching from the egg.

In the case of feeding deterrents, the development of sensitivity in *P. n. oleracea* may be more similar to that in *P. rapae*. This indigenous North American species will often lay eggs on introduced crucifers that are not suitable for larval growth and development, and deterrents are likely to be involved in restriction of larval feeding. One such plant is garlic mustard, *Alliaria petiolata*, which has become extremely invasive in eastern North America, largely because of a lack of herbivory. Feeding by *P. n. oleracea* is blocked primarily by the presence of two compounds. One of these (compound 1) inhibits feeding by neonates, whereas the other (compound 2) deters feeding by later instars (Renwick et al., unpublished results). Recent experiments have examined the effect of diet on larval responses to these compounds, and two distinct mechanisms have emerged. The responses of larvae that were reared on either wheat germ diet or cabbage plants were measured at each larval stage. For the cabbage-reared larvae, a steady decline in sensitivity to compound 1 was observed as the larvae progressed from first to third instars. On the other hand, sensitivity to compound 2 increased steadily to reach a maximum in fourth and fifth instars. Larvae that fed on a wheat germ diet followed the same pattern of responses to compound 1, but never became sensitive to compound 2. From these results, along with behavioral observations, we concluded that two distinct mechanisms are involved in blocking feeding by the larvae at different stages. Compound 1 appears to be a postingestive feeding inhibitor, whereas compound 2 is a feeding deterrent that is perceived by taste. This conclusion would suggest, therefore, that dietary experience affects the ability of the insects to taste or respond to deterrents, but has no effect on postingestive inhibitory responses. This may mean that rearing larvae on different diets can be used to determine the mechanism involved in rejection of certain foods. From an ecological point of view, this also means that discrimination between potential host plants on the basis of taste may depend on the previous diet of the insect.

VARIATION IN PLANT CHEMISTRY

The compounds in *A. petiolata* responsible for blocking feeding by *P. n oleracea* have now been identified. Compound 1, alliarinoside, is a unique cyanoglycoside (Haribal et al., unpublished data) and compound 2 is a flavone glycoside (Haribal and Renwick, 1998). These compounds can be readily detected in aqueous extracts of foliage after preliminary solvent partitioning and chromatography, and analyses of samples from different sources have revealed considerable quantitative variability. Field observations have indicated that some populations of

P. n. oleracea can survive on isolated populations of *A. petiolata* (Courant et al., 1994). A detailed study of variation in plant chemistry throughout the year has now been conducted for different populations of *A. petiolata* in New York State. The analyses revealed substantial seasonal as well as population variation in the content of these active compounds (Haribal and Renwick, unpublished data). Such variation may well be sufficient to account for observed differences in growth and survival of larvae on the plants. A general dip in concentrations of both compounds 1 and 2 from June to July might suggest that a window of opportunity exists for *P. n. olearacea* to adapt to the variable resistance of garlic mustard (Haribal and Renwick, unpublished data).

CONCLUSIONS

The importance of plant chemistry in the evolution and maintenance of specialist insect–plant associations has been well documented in the past. Here we have focused on the role of plant chemistry in providing information about a plant and the ability of the insect to respond to this information. Chemical messages that signal suitability of a plant are perceived by the insect through a variety of chemoreceptors. For lepidopterous larvae, critical information is most often obtained by the use of taste receptors, although limited ingestion may also be needed for confirmation of suitability. Specialization usually depends on recognition of specific chemicals or classes of chemicals that stimulate feeding. At the same time, negative information allows the insect to avoid plants that may be toxic or unsuitable for continued growth and development.

We now know that variation in plant chemistry may provide opportunities for an insect to feed on plants that are usually considered to be unsuitable. Such variation may be genetically controlled, or it may result from seasonal, climatic, or other environmental factors. Similarly, differences in responses of insects to plant chemicals may be genetically or environmentally based. However, it is now clear that changes in sensory physiology may also play an important role. Diet-dependent sensitivity of the insect to negative signals may affect its ability to avoid plants that are likely to be toxic. The response of larvae to genus-specific feeding stimulants can play a key role in host selection by specialists, and we have now shown that some species become dependent on such stimulants for host recognition and continued feeding. This dependence may be manifested as an innate craving, or it may develop as a result of experience, like a type of addiction. Furthermore, experiments with *M. sexta* indicate that the development of dependence involves changes in the peripheral receptors that are responsible for tasting plant compounds at the leaf surface.

The observed variation in both plant chemistry and insect responses to specific chemicals might suggest that adaptation of a native insect to an introduced plant

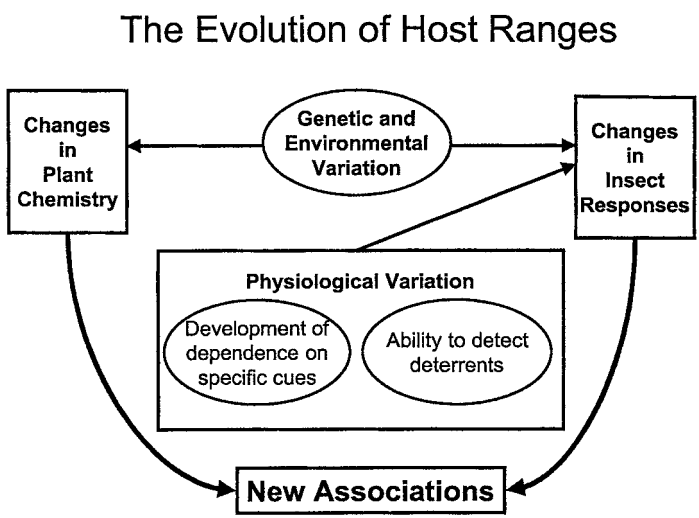


FIG. 5. The possible role of physiological variation, based on dietary experience, on the evolution of new insect–plant associations.

could occur relatively rapidly. Results of our research support the idea that the evolution of host ranges involves physiological as well as genetic and environmental factors (Figure 5). Plasticity in perception of deterrents and the development of dependence on specific chemicals undoubtedly play important roles. Future research in this area will benefit from collaborative efforts that combine experimental input from studies in sensory physiology, behavior, and phytochemistry to provide an integrated view of the processes involved.

Acknowledgments—I thank Dr. Meena Haribal and Matthew Martin-Rehrmann for help with the figures and for critical review of the manuscript . This work was supported in part by USDA CSREES grant 97-35302-4225

REFERENCES

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York.

BROWER, L. P. 1984. Chemical defence in Butterflies. *Symp. R. Entomol. Soc. London* 11:109–134.

CHEW, F. S., and RENWICK, J. A. A. 1995. Host-plant choice in *Pieris* butterflies, p. 214–238, *in* R.T. Cardé and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman and Hall, New York.

COURANT, A. V., HOLBROOK, A. E., VAN DER REIJDEN, E. D., and CHEW, F. S. 1994. Native Pierine butterfly (Pieridae) adapting to naturalized crucifer? *J. Lepid. Soc.* 48:168–170.

DAVID, W. A. L., and GARDINER, B. O. C. 1966. Mustard oil glucosides as feeding stimulants for *Pieris brassicae* larvae in a semi-synthetic diet. *Entomol. Exp. Appl.* 9:247–255.

DEL CAMPO, M. L. 1999. Behavioral, phytochemical, and developmental aspects of induction of host specificity in the tobacco hornworm to Solanaceae. Doctoral dissertation. Cornell University, Ithaca, New York.

DEL CAMPO, M. L., and RENWICK, J. A. A. 1999. Dependence on host constituents controlling food acceptance by *Manduca sexta* larvae. *Entomol. Exp. Appl.* 93:209–215.

DEL CAMPO, M. L., and RENWICK, J. A. A. 2000. Induction of host specificity in larvae of *Manduca sexta*: Chemical dependence controlling host recognition and development rate. *Chemoecology* 10:115–121.

DEL CAMPO, M. L., SCHROEDER, F. C., MILES, C. I., MÜLLER, C., BOOKER, R., and RENWICK, J. A. 2001. A host plant compound controls host recognition by the tobacco hornworm: a peripheral neural mechanism for specialization. *Nature* (in press).

DU, Y.-J., VAN LOON, J. J. A ., and RENWICK, J. A. A. 1995. Contact chemoreception of oviposition-stimulating glucosinolates and oviposition-deterrent cardenolides in two subspecies of *Pieris napi*. *Physiol. Entomol.* 20:164–174.

FELTWELL, J. 1982. The Large White Butterfly: The Biology, Biochemistry, and Physiology of *Pieris brassicae* (Linnaeus). Dr. W. Junk, Publishers, The Hague.

HARIBAL, M., and RENWICK, J. A. A. 1998. Isovitexin 6″-*O*-β-D-Glucopyranoside: A feeding deterrent to *Pieris napi oleracaea* from *Alliaria petiolata*. *Phytochemistry* 47:1237–1240.

HARIBAL, M., YANG, Z., ATTYGALLE, A. B., RENWICK, J. A. A., and MEINWALD, J. 2001. A cyanoallyl glucoside from *Alliaria petiolata* as a feeding deterrent for larvae of *Pieris napi oleracea*. *J. Nat. Prod.* 64:440–443.

HUANG, X. P., and RENWICK, J. A. A. 1994. Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi oleracea*. *J. Chem. Ecol.* 20:1025–1037.

HUANG, X. P., and RENWICK, J. A. A. 1995a. Chemical and experiential basis for rejection of *Tropaeolum majus* by *Pieris rapae* larvae. *J. Chem. Ecol.* 21:1601–1617.

HUANG, X. P., and RENWICK, J. A. A. 1995b. Cross habituation to feeding deterrents and acceptance of a marginal host plant by *Pieris rapae* larvae. *Entomol. Exp. Appl.* 76:295–302.

HUANG, X. P., and RENWICK, J. A. A. 1997. Feeding deterrents and sensitivity suppressors for *Pieris rapae* larvae in wheat germ diet. *J. Chem. Ecol.* 23:51–70.

HUANG, X. P., and RENWICK, J. A. A., and SACHDEV-GUPTA, K. 1993. Oviposition stimulants and deterrents regulating differential acceptance of *Iberis amara* by *Pieris rapae* and *P. napi oleracaea*. *J. Chem. Ecol.* 19:1645–1663.

HUGENTOBLER, U., and RENWICK, J. A. A. 1995. Effects of plant nutrition on the balance of insect relevant cardenolides and glucosinolates in *Erysimum cheiranthoides*. *Oecologia* 102:95–101.

HUGHES, P. R. 1974. Myrcene: A precursor of pheromones in *Ips* beetles. *J. Insect Physiol.* 20:1271–1275.

JOHANSSON, A. S. 1951. The food plant preference of the larvae of *Pieris brassicae* L. (Lepid., Pieridae). *Norsk Entomol. Tidsskr.* 8:187–195.

MA, W. C. 1972. Dynamics of feeding responses in *Pieris brassicae* Linn., as a function of chemosensory input: A behavioral, ultrastructural and electrophysiological study. *Meded. Landbouwhogh-esch. Wageningen* 72(11):162 pp.

MILLER, J. R., and STRICKLER, K. L. 1984. Finding and accepting host plants, pp. 127–155, *in* W. Bell and R. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

RENWICK, J. A. A. 1996. Diversity and dynamics of crucifer defenses against adults and larvae of cabbage butterflies, pp. 57–79, *in* J.T. Romeo, J.A. Saunders and P. Barbosa (eds.). Recent Advances in Phytochemistry 30. Phytochemiscal Diversity and Redundancy in Ecological interactions. Plenum Press, New York.

RENWICK, J. A. A., and HUANG, X. P. 1994. Interacting chemical stimuli mediating oviposition by Lepidoptera, pp. 79–94, *in* T.N. Ananthakrishnan (ed.). Functional Dynamics of Phytophagous Insects. Oxford & IBH Publishing, New Delhi.

RENWICK, J. A. A., and HUANG, X. P. 1995. Rejection of host plant by larvae of cabbage butterfly: Diet-dependent sensitivity to an antifeedant. *J. Chem. Ecol.* 21:465–475.

RENWICK, J. A. A., and LOPEZ, K. 1999. Experience-based food consumption by larvae of *Pieris rapae*: Addiction to glucosinolates? *Entomol. Exp. Appl.* 91:51–58.

RENWICK, J. A. A., RADKE, C. D., SACHDEV-GUPTA, K., and STÄDLER, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3:33–38.

ROWELL-RAHIER, M., and PASTEELS, J. M. 1993. Third trophic level influences of plant allelochemicals, pp. 243–277, *in* G.A. Rosenthal and M.R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites II. Ecological and Evolutionary Processes. Academic Press, San Diego.

SACHDEV-GUPTA, K., RENWICK, J. A. A., and RADKE, C. D. 1990. Isolation and identification of oviposition deterrents to cabbage butterfly, *Pieris rapae*, from *Erysimum cheiranthoides. J. Chem. Ecol.* 16:1059–1067.

SCHOONHOVEN, L. M. 1987. What makes a caterpillar eat? The sensory code underlying feeding behavior, pp. 69–97, *in* R.F. Chapman, E.A. Bernays and J.G. Stoffolano (eds.). Advances in Chemoreception and Behavior. Springer-Verlag, New York.

SCHOONHOVEN, L. M., JERMY, T., and VAN LOON, J. J. A. 1998. Insect–Plant Biology. From Physiology to Evolution. Chapman & Hall, London.

STÄDLER, E., and HANSON, F. E. 1978. Food discrimination and induction of preference for artificial diets in the tobacco hornworm, *Manduca sexta. Physiol. Entomol.* 3:121–133.

STÄDLER, E., RENWICK, J. A. A., RADKE, C. D., and SACHDEV-GUPTA, K. 1995. Tarsal contact chemoreceptor response to glucosinolates and cardenolides mediating oviposition in *Pieris rapae. Physiol. Entomol.* 20:175–187.

SZENTESI, A., and JERMY, T. 1989. The role of experience in host plant choice by phytophagous insects, pp. 39–74, *in* E.A. Bernays (ed.). Insect–Plant Interactions, Vol. II. CRC Press, Boca Raton, Florida.

THORSTEINSON, A. J. 1953. The chemotactic responses that determine host specificity in an oligophagous insect [*Plutella maculipennis*. (Curt.) Lepidoptera]. *Can. J. Zool.* 31:53–72.

VAN LOON, J. J. A., and SCHOONHOVEN, L. M. 1999. Specialist deterrent chemoreceptors enable *Pieris* caterpillars to discriminate between chemically different deterrents. *Entomol. Exp. Appl.* 91:29–35.

VERSCHAFFELT, E. 1910. The cause determining the selection of food in some herbivorous insects. *Proc. K. Ned. Akad. Wet.* 13:536–542.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

DOES EXCRETION OF SECONDARY METABOLITES ALWAYS INVOLVE A MEASURABLE METABOLIC COST? FATE OF PLANT ANTIFEEDANT SALICIN IN COMMON BRUSHTAIL POSSUM, *Trichosurus vulpecula*

S. McLEAN,[1] G. J. PASS,[1] W. J. FOLEY,[2,*] S. BRANDON,[1] and N. W. DAVIES[3]

[1] *School of Pharmacy and*
[3]*Central Science Laboratory*
University of Tasmania
Hobart Tas 7001, Australia

[2]*Division of Botany & Zoology*
Australian National University
Canberra 0200, Australia

(Received October 26, 2000; accepted February 8, 2001)

Abstract—Salicin was administered orally to six brushtail possums by incorporation in food for six days at three dose levels (0.05, 0.5, and 1.5% wet weight), giving mean ± SD daily intakes of 0.31 ± 0.09, 2.76 ± 0.75, and 6.04 ± 1.12 mmol salicin. Metabolites were identified by mass spectrometry and assayed by HPLC. Salicyl alcohol glucuronide accounted for 56–64% of urinary metabolites over the three doses, salicyluric acid 15–26%, salicin 10–18%, and there were smaller amounts of free (2–4%) and conjugated (0–6%) salicylic acid. β,2-Dihydroxyphenylpropionic acid was a minor metabolite. The hydrolysis of dietary salicin enabled reconjugation of its aglycone, salicyl alcohol, with a more polar sugar, glucuronic acid, thus enhancing its renal excretion and resulting in little net loss of substrates for conjugation and a low measurable metabolic cost of excretion.

Key Words—Salicin, metabolism, glucose conjugate, plant secondary metabolite, common brushtail possum, detoxication, costs.

INTRODUCTION

Although there is clear evidence that some plant secondary metabolites (PSMs) influence foraging of herbivores (Foley et al., 1999), the mechanisms by which

*To whom correspondence should be addressed. e-mail: william.foley@anu.edu.au

0098-0331/01/0600-1077$19.50/0

these effects are exerted remain unclear. Some compounds exert clear antifeedant effects (Lawler et al., 1998) or have a demonstrable impact on the metabolism of the animal. Yet most mammalian herbivores ingest large concentrations of many PSMs that do not appear to have major effects on their choices of foods (Palo and Robbins, 1991). Most syntheses of mammal–plant interactions place some importance on the cost of neutralizing and excreting any PSMs that are ingested (Freeland and Janzen, 1974; Foley and McArthur, 1994; Foley et al., 1999). The difficulty with assigning costs to the neutralization and excretion of PSMs is that only rarely are the pathways of metabolism known with any certainty (Freeland, 1991). Available information is generally restricted to laboratory species, and it is hard to place these studies in an ecological context. Costs that might be important in an ecological context could include the cost of maintaining enzyme systems, the cost of conjugates for excretion and the opportunity costs of lost foraging time because of the pharmacokinetic constraints of neutralizing and disposing of metabolites (Foley et al., 1999).

Although all these avenues could potentially be important, only the cost of excreted conjugates has been quantified. For example, Cork (1981) showed that glucuronic acid excretion comprised up to 25% of the fasting glucose entry rate of koalas (*Phascolarctos cinereus*). Lowry et al. (1993) showed that sheep fed diets rich in organic acids excreted 17% of their digestible N intake as benzoyl glycine (hippuric acid). Nonetheless, we think that some compounds, in particular phenolic glycosides, could be excreted with very little cost either because they are excreted unconjugated (Scheline, 1991) or because the glycoside is used as a source of metabolizable energy to compensate for the excretion of a carbohydrate-based conjugate such as a glucuronide.

We evaluated this possibility by studying the metabolism of salicin in common brushtail possums. Salicin [salicyl alcohol glucoside; 2-(hydroxymethyl)-phenyl-β-D-glucopyranoside] is a bitter-tasting phenolic glycoside found in the bark and leaves of various species of *Salix* and *Populus* (Trease and Evans, 1972). Earlier studies had implicated salicin as a cause of selective feeding on *Salix* and *Populus* by common brushtail possums (Markham, 1970; Edwards, 1978) and mountain hares (*Lepus timidus*) (Tahvanainen et al., 1985), but little was known of how such an antifeedant effect could occur. Pass and Foley (2000) examined the effect of isolated salicin on feeding in common brushtails but could find no evidence of postingestive effects and attributed the antifeedant effects of high concentrations of salicin to preingestive effects such as taste. Therefore, we undertook this study of the metabolic disposition of salicin to provide further evidence of the nature of any postingestive effects.

In the only detailed study of the metabolism of salicin, Fotsch et al. (1989a) found that rats excreted oral doses of salicin mostly as free salicylic acid and unchanged salicin. However, after a subcutaneous dose, salicin was excreted virtually unchanged (Fotsch and Pfeifer 1989). In vitro experiments showed that salicin was

hydrolyzed by bacteria in the cecum and colon of rats, indicating that salicin is extensively hydrolyzed in the rat gastrointestinal tract.

METHODS AND MATERIALS

Materials. Salicin (min. 99% purity) was purchased from Sigma. All other chemicals were of good commercial quality. Reference metabolites (salicyl alcohol, salicylic acid, salicyluric acid) were chromatographically pure. Methanol and acetonitrile were HPLC grade (Waters Associates, Sydney, Australia) and acetone was an analytical reagent grade (May & Baker).

Animals and Urine Collection. Animal work was carried out at James Cook University, Townsville, and was approved by that university's ethics committee (Pass and Foley, 2000). Six common brushtail possums were trapped in the wild and kept in an air-conditioned animal house maintained at $22 \pm 2^{\circ}$C and a 12-hr light–dark cycle. The animals were adult males weighing 1.97–2.50 kg (mean = 2.27 kg). They had been living in a mangrove forest and had not been previously exposed to willows or poplars. The animals were fed on a diet of fruit and cereals (Pass and Foley, 2000). In feeding experiments, salicin was added to the dry ingredients of this diet and mixed to give a final uniform distribution of salicin 0.05, 0.5, or 1.5% wet weight of feed. Animals were housed individually and fed each of the three salicin diets for eight days, using a balanced Latin-square design. During each eight-day diet period, 24-hr urine samples were taken on day 6 for metabolite analyses. Urine was collected into bottles immersed in solid carbon dioxide and stored frozen until analyzed. Food intake was measured, by weight, on day 5.

Identification of Metabolites. Most metabolites were identified by mass spectrometry (MS) after separation by gas chromatography (GC). After hydrolysis of conjugates with β-glucuronidase/arylsulfatase (see below), urine samples were acidified to pH 1 with 5 M HCl and extracted three times with ethyl acetate. An aliquot of the extract was then placed into an ice bath in a fume hood, ethereal diazomethane added, and the reaction allowed to stand for 30 min. Excess diazomethane was removed with a gentle stream of nitrogen. The methylated metabolites were dissolved in ethyl acetate and analyzed by GC. The instrument was a Hewlett-Packard 5890 gas chromatograph with 5970B mass-selective detector using HP 59970A Chemstation software (Hewlett-Packard, Melbourne, Australia). GC conditions were: Hewlett-Packard HP1 capillary column (0.52 μm methylpolysiloxane), 25 m $\times$ 0.32 mm ID; oven 100–190°C at 5°C/min, then 190–200°C at 20°C/min; carrier He at 12 psi; injector 250°C; detector 300°C.

Salicyl alcohol glucuronide was isolated by liquid chromatography to enable its identification by high-resolution MS. Salicin and salicyl alcohol glucuronide were separated from urine on a column of Amberlite XAD-4 resin (Serva) using

a method adapted from that of White and Schwartz (1980). The resin (10 g) was washed successively with methanol, water, acetone, acetone–water, methanol, and water. An aliquot of urine (50 μl) was diluted in 75 ml water and passed through the column at 3 ml/min. The column was then washed with 80 ml water followed by successive 30 ml volumes of acetone–water (1:1). Eluted fractions were concentrated to approximately 1–2 ml by rotary evaporation at 40°C and analyzed by HPLC. Salicyl glucuronide eluted with the aqueous wash and gave no HPLC peak for salicin or salicyl alcohol, but after enzyme hydrolysis, produced salicyl alcohol. Salicin eluted with the acetone–water fractions, and enzyme hydrolysis gave salicyl alcohol. The structures of urinary salicyl glucuronide and salicin were confirmed by solid probe mass spectrometry. The instrument (Kratos Concept ISQ, Manchester, UK) used a Cs ion gun at 10 kV for FAB mass spectrometry in a glycerol matrix.

Structures of conjugates were confirmed by liquid chromatography–mass spectrometry (LC-MS) with a Waters Alliance HPLC and Finnegan LCQ mass spectrometer using LCQ Navigator software. Diluted urine samples were chromatographed on a reverse-phase C-18 Nova-Pak column (3.9 mm × 150 mm; Waters Associates) with a mobile phase starting with 90% water–10% methanol containing 2% acetic acid in each and programmed to 100% methanol after 40 min at 0.8 ml/min. Peaks were detected by negative electrospray ionization (ESI). MS-MS analysis confirmed the origin of daughter ions. Retention times were: salicyl alcohol glucuronide, 4.89 min; salicylic acid glucuronide, 6.49 min; and salicyluric acid, 9.93 min. Salicin does not produce a negatively charged ion under the conditions used and was not detected.

Quantification of Metabolites by HPLC. A Varian 9010 solvent delivery system with Rheodyne injector model 7161 was connected to a Varian 9050 UV-VIS absorbance detector and Star workstation (Varian Instruments, Melbourne, Australia). The column was a reverse-phase C-18 Nova-Pak (3.9 mm × 150 mm; Waters Associates) and solvents were pumped at 0.9 ml/min. The injection volume was 10 μl.

Salicin and salicyl alcohol were analyzed with methanol–water (25:75) at a wavelength of 213 nm (HPLC system 1). Calibration curves were prepared using 400 μl predose urine, to which were added known amounts of salicin and salicyl alcohol in methanol (0–500 μl), internal standard (1.32 mg benzyl alcohol in 1 ml 25% aqueous methanol), and water to 5 ml. Elution times were: salicin, 2.34 min; salicyl alcohol, 3.59 min; and benzyl alcohol, 6.49 min. Calibration curves were constructed using the concentration of the final solution and peak areas and were linear for salicin (0–1.76 μmole/ml; $r^2 = 0.998$) and salicyl alcohol (0–4.12 μmol/ml; $r^2 = 1.000$). Urinary concentrations were calculated using dilution factors to account for the varying volumes of urinary aliquots (200–500 μl) that were required to accommodate the large range of metabolite concentrations found at the different doses.

Salicylic acid and salicyluric acid were analyzed with acetonitrile (27%)–acetic acid (2%) in water (73%) at a wavelength of 313 nm (HPLC system 2). Calibration curves were prepared as before using predose urine spiked with standards. The internal standard was *o*-anisic acid (600 μg in 100 μl 25% aqueous methanol), urine volumes were 100–900 μl, and the final volume was made to 1 ml with water. Elution times were: salicyluric acid, 2.30 min; *o*-anisic acid, 2.85 min; and salicylic acid, 4.29 min. Calibration curves were linear for salicylic acid (0–3.62 μmoles/ml; $r^2 = 1.000$) and salicyluric acid (0–2.56 μmoles/ml; $r^2 = 0.999$), using the final concentration as above.

Hydrolysis of Conjugates. To analyze for conjugates of salicyl alcohol, a 500-μl aliquot of urine was mixed with 250 μl water, 200 μl acetate buffer (1.1 M, pH 5.2), and 50 μl of *Helix pomatia* extract (5.5 units β-glucuronidase and 2.6 units arylsulfatase in 1 ml) (Boehringer Mannheim, Germany). After overnight incubation at 37°C, an aliquot was transferred to a vial, 500 μl of internal standard solution (benzyl alcohol, as above) added, and the volume made to 2.5 ml with water before analysis by HPLC system 1. To analyze for conjugates of salicylic acid, 200–600 μl of urine hydrolysate was transferred to a vial with 100 μl internal standard solution (*o*-anisic acid, as above) and made to 1.0 ml with water before analysis by HPLC system 2.

RESULTS

Identification of Metabolites. Metabolites were identified by their chromatographic behavior and comparison with standards. Their structures were confirmed by mass spectrometry. Initial analysis of unhydrolyzed urine using HPLC system 1 showed a small peak for salicin, but no salicyl alcohol. Acid hydrolysis (pH 1, boiling water bath, 30 min) converted the salicin into an equivalent amount of salicyl alcohol. However, after enzymatic hydrolysis, the salicin disappeared, and there was a very large amount of salicyl alcohol, more than could be accounted for by the hydrolysis of salicin alone. This indicated that there was another conjugate of salicyl alcohol that was not detected by HPLC analysis. A glucuronide conjugate subsequently was isolated from urine using a column of XAD-4 resin (see Methods and Materials for details). FAB MS showed a protonated molecular ion (MH^+) at 301.0 and several adducts with Na^+ and K^+ whose structures were supported by high-resolution data, confirming the formula $C_{13}H_{16}O_8$ that corresponds to salicyl glucuronide (Table 1). Salicin, which was found in later fractions eluting from the XAD-4 column, did not give a molecular ion, but high-resolution analysis of ions with masses corresponding to the mono- and di- Na^+ adducts agreed closely with expected values, confirming the formula of $C_{13}H_{18}O_7$.

Salicyl alcohol, salicylic acid, and salicyluric acid were identified in urine by GC-MS analysis of their methylated derivatives (Table 1). Some urine samples

TABLE 1. MASS SPECTRAL DATA OF METABOLITES FOUND IN BRUSHTAIL POSSUM URINE.

Metabolite	Derivative[a]	R_t (min)[b]	Significant EI ions [m/z (% *abundance*)]				
Salicyl alcohol		6.72	124 (M^+) (27)	106 (42)	78 (100)		
Salicylic acid	Methyl	5.75	152 (M^+) (*42*)	120 (*100*)	92 (*87*)		
Salicyluric acid	Methyl	13.27	209 (M^+) (*16*)	121 (*100*)	92 (*24*)		
β,2-Dihydoxy-phenyl propionic acid	Methyl	12.64	196 (M^+) (*66*)	178 (*3*)	123 (*100*)	121 (*34*)	95 (*90*)
		Significant FAB ions (m/z)[c]					
Salicin		309.09541 $[M + Na]^+$		331.1 $[M + 2\,Na - H]^+$			
Salicyl alcohol glucuronide	301.0 $[M + H]^+$	323.07365 $[M + Na]^+$		339.04778 $[M + K]^+$			
		345.0 $[M - H + 2\,Na]^+$		361.0 $[M - H + Na + K]^+$			

[a] Methyl ester.
[b] Retention time in GC-MS analysis (see Methods and Materials for chromatography conditions).
[c] FAB MS using probe insertion.

also showed traces of gentisic acid by GC-MS. All urine samples contained small amounts of β,2-dihydroxyphenylpropionic acid, which gave a mass spectrum similar to that of β-hydroxyphenylpropionic acid but with ions 16 amu heavier. β-Hydroxyphenylpropionic acid is a urinary metabolite of benzoic acid (Marsh et al., 1982; Awaluddin and McLean, 1985) and β,2-dihydroxyphenylpropionic acid is the analogous metabolite of salicylic acid, containing one extra oxygen atom. β,2-Dihydroxyphenylpropionic acid was not quantified because of its small amount and interference from the hippuric acid peak.

Conjugates were confirmed by LC-MS. Salicyl alcohol glucuronide formed a dimer at m/z 599 (a feature of negative ESI) and showed a deprotonated molecular ion $[M\text{-}H]^-$ at 299. MS-MS showed significant daughter ions at *m/z* 175 and 113 (characteristic of glucuronic acid) and 123 (the anion from salicyl alcohol). Salicylic acid glucuronide showed an $[M\text{-}H]^-$ ion at *m/z* 313, glucuronic acid ions at *m/z* 175 and 113 and a salicylic acid anion at *m/z* 137. Salicyluric acid gave ions at *m/z* 194 $[M\text{-}H]^-$ and 150 $[M\text{-}H\text{-}CO_2]^-$.

Quantification of Metabolites. Predose urine gave no interfering HPLC peaks. The 24-hr recovery of urinary metabolites of salicin for the three consumption levels of salicin is shown in Table 2. The dose of salicin was calculated from the food intake on day 5 and the percentage salicin in the food. Only two thirds of the estimated dose of salicin was recovered in urine as salicin and its metabolites, except at the lowest intake of salicin when recovery was complete. Excretion of individual metabolites is expressed as the percentage of total recovered metabolites in order to enable direct comparisons between animals and doses. The proportion found as unchanged salicin varied from 10% to 18% of total recovered metabolites, while the major metabolites were salicyl alcohol glucuronide (56–64%) and salicyluric acid (15–26%). There were only traces of free salicyl alcohol and small amounts of free and conjugated salicylic acid. There was a significantly greater proportion of salicin excreted as salicyluric acid at the lowest consumption rate compared to the higher doses, but no other significant dose-related differences were found.

DISCUSSION

The major urinary metabolite of salicin in the common brushtail possum was the glucuronide of its aglycone, salicyl alcohol, while very little unchanged salicin was excreted. Thus after an oral dose the glucoside of salicyl alcohol is excreted largely as its glucuronide. The metabolic pathways are shown in Figure 1.

This suggests that there is little measurable metabolic cost in the excretion of metabolites of salicin because, although the compound has been transformed, there is little net loss of substrate in the conjugation reactions. We recognize that the enzyme systems and intermediate reactions leading to the hydrolysis of salicin and the formation of the glucuronide require energy to initiate and maintain. Similarly,

TABLE 2. URINARY METABOLITES OF SALICIN EXCRETED IN 24 HOURS AFTER THREE DIFFERENT DIETARY CONCENTRATIONS

Salicin in diet (%)	Salicin intake (μmol)[a]	Fraction recovered in urine (mean ± SD)	Urinary metabolites as % total recovered (*mean* ± *SD*)					
			Salicin	Salicyl alcohol glucuronide	Salicyluric acid	Free salicyl alcohol	Free salicylic acid	Conjugated salicylic acid
1.5	6037 ± 1115	0.64 ± 0.13	9.9 ± 3.7	63.7 ± 7.0	16.7 ± 5.4	0.2 ± 0.5	3.5 ± 2.5	5.9 ± 3.0
0.5	2756 ± 745	0.68 ± 0.25	17.8 ± 10.9	63.0 ± 9.6	14.8 ± 2.4	0.9 ± 1.4	3.5 ± 2.4	0.1 ± 0.6
0.05	303 ± 85	1.02 ± 0.47	16.5 ± 12.3	56.3 ± 19.1	25.8 ± 10.5	0	1.9 ± 0.9	0
P[b]		0.129	0.077	0.43	0.008			

[a] Calculated from the food consumed during the 24 hr prior to urine collection. ($N = 6$).
[b] Significance of differences between doses, repeated measurements ANOVA.

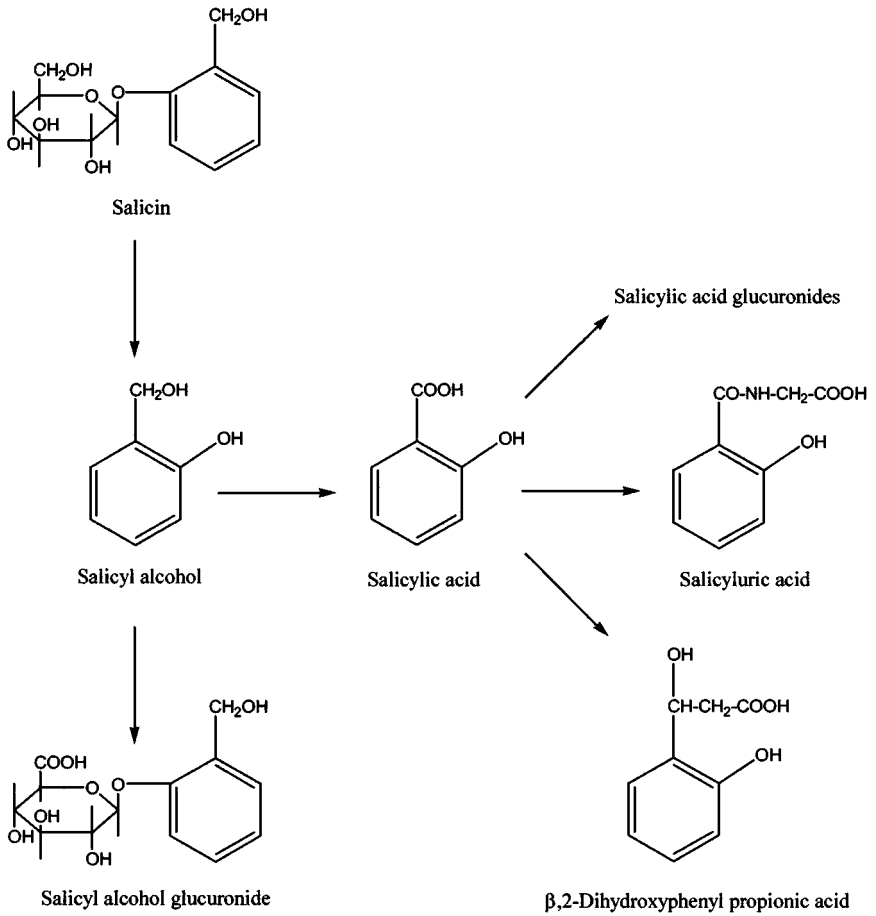


FIG. 1. Metabolic pathways of salicin in the brushtail possum.

there may be unmeasured cell damage or other metabolic disruptions that could constitute a cost, but this seems unlikely in view of the findings of Pass and Foley (2000) that ingestion of salicin did not cause measurable postingestive effects on possums. In an ecological context, the net result is that the phenolic moeity is ingested with a simple sugar derivative and excreted with a simple sugar derivative and the net cost is minimal.

The only other study of the metabolic fate of salicin used rats that were given repeated oral doses (1000 μmol/kg daily) (Fotsch et al., 1989a). Only about half (0.52) of the dose was recovered in urine, somewhat less than found in the present study after comparable doses (Table 2). The major urinary metabolites in the rat were free salicylic acid (57% of total urinary metabolites) and salicin (27%), with

smaller amounts of conjugated salicylic acid (10%), gentisic acid (4%), salicyluric acid (0.2%), and salicyl alcohol (0.2%). Apart from salicin, no urinary conjugates of salicyl alcohol were found. Thus, the metabolic fate of salicin appears to be quite different in the two species, with large amounts of salicyl alcohol glucuronide and salicyluric acid being excreted in the possum whereas salicylic acid was the major rat urinary metabolite. However, this comparison must be treated cautiously because other factors, such as diet, can influence the extent of metabolism (Mulder et al., 1990; Hutt and Caldwell, 1990).

The absorption and excretion of intact salicin may seem surprising, since it is well known that substances must be lipophilic to cross cell membranes, except where specific transport systems exist (Pratt, 1990). Salicin does not appear to interact with rabbit Na^+/glucose cotransporters (Lostao et al., 1994), whereas some other phenylglycosides (e.g., arbutin) are transported. However, Matsumoto et al. (1993) showed that salicin and other monosaccharides are able to permeate the cell membranes of human erythrocytes, although they do so more slowly than the more lipophilic aglycones. Whether this occurs by a paracellular pathway is not known.

After subcutaneous administration of salicin to rats, most of the dose was recovered unchanged in urine, indicating that the hydrolysis of oral doses occurs in the gut (Fotsch and Pfeifer, 1989). In vitro experiments by these authors showed that salicin was hydrolysed by rat caecum or colon contents, but not by liver homogenates.

A broad-specificity β-glucosidase has been found in guinea pig liver that is capable of hydrolyzing various plant glucosides including salicin (LaMarco and Glew, 1986; Gopalan et al., 1992). These authors postulated that the hepatic glucosidase may have evolved as a response of herbivores to the presence of toxic plant glucosides, such as the cyanogenic glycosides amygdalin and prunasin, and vicine, which can cause hemolytic anemia. Gopalan et al. (1992) showed that these three plant glucosides were able to cross segments of guinea pig jejunum in vitro without hydrolysis. Since lipid-soluble substances are reabsorbed from the renal tubules (Pratt, 1990), glucosides with sufficient hydrophobic character to be absorbed from the gut are also liable to be reabsorbed from the renal tubules, resulting in a low renal clearance. However, after hydrolysis, the aglycone can be conjugated with a more polar sugar, glucuronic acid, enabling more efficient renal excretion. Although cecal microorganisms can hydrolyze glucosides, any that are absorbed from the small intestine will escape microbial hydrolysis (unless secreted in the bile). The hepatic glucosidase would be in position to hydrolyze absorbed glucosides, enabling their reconjugation with glucuronic acid. Our data showing the hydrolysis of salicin and reconjugation of its aglycone to salicyl alcohol glucuronide are consistent with this proposed mechanism.

A simple oxidation reaction could convert salicin directly to the corresponding glucuronide, but there is no evidence that this reaction occurs. The glucuronic

acid in conjugates is formed by a specific enzymatic oxidation of UDP-glucose (Mulder et al., 1990), indicating that other glucosides would not be suitable substrates. It is also unlikely that salicyl alcohol is reconjugated with glucose, as β-glucoside formation is very uncommon in mammals, although an unusual α-glucoside formation has been reported in rat liver (Kamimura et al., 1992). When 1-naphthol-β-glucuronide was administered orally to mice, no glucoside was excreted, but administration of 1-naphthol-β-glucoside resulted in the excretion of both the glucoside and glucuronide (Chern and Dauterman, 1983).

Although little has been published on the fate of salicyl alcohol or salicin, there is a vast literature on the metabolism of salicylic acid, the oxidation product of salicyl alcohol (Scheline, 1991). There is considerable variability between and within species in the urinary metabolites of salicylic acid (Scheline, 1991). In the brushtail possum, only traces of free salicyl alcohol appeared in the urine, with small amounts of free and conjugated salicylic acid (presumably the glucuronide), while the major salicylate was the glycine conjugate, salicyluric acid (Table 2). There was relatively less salicyluric acid excreted after the higher doses of salicin, suggesting that the glycine conjugation pathway may be readily saturable in the brushtail possum, as in other species (Hutt and Caldwell, 1990). Other factors including diet and gut microorganisms also can be important determinants of glycine conjugation (Phipps et al., 1998).

This is the first report of the formation of β,2-dihydroxyphenylpropionic acid as a salicylate metabolite. It was presumably formed from the acyl-CoA of salicylic acid, in an analogous fashion to the postulated formation of β-hydroxyphenylpropionic acid from benzoic acid (Marsh et al., 1982). This may represent a general reaction of acyl-CoAs, as it has now been seen for benzoic acid in the horse and rabbit (Marsh et al., 1982), as well as in rats, seven marsupials (including the brushtail possum), and the echidna (a monotreme) (Awaluddin and McLean, 1985). Acyl-CoAs are intermediates in the formation of amino acid conjugates (Hutt and Caldwell, 1990), and the β-hydroxy metabolite has always been seen in animals that form acylglycine conjugates.

Although alcohols and phenols can form sulfate conjugates, there was no evidence of this occurring. Sulfate conjugates have not been reported for salicylic acid, and the brushtail possum is considered to have a low capacity for sulfatation (Baudinette et al., 1980).

Both Fotsch et al. (1989a) and we, in the present study, could recover only part of the salicin consumed. In the present study, some errors may be due to uncertainty in estimating the dose consumed and loss of some of the urine. Some metabolites may have been excreted in bile and eliminated in the feces. There is also the possibility of the formation of unknown metabolites. Chern and Dauterman (1983), using a ^{14}C label, found that oral administration of 1-naphthol-β-glucoside to mice resulted in the urinary excretion of a considerable amount (15–33% dose) of unidentifiable polar material, suggesting the formation of unknown metabolites.

This was not seen after administration of 1-naphthol or its glucuronide. As in the present study, they also found an inverse relationship between the dose of glucoside and both the fraction of dose recovered, and the proportion excreted unchanged in the urine.

Acknowledgments—We thank Mr Robert Gegg for assistance with maintenance of captive animals.

REFERENCES

AWALUDDIN, A. B., and MCLEAN, S. 1985. Conjugation of benzoic acid in marsupials. *Aust. J. Zool*. 33:693–698.

BAUDINETTE, R. V., WHELDRAKE, J. F., HEWITT, S., and HAWKE, D. 1980. Metabolism of [^{14}C] phenol by native Australian rodents and marsupials. *Aust. J. Zool*. 28:511–520.

CHERN, W. H., and DAUTERMAN, W. C. 1983. Studies on the metabolism and excretion of 1-naphthol, 1-naphthyl-β-D-glucuronide, and 1-naphthyl-β-D-glucoside in the mouse. *Toxicol. Appl. Pharmacol*. 67:303–309.

CORK, S. J. 1981. Digestion and metabolism in the koala *(Phascolarctos cinereus* Goldfuss): An arboreal folivore. PhD thesis. University of New South Wales, Australia.

EDWARDS, W. R. N. 1978. Effect of salicin content on the palatability of *Populus* foliage to opossum (*Trichosurus vulpecula*). *N. Z. J. Sci*. 21:103–106.

FOLEY, W. J., and MCARTHUR, C. 1994. The effects and costs of ingested allelochemicals in mammals: An ecological perspective, pp. 370–391, *in* D. J. Chivers and P. Langer (eds.). The Digestive System in Mammals: Food, Form and Function. Cambridge University Press, Cambridge, UK.

FOLEY, W. J., IASON, G., and MCARTHUR, C. 1999. Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores—how far have we come in 25 years? pp. 203–274, *in* H.-J. G. Jung and G. C. Fahey, Jr. (eds.). Nutritional Ecology of Herbivores; Vth International Symposium on the Nutrition of Herbivores. American Society of Animal Science, Savoy, IL.

FOTSCH, G., and PFEIFER, S. 1989. Die Biotransformation der Phenolglycoside Leiocarposid und Salicin-Beispiele für Besonderheiten von Absorption und Metabolismus glycosidischer Verbindungen, *Pharmazie* 44:710–712.

FOTSCH, G., PFEIFER, S., BARTOSZEK, M., FRANKE, P., and HILLER, K. 1989. Biotransformation der Phenolglycoside Leiocarposid und Salicin. *Pharmazie* 44:555–558.

FREELAND, W. J. 1991. Plant secondary metabolites: Biochemical coevolution with herbivores, pp. 61–68, *in* R. T. Palo and C.T. Robbins (eds.). Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:269–287.

GOPALAN, V., PASTUSZYN, A., GALEY, W. R., and GLEW, R. H. 1992. Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic β-glucosidase. *J. Biol. Chem.* 267:14027–14032.

HUTT, A. J., and CALDWELL, J. 1990. Amino acid conjugation, pp. 273–305, *in* G. J. Mulder (ed.). Conjugation Reactions in Drug Metabolism. Taylor and Francis, London.

KAMIMURA, H., OGATA, H., and TAKAHARA, H. 1992. α-Glucoside formation of xenobiotics by rat liver α-glucosidases. *Drug Metab. Dispos.* 20:309–315.

LAMARCO, K. L., and GLEW, R. H. 1986. Hydrolysis of a naturally occurring β-glucoside by a broad-specificity β-glucosidase from liver. *Biochem. J.* 237:469–476.

LAWLER, I. R., FOLEY W. J., ESCHLER B., PASS D. M., and HANDASYDE, K. 1998. Intraspecific variation in secondary metabolites determines food intake by folivorous marsupials. *Oecologia* 116:160–169.

LOSTAO, M. P., HIRAYAMA, B. A., LOO, D. D. F., and WRIGHT, E. M. 1994. Phenylglucosides and the Na^+/glucose co-transporter (SGLT1): Analysis of interactions. *J. Membr. Biol.* 142:161–170.

LOWRY, J. B., SUMPTER, E. A., MCSWEENEY, C. S., SCHLINK, A. C., and BOWDEN, B.1993. Phenolic acids in the fibre of some tropical grasses; effect on feed quality and their metabolism by sheep. *Aust. J. Agric. Res.* 44:1123–1133.

MARKHAM, K. R. 1970. A chemotaxonomic approach to the selection of opossum resistant willows and poplars for use in soil conservation. *N. Z. J. Sci.* 14:179–186.

MARSH, M. V., CALDWELL, J., HUTT, A. J., SMITH, R. L., HORNER, M. W., HOUGHTON, E., and MOSS, E. 1982. 3-Hydroxy- and 3-keto-3-phenylpropionic acids: novel metabolites of benzoic acid in horse urine. *Biochem. J.* 31:3225–3230.

MATSUMOTO, Y., OHSAKO, M., TAKADATE, A., and GOTO, S. 1993. Reduction of erythrocyte membrane permeability and protein binding of low-molecular-weight drugs following glucoside derivitization. *J. Pharm. Sci.* 82:399–403.

MULDER, G. J., COUGHTRIE, M. W. H., and BURCHELL, B. 1990. Glucuronidation, pp. 51–105, *in* G. J. Mulder (ed.). Conjugation Reactions in Drug Metabolism. Taylor and Francis, London.

PALO, R. T., and ROBBINS, C. T. 1991. Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

PASS, G. J., and FOLEY, W. J. 2000. Plant secondary metabolites as mammalian feeding deterrents: Separating the effects of the taste of salicin from its post-ingestive consequences in the common brushtail possum (*Trichosurus vulpecula*). *J. Comp. Physiol. B.* 170:185–192.

PHIPPS, A. N., STEWART, J., WRIGHT, B., and WILSON, I. D. 1998. Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between the diet, gut microflora and substrate specificity. *Xenobiotica* 28:527–537.

PRATT, W. B. 1990. The entry, distribution and elimination of drugs, pp. 201–296, *in* W. B. Pratt and P. Taylor (eds.). Principles of Drug Action. Churchill, New York.

SCHELINE, R. R. 1991. CRC Handbook of Mammalian Metabolism of Plant Compounds. CRC Press, Boca Raton, Florida.

TAHVANAINEN, J., HELLE, E., JULKUNEN-TIITTO, R., and LAVOLA, A. 1985. Phenolic compounds of willow bark as deterrents against feeding by mountain hare. *Oecologia* 65:319–323.

TREASE, G. E., and EVANS, W. C. 1972. Pharmacognosy. Balliere Tindall, London.

WHITE, J. D., and SCHWARTZ, D. P. 1980. Evaluation of Amberlite XAD-4 for recovery of glucuronides and sulfates from aqueous solution. *J. Chromatogr*. 196:303–308.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF *Arhopalus tristis* TO BURNT PINE AND OTHER STIMULI

D. M. SUCKLING,[1,*] A. R. GIBB,[1] J. M. DALY,[1] X. CHEN,[1] and E. G. BROCKERHOFF[2]

[1]*HortResearch*
PO Box 51, Lincoln, New Zealand
[2]*Forest Research*
PO Box 29237, Christchurch, New Zealand

(Received July 5, 2000; accepted February 3, 2001)

Abstract—The exotic longhorn beetle *Arhopalus tristis* is a pest of pines, particularly those damaged by fire, and a major export quarantine issue in New Zealand. Actinograph recordings of caged individuals showed that males and females were most active from dusk to midnight. Olfactometer experiments indicated that females moved upwind toward odors from burnt pine (80%, $N = 75$), compared to unburnt pine (20%). Oviposition choice tests showed that eggs were predominantly laid on burnt logs (79%, $N = 20$), compared to unburnt logs. Beetles were trapped by funnel traps baited with burnt (mean catch per trap 7.8) and unburnt (mean catch 4.1 per trap) pine bark from inside a screen cage (4×3 m), while unbaited traps had a mean catch 0.1 beetles ($N = 8$ replicates). The treatment of burnt pine bark with a 1:1:2 mixture of green leaf volatiles (*E*)-2-hexen-1-ol and (*E*)-2-hexenal) in mineral oil as a repellent reduced trap catch by fivefold in a similar experiment (mean catches of 1.2 beetles per trap to burnt pine bark plus repellent treatment and 6.2 beetles per trap to burnt pine bark alone). The treatment of burnt pine bark with this solution also reduced oviposition by 98.5% (mean eggs per log of 11.1 on burnt pine and 0.3 on burnt pine plus repellent), indicating that oviposition cues have the potential to be significantly disrupted. The electrophysiological responses of adult beetles were recorded to a range of odorants. Normalized responses to monoterpenes known to occur in *Pinus radiata* ranged from about 20 to about 150, with α-terpineol giving the greatest responses in both sexes. Green leaf volatiles also gave high responses. The potential exists to improve the management of this insect using chemical cues in various ways.

Key Words—Attractant, *Pinus radiata*, *Arhopalus tristis*, fire, smoke, trap, Cerambycidae, oviposition, bark, repellent.

*To whom correspondence should be addressed.

The burnt pine longhorn beetle, *Arhopalus tristis* (F.) (Coleoptera: Cerambycidae), is native to Europe, Asia, and North Africa (Villiers, 1978). This species was accidentally introduced to New Zealand, probably in the 1950s, and it has since spread throughout the country (Hosking and Bain, 1977). Most references from New Zealand refer to *A. ferus* (Mulsant), but this is an older synonym of *A. tristis*.

Arhopalus tristis has been recorded to attack logs, stumps, and standing dead or damaged trees of pine species (*Pinus* spp.) and, less commonly, Norway spruce [*Picea abies* (L.) Karst] (Freude et al., 1966; Brockerhoff and Hosking, 2001). However, *A. tristis* is best known for its spectacular attacks of scorched trees within a few days to weeks of a forest fire (Hosking and Bain, 1977; Bradbury, 1998), a habit that is shared with other Palaearctic and Nearctic species of *Arhopalus* (e.g., Eaton, 1959; Wickman, 1964; Ehnström et al., 1995). The life cycle of *A. tristis* and the damage caused in New Zealand were described by Hosking and Bain (1977), Brockerhoff and Hosking (2001), and Bradbury (1998). Eggs are usually laid in bark crevices and the larvae feed first in the phloem area but later tunnel up to 10 cm deep into the wood. The risk of timber damage resulting from tunneling by *A. tristis* greatly reduces the salvage time for fire-damaged trees. In addition, *A. tristis* is a major vector of sapstain fungi such as *Ophiostoma* sp., *Sporothrix* sp., and *Graphium* sp. (Bradbury, 1998; Suckling et al., 1999), which further reduce the value of salvaged wood. Aside from this direct damage, *A. tristis* is frequently reported as sheltering in sawn timber at sawmills and in ports. Sawn timber destined for export is fumigated to meet quarantine regulations (Brockerhoff and Hosking, 2001; Cross, 1991). However, the fumigant currently in use (methyl bromide) (Cross, 1991), is being phased out. Alternative treatments or other pest management methods are therefore urgently needed to support timber exports.

The management of other forest and timber pests, for example, bark and ambrosia beetles, often involves the use of attractants such as pheromones and host plant volatiles (e.g., Borden, 1995; Jones, 1998). A strong attraction to odors emanating from forest fires or burnt trees has been documented for some cerambycids and buprestids specializing in the exploitation of postfire habitats (Gardiner, 1957; Parmelee, 1941; Ross, 1960; Evans, 1972, 1973). Circumstantial evidence that *A. tristis* is attracted by odors emitted by burnt pine, peeled bark, and sawn timber suggests that odorants could possibly be used for monitoring and managing populations of this beetle in ports, sawmills, and after forest fires. This could be achieved with an attractant for *A. tristis*, which could also be used in novel pest management tactics such as lure and kill (Suckling and Karg, 2000). Recently it has been established that antennae of a beetle attracted to postfire habitats are specifically tuned to perceive fire-associated volatile compounds (Schütz et al., 1999). Thus, we hypothesize that *A. tristis* also shows an olfactory response to fire-associated compounds and that the olfactory system is tuned to these. Here, we report results of a study investigating behavioral responses from *A. tristis* to

burnt pine odors. We also report results of electroantennogram (EAG) studies investigating responses to individual compounds typically emitted by pines, along with a range of other odorants. In addition, results on the daily activity pattern and local abundance within sawmills are reported.

METHODS AND MATERIALS

Insects. A preliminary experiment was conducted to determine relative beetle density at pine forest and sawmill locations, in order to focus future efforts at beetle collection. Passive funnel traps, consisting of two clear plastic cross vanes mounted above a plastic funnel held above a collecting jar were placed at three sawmills and two sites in *P. radiata* stands at Ashley forest (three replicates per site) near Christchurch, New Zealand. Traps were checked weekly. Based on the greater abundance of beetles at the sawmills, collections for experiments were made at two sawmills. Beetles were collected using passive funnel traps and manual collection after dark (20:00–22:00 hr, see below). To establish the relative abundance of beetles within sawmill sites, the location of collected beetles was recorded from the following areas: old and new sawn timber stacks, around a freshly peeled bark stack, and around drying kilns. The number of beetles present was recorded in the following abundance classes: <5, 6–20, 21–50, 51–80 (hence standard errors are not presented with these data). Beetles were placed individually in plastic Petri dishes (60 mm diam.) with a damp cotton wad to provide sufficient humidity, and kept outdoors under a shelter until used in bioassays. The use of individual containers avoided damage due to fighting, especially among males. Only fit beetles showing normal responses to disturbance were used for bioassays.

Diurnal Activity. The aim of this experiment was to determine the natural period of activity and therefore the best time for conducting behavioral assays with beetles and making field collections. An activity recorder was constructed from a rectangular stainless steel gauze mesh cage $80 \times 70 \times 60$ mm mounted over a sensor window (60×40 mm and shielding an infrared movement detector) as the floor of the cage. The size of the cage was determined by the need to keep the beetles within range of the detector. The apparatus was maintained outdoors under a lean-to roof with natural light. The detector was linked to a data logger (model CR10, Campbell, Logan, Utah), which recorded the number of movement events large enough to trigger the sensor, in successive 1-min periods. Male ($N = 12$) or female ($N = 12$) beetles were placed individually in the cage in the afternoon and records obtained over the following 24 hr.

Olfactometer. An apparatus was constructed from a 20-liter food-grade inert plastic cylinder, 400 mm long $\times$ 300 mm diam., with a plastic funnel attached to each end. Each funnel led to a plastic pipe (35 mm diam.) with a right angle bend ending in the middle of a trap jar with a gauze base and smooth sides, designed

as a one-way system (Figure 2 below). Air was drawn from the geometric center of the cylinder at around 12 liters/min, and the olfactometer inlets passed either over strips of freshly peeled bark or over freshly peeled burnt bark before entering via the two opposing traps. Beetles were unable to touch the bark before making an irreversible choice of trap. The orientation of the apparatus was reversed after each run to control for any potential biases. Groups of 15 quiescent female beetles were placed in the center of the barrel at 16:00 hr (before activity commenced with scotophase) and the pump was programmed with a time switch to run for the first 4 hr of darkness (20:00–24:00 hr), based on the activity cycle determined earlier. The pump was therefore switched on 1 hr before significant beetle activity began. The following morning, the number of beetles in each trap was counted. This approach minimized the duration of pump operation, while operating during the period of most beetle activity. All beetles able to do so made a choice. The exceptions were three beetles that died in the center of the barrel, and two that were trapped there.

Oviposition Choice. A choice test for oviposition site preference was performed using a covered cylindrical arena made from metal gauze (190 mm diam. × 160 mm high), placed over a base of moist sand. Two short sections of *P. radiata* branch (ca. 15 cm long × 5 cm diam.) were placed vertically on the sand on opposite sides of each arena, touching the mesh on the outside of the arena. One branch (treatment), was freshly burnt with a gas torch on the side adjacent to the arena until the outside bark surface was scorched and one (control), was left unburnt. Branch sections were cut from living *P. radiata* and were used within two days. Adjacent sections were used for paired treatments in each arena so that differences in bark roughness or other characteristics were minimized. Two field-collected beetles were placed in the arena, and the top of each arena was covered with a Perspex sheet. Branch positions were alternated between adjacent arenas and also between successive replicates with the same arena. The minimum distance between adjacent arenas was 20 cm.

The arenas were set up in a darkroom with an exhaust fan, expelling air through a 30-cm-diameter duct at 1.8 m/sec, ensuring regular slow clean air replacement. Arenas were set up between 16:00 and 18:00 hr, and eggs were counted the following morning. The room was kept in darkness once the assays were set up, until the following morning when the number of eggs laid on or adjacent to burnt and unburnt *Pinus* sections was counted under a binocular stereo microscope. Eggs laid at the base of each log, between the log and the sand surface (ca. 20% of total eggs laid) were counted, along with those laid on the bark of the log itself. Between replicates, the sand surface was scraped clean to remove any eggs and was moistened again if necessary. The gauze cylinders were brushed clean of any attached eggs and rotated, to present different sectors adjacent to logs in successive trials.

Oviposition Repellency. The above oviposition choice test was repeated in an exploratory experiment on repellency, except that both logs in the choice test were

burnt, and a repellent was applied to one of the logs, before the experiment. The choice of two green leaf volatile compounds tested as a repellent was based on the knowledge that such green leaf volatiles have been found acting as repellents in other species (Dickens et al., 1992; Wilson et al., 1996), in conjunction with the observation that these particular compounds were active in the EAG tests. The repellent mixture was made up of 25 ml each of (*E*)-2-hexen-1-ol and (*E*)-2-hexenal and 50 ml of mineral oil (1:1:2), applied as a liquid at about 35 ml of green leaf volatiles per square meter of log surface ($N = 7$ replicates). The use of mineral oil would have considerably reduced the release rate (Brockerhoff and Grant, 1999).

Field Cage Experiments. These experiments were designed to determine the relative attractiveness of burnt compared to unburnt bark as a lure in a trap, using a known and confined population of beetles. Cylindrical Perspex funnel traps (300 mm × 120 mm diam., with a screen funnel at each end) were used. Burnt (a) and unburnt (b) bark strips were used as lures and catches were compared with unbaited controls containing no bark (c and d). A screened field cage (3 × 4 m) with a roof and floor was used for the trials. Trap cylinders were lined with Mylar sheets, which were replaced after each trial to reduce the possibility of odor contamination of the traps. The four traps (a–d) were placed on the ground, one in each corner of the cage (ca. 300 mm from the wall). Beetles ($N = 20$) were placed on the floor in the center of the field cage at 16:00 hr. Beetles could enter the trap through holes at the apex of each cone but were then unable to leave the trap. Beetles were not able to touch the bark lure inside the trap until after they had entered. The location of trapped beetles was recorded the following morning. The array of four traps was rotated 90° between trials to control for any inherent directional biases, such as sources of light or external odors. The bark strips were replaced after two days. Each bark lure consisted of three strips, each 100 × 40 mm, and the burnt strips were burned as described above. Bark strips were cut from smooth bark on the surface of logs less than 150 mm diam. within 14 days of the trees being felled.

Repellency Test. The trapping test described above was repeated in another exploratory test of repellency, except that the repellent [1 : 1 : 2 of (*E*)-2-hexen-1-ol and (*E*)-2-hexenal in mineral oil as above] was applied to the bark strips in one of the treatments, before the traps were baited ($N = 6$ replicates).

Electroantennograms. EAGs were recorded using excised antennae. These were stimulated with a range of monoterpenes known to be constituents of *P. radiata* bark headspace (Zabkiewicz and Allan, 1975) or oleoresin (Burdon et al., 1992) as well as several other widely available compounds, included to broaden the range screened. Filter papers (5 × 20 mm) were loaded with 10 μl of 10% solutions of each compound in hexane. Antennae were stimulated three times with each substance at 30-sec intervals. The reference standard (α-pinene) was tested at the beginning and end of each series and after every two test substances. The

sequence of substances was changed for each antenna ($N = 10$ for males and 10 for females). Electroantennogram recordings were made using an INR-02 recording unit with amplifier and computer board (Syntech, Hilversum, The Netherlands) and stored on hard disk. Responses were normalized to the reference standard by dividing sample responses by the mean of temporally adjacent responses to the reference standards. Preliminary tests indicated EAG responses to the odors from burnt wood, but no pure chemicals derived from this source were available.

Chemicals. The source and listed purity of the compounds used are shown below. Sigma: farnesol (96%), *n*-decyl alcohol (99%), phytol (97%), (*E*)-2-hexenal (98%), nerol (97%), γ-terpinene (97%), α-humulene (>90%), (−)-(*E*)-caryophyllene (>90%), eugenol (>90%), nerolidol (>90%), (+)-limonene (97%), benzaldehyde (>90%), (±)-2-octanol (>90%), 1-hexanol (98%), 1-heptanol (99%), 1-octanol (98%), 1-nonanol (98%), β-myrcene (90%), α-terpinene (85%), citral (95%), (±)-linalool (95–97%), geraniol (98%), citronellal (85–90%), butanol (99%), (*Z*)-3-hexen-1-ol (>90%); Aldrich Chemical Co.: 3-carene (90%), (1*S*)-(−)-β-pinene (99%), (1*R*)-(+)-α-pinene (98%), terpineol (mixed isomers, approx. 95% α-terpineol); (*Z*)-3-hexyl acetate (99%), amyl acetate (99%), amyl alcohol (99%), benzyl alcohol (98%), (*E*)-2-hexen-1-ol (96%); Merck: α-phellandrene (>80%).

Statistics. Trap catch data were analyzed by *t* test. Olfactometer, repellency, and oviposition choice data were analyzed by paired *t* test. ANOVA was conducted on beetle catches in the field cage tests, followed by a Tukey test to separate treatments. The relationship of male and female EAG responses was examined by correlation analysis. Vertical bars show standard errors of means throughout. Data were analyzed using Minitab or SYSTAT 9.

RESULTS AND DISCUSSION

Diel Periodicity. Both sexes were most active from 21:00 to 24:00 hr, although females showed a broader activity period, including parts of the photophase (Figure 1). There was also some evidence of a secondary peak in male activity around dawn. This primary activity pattern during the early scotophase was confirmed by video analysis of beetle behavior in larger cages, which indicated long periods of continuous movement over this diel period (unpublished data). Bioassays were carried out in the early scotophase, based on these findings.

Field Occurrence. Passive traps used to assess the occurrence of *A. tristis* populations at several locations revealed that mean catches were much higher near sawmills [85.7 ± 29.3 beetles (SEM)] than in pine forests (4.5 ± 3.5 beetles; *t* test on log transformed data: $t = 3.95$, $P = 0.02$; $df = 3$). This led to the focus on sawmills for subsequent beetle collections. It is not known whether *A. tristis* were

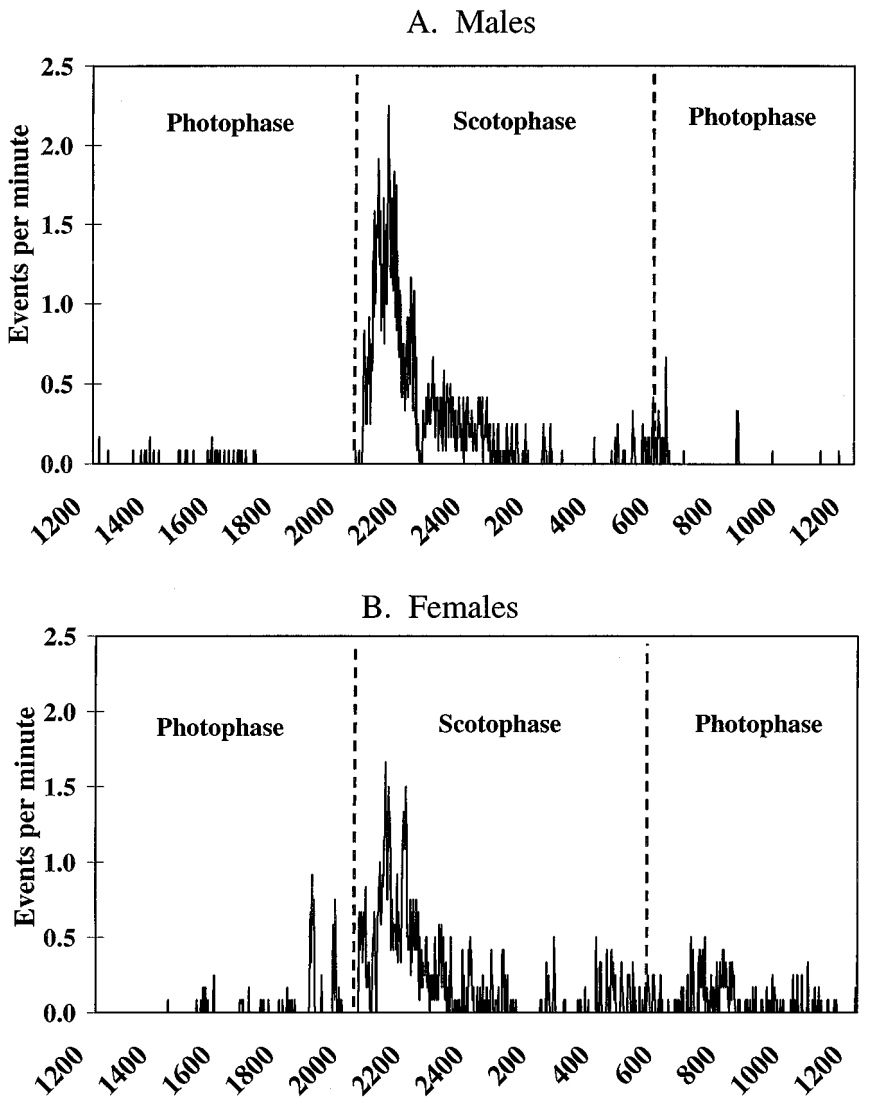


FIG. 1. Diel periodicity of male (A) and female (B) *Arhopalus tristis* movement activity outdoors under natural light as determined by a motion sensor.

brought with logs from the forest to the sawmills and accumulated there or whether they were attracted to the sawmills from surrounding forest areas, independently of log movements. It has also been observed that *A. tristis* breeds in reject logs and other suitable material on site (G. Hosking, personal communication). Most

beetles collected manually during the scotophase at the sawmill were either walking around on the ground or resting on vertical surfaces of timber stacks or wooden structures in the yard. Only three beetles were observed flying, out of a total of nearly 1000 collected individually. Most beetles were collected either near the drying kilns (36 beetles per night) or piles of freshly peeled bark (36 beetles per night), with few collected near sawn timber stacks (5 beetles per night). The first two areas were both concentrated sources of pine volatiles, clearly perceptible to the authors. These results suggest that olfaction might be playing an important role in the beetle behavior.

Olfactory Orientation. In olfactometer bioassays, female beetles showed a very strong preference (80%) for odors associated with burnt over unburnt bark (Figure 2) (paired t test, $t = 2.57$, $P = 0.0002$, $df = 5$). In the field cage experiments, females clearly responded to odorants from both burnt and unburnt bark, and very few beetles were found in the blank traps, which were then pooled (Figure 3; $F_{2,29} = 26.0$, $P < 0.0001$). In this case, the choice of burnt over unburnt bark was only marginally significant (paired t test, burnt versus unburnt $t = 2.01$, $P = 0.07$, $df = 11$). The bark strips used for the field cage trapping experiment had been freshly peeled from recently cut logs, suggesting the release of volatiles from the torn cambium may have been high from both burnt and unburnt strips. However, the composition of volatiles emitted from burnt bark will undoubtedly be different from that of fresh bark (Lomax et al., 1991). A related North American species,

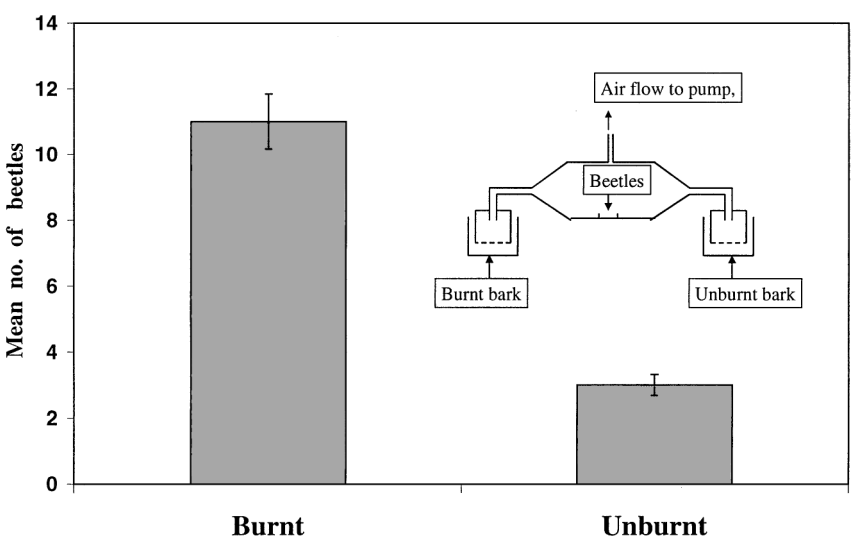


FIG. 2. Preference of female *A. tristis* in an olfactometer test (schematic) between burnt and unburnt bark of *P. radiata* ($N = 5$ replicates).

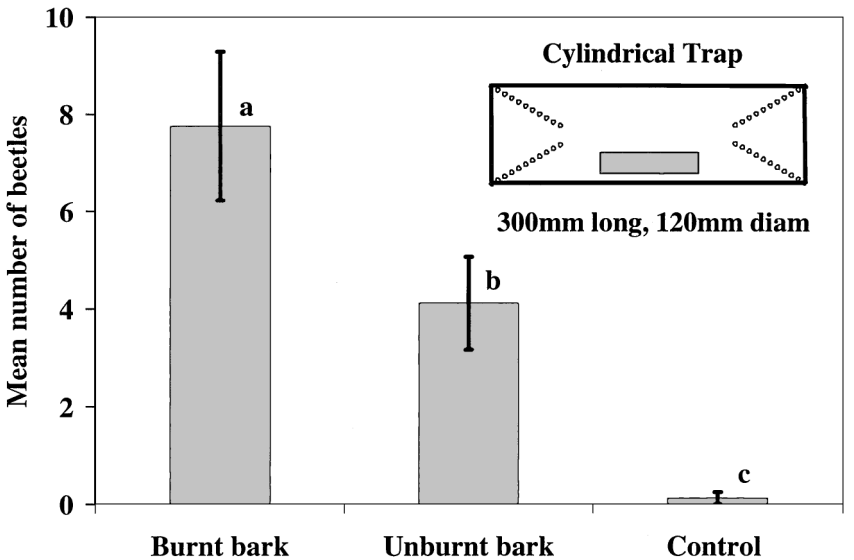


FIG. 3. Number of female *A. tristis* trapped in cylinder traps (schematic) to burnt and unburnt bark of *P. radiata* ($N = 8$ replicates) in a field cage. Treatments with different letters are significantly different according to a Tukey test.

A. asperatus (LeConte), is also known to be particularly attracted by freshly scorched trees (Wickman, 1964).

Repellency Test. Because green leaf volatiles have been reported to disrupt the attraction of beetles to odorants (Dickens et al., 1992; Wilson et al., 1996), we examined whether they may act as repellents for *A. tristis*. The repellent significantly reduced the proportion of beetles trapped in the field cage to treated burnt bark ($N = 88$ beetles in total), compared to burnt bark without repellent ($t = -3.97$, $P = 0.0014$, $df = 14$), by a factor of approximately five (Figure 4). This result suggests that the repellent is active, and hence it may be possible to reduce host location behaviors in the field. The rate used here was very high, and further experiments would need to explore the dose response.

Oviposition Choice. In choice tests, female beetles laid the majority of their eggs (87.6%) on burnt rather than unburnt pine bark (Figure 5) ($N = 558$ eggs laid by 20 female *Arhopalus*, summed over two nights per insect). This difference was highly significant (paired t test on log transformed data, $t = -3.35$, $P = 0.002$, $df = 37$). Because these assays were conducted as choice tests, differences in fecundity among individual females could not have biased the results. Beetles were observed to prefer to lay eggs in areas that were lightly rather than fully scorched, although this was not specifically quantified. This suggests that the attraction of *A. tristis* to

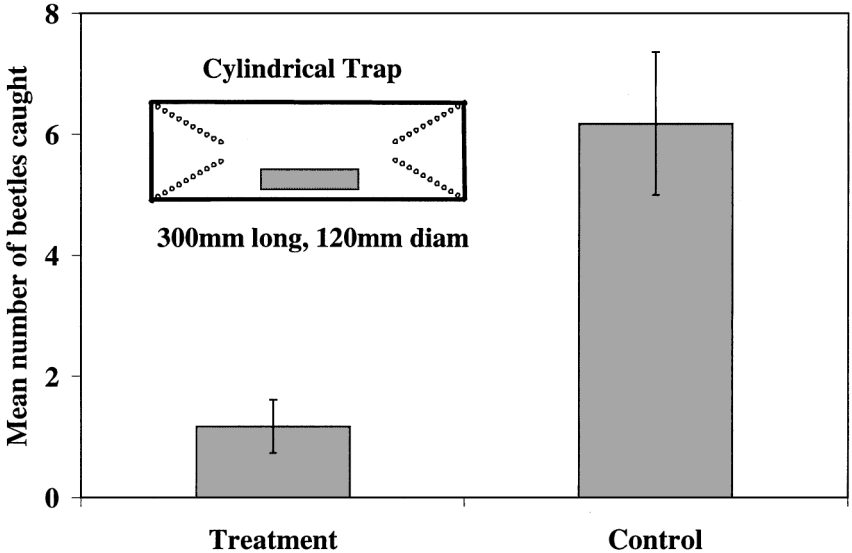


FIG. 4. Effect of green leaf volatile-based repellent treatment applied in mineral oil to strips of burnt *P. radiata* bark on the number of *A. tristis* trapped with strips of treated and untreated burnt bark in field cage trials ($N = 6$ replicates).

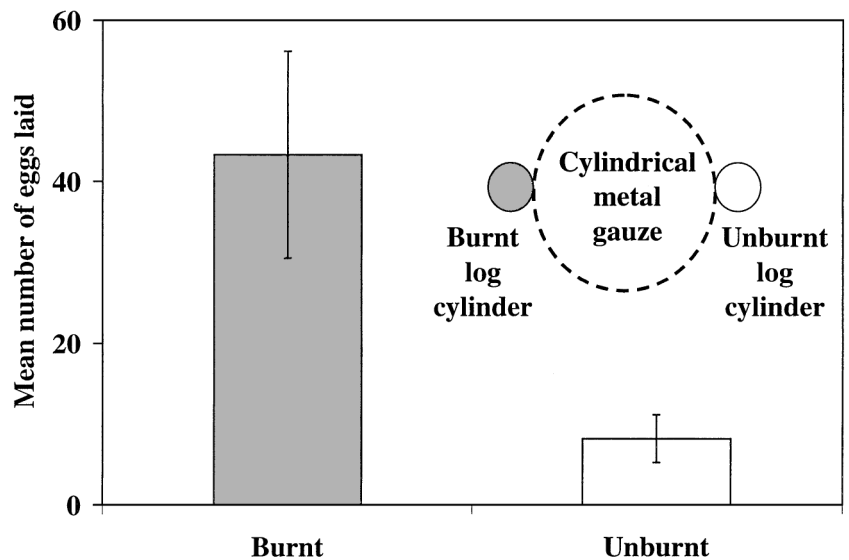


FIG. 5. Preference of female *A. tristis* for oviposition in a choice test of burnt and unburnt logs of *P. radiata* ($N = 5$ tests).

burnt trees may be related to such habitats representing the occurrence of a favored oviposition substrate.

Oviposition Repellency. Green leaf volatiles were also used as potential repellents in the oviposition bioassay. The oviposition test indicated a 98.5% reduction in number of eggs laid on burnt logs treated with the repellent, compared to burnt logs without the repellent (Figure 6). This result indicates that the repellent mixture reduced the suitability of the bark surface for oviposition (paired t test on log transformed data, $t = 4.82$, $P = 0.0001$, $df = 8$). If the repellent was as effective in the field at economically viable application rates, a reduction in egg laying of this magnitude could be expected to have significant potential for pest management, by reducing colonization and potentially vectoring of sapstain.

Electroantennograms. Males generally showed higher EAG responses than females, although there was a high correlation between sexes in olfactory response ($r^2 = 0.948$, $df = 35$). This was despite the considerable differences in the number of several types of olfactory sensillae on antennae of male and female *A. tristis* (Hosking, 1982). Males have more sensillae chaetica, suggesting this receptor type may be most relevant to the perception of monoterpenes and green leaf volatiles. Both sexes showed strong EAG responses to the monoterpenes recorded from *P. radiata*, with the highest responses to α-terpineol and (1*R*)-(+)-α-pinene (normalized values of 100.0 $\pm$ 0.0 and 102.1 $\pm$ 7.4 for females and 100.0 $\pm$ 0.0

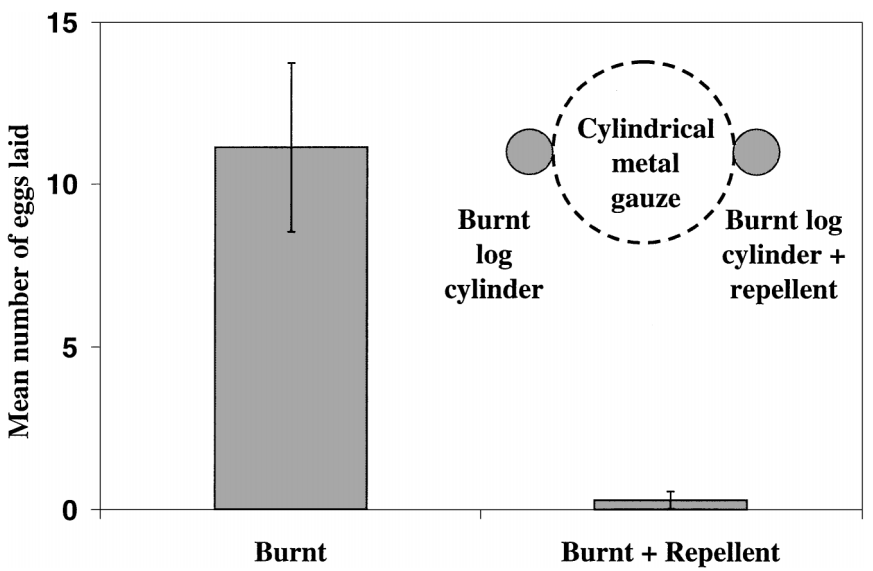


FIG. 6. Effect of green leaf volatile-based repellent treatment applied in mineral oil to burnt logs on the number of *A. tristis* eggs laid on treated or untreated burnt *P. radiata* logs in field cage trials ($N = 6$ trials).

and 149.8 ± 24.2 for males, for the compounds, respectively). Monoterpenes were among the compounds found in volatiles from smoldering wood to which *Melanophila acuminata* DeGeer (Buprestidae), a beetle also attracted to burnt pines, showed high EAG responses (Schütz et al., 1999). However, volatiles from burnt pine wood contain additional, fire-specific compounds that appear to be more important during habitat location by *M. acuminata* (Schütz et al., 1999). The green leaf volatile (*Z*)-3-hexen-1-ol, which was in the repellent mixture tested in reduced attraction and oviposition assays, elicited strong EAG responses (82.1 ± 12.2 and 108.0 ± 12.7 for females and males, respectively), indicating that antennae have receptors for such compounds. Detection of such green leaf compounds could have a functional role for the insect as an indicator of habitat quality, being normally low in the atmosphere immediately after a fire (when host tree quality appears to be most suitable for colonization), but rising as the fire-damaged area was colonized by other plants, indicating a probable decline in the beetles' preferred ecological niche. The least active compounds included phytol, *n*-decyl alcohol, and farnesol (EAG values for both sexes in the range of 15–25% of the standard, α-pinene).

These experiments have demonstrated that olfactory cues are involved in the attraction of *A. tristis* to areas of forest affected by fire or areas with high levels of volatiles associated with burnt or unburnt logs and with sawmills. Numerous ecological adaptations can be observed in insects occurring in forest ecosystems that are naturally prone to regularly experience fires such as boreal forests (McCullough et al., 1998). Our experiments provide further evidence of how such insects are behaviorally and physiologically adapted to exist in such an environment and even exploit these conditions to their advantage. Prospects for applying attractants in the management of *A. tristis* in the forest, at ports, or in sawmills are illustrated by the example of successful trapping of a North American species, *A. rusticus obsoletus* (Rand.), using natural turpentine and ethanol as a lure (Fatzinger et al., 1987). However, attempts to attract *A. tristis* with a comparable blend have so far been unsuccessful (unpublished data). Better traps could be devised if the active components in the blend of volatiles were known (e.g., Schütz et al., 1999), but more experimentation is needed to establish this for *A. tristis*. There are also prospects of using green leaf volatiles as repellents for *A. tristis*. Our results suggest such compounds reduce the attraction of beetles to otherwise attractive host material, and they also reduce oviposition stimuli associated with burnt bark. The ecological significance of these findings could be comparable to that suggested for bark beetles, which presumably perceive green leaf volatiles as nonhost indicators (i.e., broadleaved tree species) (Dickens et al., 1992; Wilson et al., 1996). This repellent could potentially be used to avoid infestations after fires, by reducing postfire colonization and oviposition, and may reduce quarantine problems with export timber products.

Attractants could be useful for pest management by helping forest managers estimate field populations in order to determine the appropriate response to forest fires, etc. Improved trapping systems could also aid in the quarantine risk management of export logs destined for markets where this insect is not present.

Acknowledgments—We would like to acknowledge the help of the managers and staff of Keighleys Waimak Sawmill and McVicars Sawmill of Christchurch, New Zealand, for their cooperation in providing access to their sites for *Arhopalus* trapping and collection, and to the Selwyn Plantation Board for providing logs. We thank G. Hosking, D. Rowan, and Q. Wang for helpful comments on the manuscript. This work was supported by the Foundation for Research, Science and Technology under CO6630.

REFERENCES

BORDEN, J. H. 1995. Development and use of semiochemicals against bark and timber beetles, pp. 431–449, *in* J. A. Armstrong and W. G. H. Ives (eds.). Forest Insect Pests in Canada. Natural Resources Canada, Canadian Forest Service, Ottawa, Canada.

BRADBURY, P. M. 1998. The effects of the burnt pine longhorn beetle and wood-staining fungi on fire damaged *Pinus radiata* in Canterbury. *N.Z. For*. 43:28–31.

BROCKERHOFF, E. G., and GRANT, G. G. 1999. Correction for differences in volatility among olfactory stimuli and effect on EAG responses of *Dioryctria abietivorella* (Lepidoptera: Pyralidae) to plant volatiles. *J. Chem. Ecol*. 26:1353–1368.

BROCKERHOFF, E. G., and HOSKING, G. P. 2001. *Arhopalus tristis* (F.) [= *Arhopalus ferus* (Mulsant)] (Coleoptera: Cerambycidae), Burnt pine longhorn. Forest and Timber Insects in New Zealand No. 27. Forest Research, Rotorua, New Zealand. (in press).

BURDON, R. D., ZABKIEWICZ, J. A., and ANDREW, I. A. 1992. Genetic survey of *Pinus radiata*. 8: Population differences in monoterpene composition of cortical oleoresin. *N.Z. J. For. Sci*. 22:257–273.

CROSS, D. J. 1991. Penetration of methyl bromide into *Pinus radiata* wood and its significance for export quarantine. *N.Z. J. For. Sci*. 21:235–245.

DICKENS, J. C., BILLINGS, R. F., and PAYNE, T. L. 1992. Green leaf volatiles interrupt aggregation pheromone response in bark beetles infesting southern pines. *Experientia* 48:523–524.

EATON, C. B. 1959. Observations on the survival of *Arhopalus productus* (LeConte) larvae in Douglas-fir lumber. *Pan-Pac. Entomol.* 35:114–116.

EHNSTRÖM, B., LÅNGSTRÖM, B., and HELLQVIST, C. 1995. Insects in burnt forests—forest protection and faunal conservation (preliminary results). *Entomol. Fenn*. 6:109–117.

EVANS, W. G. 1972. The attraction of insects to forest fires, pp. 110–127, *in* Proceedings Annual Tall Timbers Fire Ecology Conference 10, Tall Timbers Research Station, Tallahassee, Florida.

EVANS, W. G. 1973. Fire beetles and forest fires. *Insect World Dig*. 1:14–18.

FATZINGER, C. W., SIEGFRIED, B. D., WILKINSON, R. C., and NATION, J. L. 1987. Trans-verbenol, turpentine, and ethanol as trap baits for the black turpentine beetle, *Dendroctonus terebrans*, and other forest Coleoptera in north Florida. *J. Entomol. Sci*. 22:201–209.

FREUDE, H., HARDE, K. W., and LOHSE, G. A. 1966. Die Käfer Mitteleuropas. Vol. 9, Cerambycidae. Goecke and Evers, Krefeld, Germany.

GARDINER, K. M. 1957. Deterioration of fire-killed pine in Ontario and the causal wood-boring beetles. *Calif. Entomol*. 89:241–263.

HOSKING, G. P. 1982. *Arhopalus ferus* antennal structure examination using the SEM. Unpublished project record. Forest Research Institute, Rotorua, New Zealand.

HOSKING, G. P., and BAIN, J. 1977. *Arhopalus ferus* (Coleoptera: Cerambycidae); its biology in New Zealand. *N.Z. J. For. Sci*. 7:3–15.

JONES, O. T. 1998. Practical applications of pheromones and other semiochemicals, pp. 263–355, *in* P. E. Howse, I. D. R. Stevens, and O. T. Jones (eds.). Insect Pheromones and Their Use in Pest Management. Chapman and Hall, London.

LOMAX, T. D., FRANICH, R. A., and KROESE, H. W. 1991. Pyrolysis products of *Pinus radiata* bark. *N.Z. J. For. Sci*. 21:111–115.

MCCULLOUGH, D., WERNER, R. A., and NEUMANN, D. 1998. Fire and insects in northern and boreal forest ecosystems of North America. *Annu. Rev. Entomol*. 43:107–127.

PARMELEE, F. T. 1941. Longhorned and flatheaded borers attacking fire-killed coniferous timber in Michigan. *J. Econ. Entomol*. 105:795–806.

ROSS, D. A. 1960. Damage by long-horned wood borers in fire-killed white spruce, central British Columbia. *For. Chron*. 36:355–361.

SCHÜTZ, S., WEISSBECKER, B., HUMMEL, H. E., APEL, K. H., SCHMITZ, H., and BLECKMANN, H. 1999. Insect antenna as a smoke detector. *Nature* 398:298–299.

SUCKLING, D. M., and KARG, G. 2000. Pheromones and semiochemicals, pp. 63–99, *in* J. Rechcigl and N. Rechcigl (eds.). Biological and Biotechnical Control of Insect Pests. CRC Press, Boca Raton, Florida.

SUCKLING, D. M., GIBB, A. R., KAY, S., PARRY, F., and ROHITHA, H. 1999. Are insects vectors of sapstain fungi in New Zealand? pp. 117–121, *in* B. Kreber (ed.). Proceedings, 2nd New Zealand Sapstain Symposium, Rotorua. Forest Research Bulletin 215.

VILLIERS, A. 1978. Faune des Coléoptères de France. I. Cerambycidae. Edition Lechevalier S. A. R. L., Paris, pp. 231–232.

WICKMAN, B. E. 1964. Freshly scorched pines attract large numbers of *Arhopalus asperatus* adults. *Pan-Pac. Entomol*. 40:59–60.

WILSON, I. M., BORDEN, J. H., GRIES, R., and GRIES, G. 1996. Green leaf volatiles as antiaggregants for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *J. Chem. Ecol*. 22:1861–1875.

ZABKIEWICZ, J. A., and ALLAN, P. A. 1975. Monoterpenes of young cortical tissues of *Pinus radiata*. *Phytochemistry* 14:211–212.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

DIFFERENTIAL NEUROSENSORY RESPONSES OF ADULT COLORADO POTATO BEETLE, *Leptinotarsa decemlineata*, TO GLYCOALKALOIDS

BENEDICT HOLLISTER, JOSEPH C. DICKENS,* FRANCES PEREZ, and KENNETH L. DEAHL

USDA, ARS, Beltsville Agricultural Research Center
Plant Sciences Institute, Vegetable Laboratory
Bldg. 010A, Rm. 240, BARC-West
Beltsville, Maryland 20705

(Received November 7, 2000; accepted February 12, 2001)

Abstract—Neurons from chemosensory hairs on the galeae of adult Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), were investigated for responses to glycoalkaloids of the family Solanaceae. While solanine and tomatine elicited irregular firing by multiple neurons and bursting activity at 1 mM concentration in most sensory hairs, stimulation with leptine I resulted in consistently high-frequency, slowly adapting responses with a dose-dependent effect between 0.03 and 0.3 mM concentrations. Responses to a mixture of solanine and leptine I suggested possible modification of the leptine I response by other glycoalkaloids, resulting in reduced neural activity relative to leptine I alone. These results establish a method for specifically evaluating leptine I and other glycoalkaloids for effects on feeding behavior of CPB and provide a sensory component for incorporating deterrent chemistry into biorational control methods for the CPB.

Key Words—Gustation, taste, feeding deterrent, leaf beetle, potato, receptor neuron, glycoalkaloids, Colorado potato beetle, Chrysomelidae.

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), is a major pest of potato, *Solanum tuberosum* (L.), throughout the world. Adaptability of the CPB and heavy use of synthetic pesticides for control has led to development of insecticide resistance in the insect and a shifting focus towards alternative,

*To whom correspondence should be addressed. e-mail: dickensj@ba.ars.usda.gov

0098-0331/01/0600-1105$19.50/0

multifaceted control options. For example, information on feeding deterrents (Murray et al., 1996) and attractants (Dickens, 1999, 2000) could be incorporated into attracticide bait formulations and "push-pull" strategies of control, where insects are repelled by resistant or deterrent plants in combination with attracticide baits. Glycoalkaloids in potato have long been implicated as resistance factors against the CPB, specifically the leptines associated with *Solanum chacoense* Bitt. (Stürckow and Löw, 1961; Tingey, 1984), and breeding efforts have attempted to incorporate this natural resistance mechanism into control programs for CPB (Lawson et al., 1993; Tingey and Yencho, 1994).

Evidence continues to accumulate associating resistance with leptines. Significant differences between high- and low-leptine *Solanum chacoense* Bitt. clones for five resistance parameters (adult counts, adult feeding damage in the field, adult feeding on leaf disks, larval development, and larval survival) were directly related to concentration of foliar leptines in a study by Sinden et al. (1986). Sikinyi et al. (1997) showed reduced leaf area consumption and increased larval mortality on *S. chacoense* vs. *S. tuberosum*. Larval growth and development were reduced in a dose-dependent manner on artificial diets containing leptine I (Kowalski et al., 1999), although overall purity of the compound was only 60%.

Although there is evidence for a relationship between foliar leptine levels and resistance to CPB for *S. chacoense*, the mechanism or mode of action through which resistance is imparted has remained an open question. Stürckow and Löw (1961) first observed reduced feeding on potato foliage infused with leptine I. Tingey and Yencho (1994) labeled the leptines as potent antifeedants based on reduced feeding rates of adults on potato leaf disks from susceptible (*S. tuberosum* var. Kennebec) and resistant (*S. chacoense*) clones (from Sinden et al., 1986, 1988). Behavioral effects for adults and larvae in the field have been related to leptine levels alone among a number of glycoalkaloids (solanine, chaconine, leptinine I, and leptinine II) analyzed, indicating a feeding deterrent effect (Yencho et al., 2000). The relationship between the observed feeding effects and sensory detection of glycoalkaloids, however, has remained unclear. Leptines have been implicated as feeding deterrents and resistance has been attributed to the presence of these compounds, but these conclusions are based upon partial analysis of whole leaf chemistry. Since leptines of high purity have not been individually tested, the observed effects could be due to synergism with unknown components or other unidentified compounds in the foliage.

Harrison and Mitchell (1988) performed detailed behavioral observations on sampling and first meal feeding behavior of adult CPB on various host plants differing in glycoalkaloid content. Although they did not investigate *S. chacoense*, they questioned the assumption that glycoalkaloids act directly on the sensory system to inhibit feeding, raising the point that such conclusions have been based upon long-term feeding experiments. The possibility of postingestive effects on feeding behavior was discussed, as they found no inhibition of first meal feeding or

altered sampling behavior associated with the glycoalkaloid tomatine, concluding there was no evidence to indicate tomatine acted on the sensory system. The only studies investigating the effect of glycoalkaloids on CPB chemosensory neurons illustrated irregular firing by several neurons housed within sensilla on the tibia and tarsus (Stürckow, 1959) or the galea (Mitchell and Harrison, 1985; Mitchell, 1987). The firing pattern was often burstlike for glycoalkaloids, with the first burst of activity delayed by several seconds after stimulation. They concluded that there was no specialized chemoreceptor in CPB for the glycoalkaloids tested: solanine, chaconine, and tomatine. A survey of responses to seven other alkaloids (strychnine, caffeine, quinine, papaverine, sparteine, atropine, and arecoline) in adult CPB galeal chemosensilla also revealed no specific deterrent receptor for this class of compounds (Mitchell, 1987). There were no cases of phasic–tonic responses for these alkaloids, and bursting activity was observed with strychnine and quinine at concentrations of 5 and 10 mM. The specialized case of the leptines was discussed by Mitchell (1994), who indicated that despite the fact that no differential sensory effect among glycoalkaloids has been measured to date, the leptines deserved further study, as they had yet to be tested in sensory physiological assays.

Our objective here was to determine the nature of chemosensory responses to purified (100%) leptine I. We demonstrate for the first time specific chemosensory neural responses to a glycoalkaloid, leptine I, in adult CPB galeal taste hairs.

METHODS AND MATERIALS

Insects. Adult Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), were obtained from a colony with all life stages reared on *Solanum tuberosum* var. Kennebeck. Emerging adults were collected daily, sexed, and isolated in Petri dishes with moistened filter paper and fresh potato foliage that was replenished daily. Insects were kept in an environmental chamber (16L: 8D) at 25°C until use. Six-day-old insects were starved at least 4 hr prior to preparation for electrophysiological recordings.

Chemicals. Glycoalkaloids used in this study are illustrated in Figure 1. Tomatine (> 98% purity) and solanine (ca. 95%) were obtained from Sigma Chemical Co., St. Louis, Missouri. Leptine I was purified by using methodology modified from Kowalski et al. (2000).

Partially purified leptine I was obtained by normal-phase and reverse-phase chromatography from crude *Solanum chacoense* leaf extract. The sample was dissolved in methanol, and dissolution of the leptine I powder was aided by alternately heating a capped tube in a water bath, sonicating, and vortexing for a few seconds. A 1-ml aliquot of the sample was taken and transferred to a clean hydrolysis tube. The original sample tube was dried and stored in the cold for future use. Further

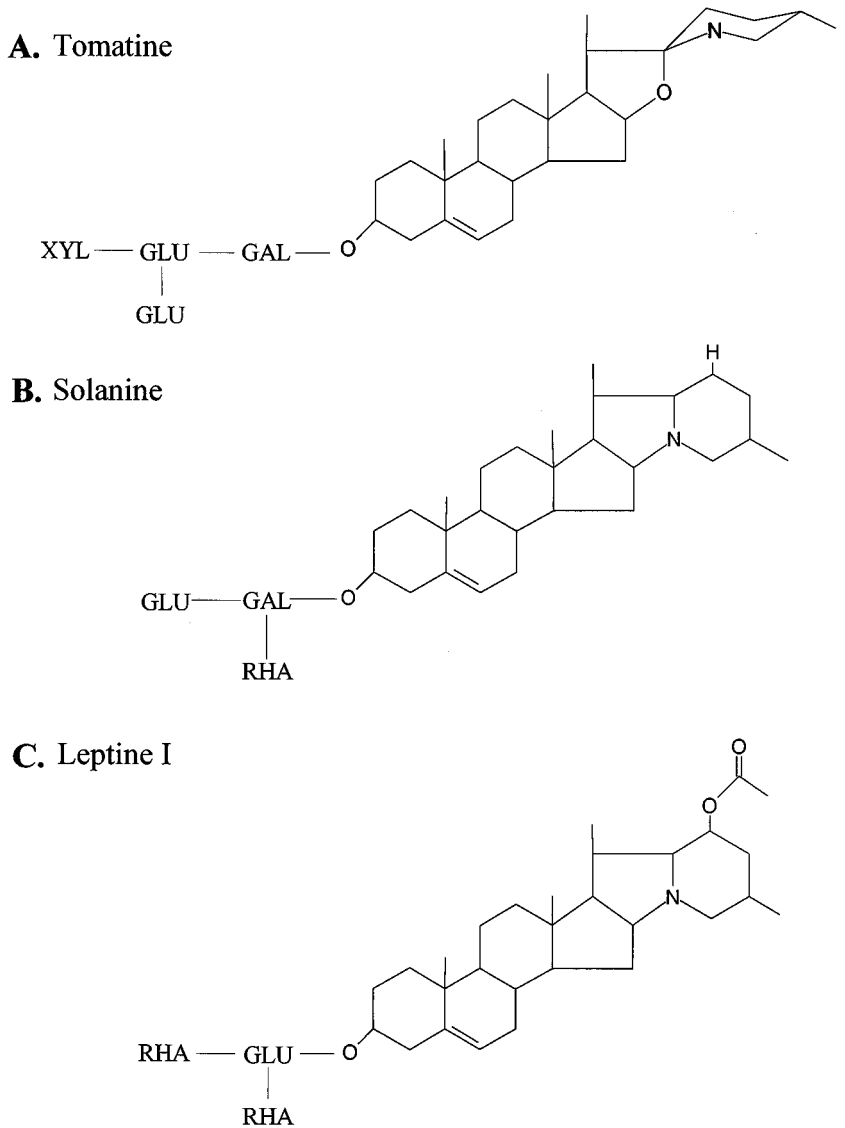


FIG. 1. Molecular structures of the glycoalkaloids used in this study: (A) tomatine, (B) solanine, and (C) leptine I. Abbreviations for sugar groups: GLU = glucose, GAL = galactose, XYL = xylose, RHA = rhamnose.

purification involved high-pressure liquid chromatography (HPLC) with a Waters 510 pump, Lambda-Max 481 wavelength detector, Waters automated gradient controller, Waters 745 data module, and a Waters 717 plus autosampler. A 20-μl volume of the leptine I aliquot was injected into a 3.9 $\times$ 300 mm Steel Resolve 5-μm spherical reverse-phase C_{18} column fitted with a Guard-pak C_{18} column insert to determine leptine I elution time and conditions for the fraction collector. HPLC condition was set at isocratic, with a mobile phase consisting of 1:1 acetonitrile (HPLC grade) –water supplemented with 5 ml 0.1 N phosphoric acid and 0.94 g 1-heptane sulfonic acid per liter. Flow rate was set at 0.7 ml/min and peak area detection was at 205 nm. Based on this initial injection, leptine I elution occurred about 8–9 min after sample injection. Thus, a Gilson FC 205 fraction collector in line with the HPLC was programmed to collect leptine I fractions from 8 and 12 min after sample injection. Fractions were eluted at a rate of 0.28 min per collection tube. Sample injections were done in 20-μl aliquots. Aliquots (5–10 μl) from these fractions were spotted on a Whatman flexible-backed polyester silica plate. Leptine I was visualized by spraying the plate with 0.11 M potassium iodide and 0.6 mM bismuth subnitrate in 3.5 M acetic acid (Dragendorff's spray reagent). Fractions containing leptine I were individually transferred to a new and clean test tube. The total volume in each tube was marked with a pen, and each tube was dried down to half the volume by blowing off acetonitrile. Acetonitrile was evaporated in the fume hood by gently heating the tubes in a beaker containing water set on a hot plate and by applying air into each tube while heating. The remaining solution from each tube was subjected to a final solid-phase extraction by using a vacuum manifold and classic C_{18} Sep-pak cartridges. This process involved conditioning the cartridges with 3 ml methanol followed by 5 ml of the aqueous heptane sulfonic acid extraction reagent. The fractions were loaded on to each conditioned Sep-pak cartridge, followed by a 5-ml acetonitrile: water (20:80) rinse. Leptine I was eluted with 2 ml methanol. Quantification and purity of leptine I was determined by analytical HPLC. α-Solanine (0.1 mg/ml methanol) served as a standard.

Solutions of glycoalkaloids were prepared for electrophysiology with 0.01 M NaCl as reference. Glycoalkaloids were solubilized in 0.01 M NaCl with lowered pH ($\sim$2–3) with HCl, then brought to pH 5.2–6.3 with NaOH. Reference electrolyte (0.01 M NaCl) was treated similarly.

Electrophysiology. Responses of galeal chemosensilla were obtained by using a standard tip-recording technique (Hodgson et al., 1955). A whole body preparation was used with adult CPB affixed inverted on cork with tape, and the head was immobilized with a tungsten collar. The head was pulled to a prognathous position with a tungsten post pressed into the cork that hooked and immobilized the mandibles. A galea was then pulled laterally with a fine nylon thread roped around the maxillary palp and immobilized by pinning between two fine

tungsten needles inserted into the cork. Use of a whole-body preparation and restriction of insect movement allowed us to identify individual sensilla and record from most, if not all ~16 chemosensory sensilla present on individual galea. A sharpened tungsten electrode inserted into the abdomen of the insect served as a reference electrode. Silver wire inserted into a glass capillary, pulled and sized to fit over the tip of one galeal chemosensory hair and filled with treatment chemicals in 0.01M NaCl, served as a stimulating/recording electrode. Both electrodes were connected to a Grass P15D AC amplifier. Chemosensory responses were viewed on an oscilloscope, monitored with a loudspeaker, and digitized for storage and analysis on a computer with Sapid (Smith et al., 1990) and AutoSpike (Syntech, Hilversum, The Netherlands) software. Numbers of nerve impulses within the first 500msec following the onset of stimulation were counted for analyses.

Experimental Protocol. In the first experiment, individual glycoalkaloids and 0.01 M NaCl reference stimulus treatments were tested successively on 5–16 hairs per galea. At least 3 min were allowed between stimulation of individual hairs. Concentrations of glycoalkaloids tested were 0.01, 0.1, and 1 mM for both male and female CPB. Leptine I concentrations tested also included 0.03 and 0.3 mM for construction of an expanded dose–response curve for female CPB. All leptine I concentrations were tested on at least 18 hairs from two to four female CPB galea, with the exception of 0.03 mM (10 hairs from one female CPB). For 1 mM solanine, 70 hairs from five females and three males were recorded; for 1 mM tomatine, 60 hairs from four females and two males were sampled. Contact with sensory hairs was maintained for at least 30 seconds to detect any glycoalkaloid-induced bursting of sensory neurons. Time to first burst was noted, and stimulation ended after a 60-sec period.

The second experiment investigated the effect of solanine on responses to leptine I. Treatments for this experiment were tested in the following order: 0.01 M NaCl, 0.3 mM leptine I (peak of dose–response curve), 1 mM solanine, 0.3 mM leptine I + 1 mM solanine, 0.3 mM leptine I. Records were obtained from nine hairs for one female CPB.

One-way analysis of variance was performed on log $(x + 1)$ transformed data to test for treatment effects on nerve impulse frequency. Duncan's multiple range test at 5% confidence level was used to test for differences between treatment means (Snedecor and Cochran, 1967).

RESULTS

Of the three solanaceous glycoalkaloids tested, only leptine I elicited a normal chemoreceptor response from a single neuron (Figure 2B). Responses increased

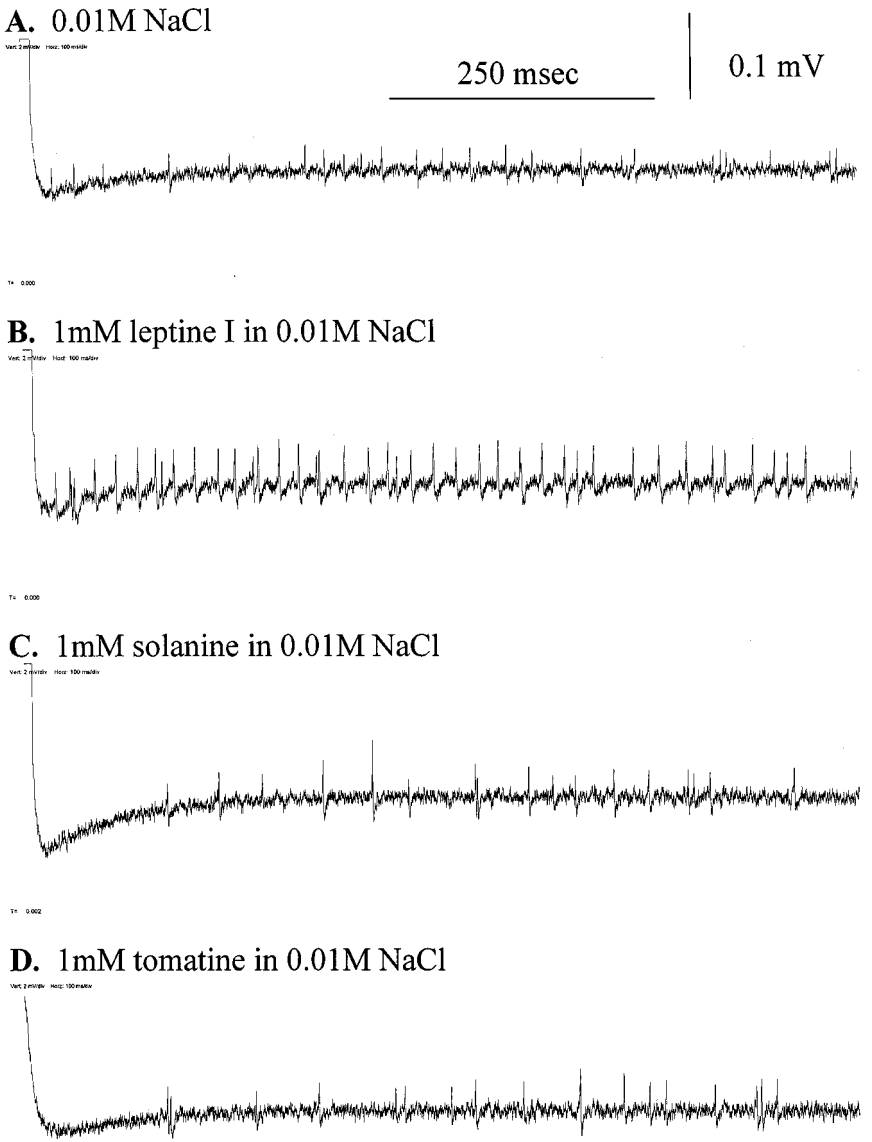


FIG. 2. Neural responses from chemosensory hairs on adult CPB maxillary galea to glycoalkaloids. Traces represent 1 sec after stimulation of sensory hair with: (A) 0.01 M NaCl reference solution, and (B) 1 mM leptine I, (C) 1 mM solanine, and (D) 1 mM tomatine, all in 0.01 M NaCl.

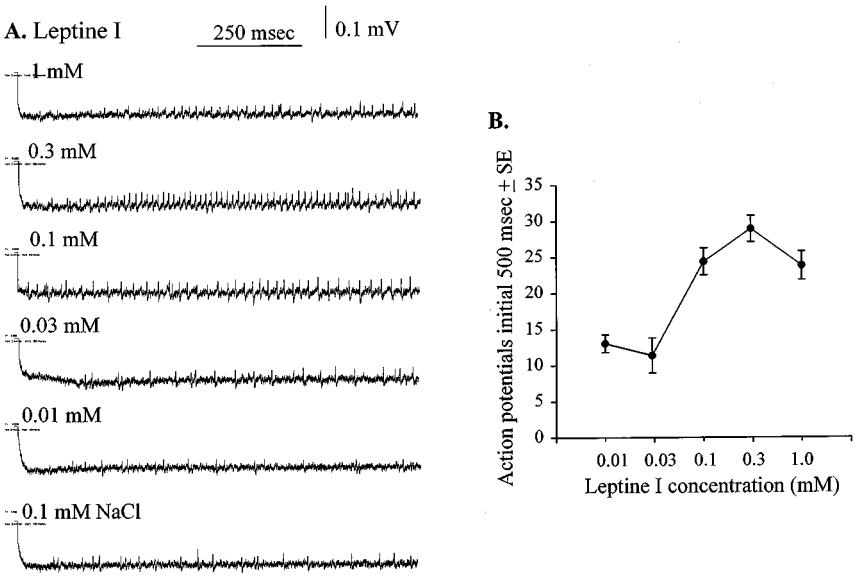


FIG. 3. (A) Representative neural responses to increasing concentration of leptine I. Traces represent 1 sec after stimulation of sensory hair with leptine I. (B) Dose-response curve for adult female CPB galeal chemosensory neurons in response to increasing concentrations of leptine I in 0.01 M NaCl. Action potentials were counted for the initial 500 msec following stimulation. $N = 10$–44 replicates/dose.

in a dose-dependent manner (Figure 3A and B), with a threshold at or above 0.03 mM and maximal response at 0.3 mM concentration. Leptine I did not elicit bursting activity below 1 mM concentration; at 1 mM weak bursting activity was observed in 9% (3/35) of hairs recorded. Percent of hairs stimulated responding to the leptine I concentrations were 12% (3/25) at 0.01 mM, 50% (5/10) at 0.03 mM, 88% (22/25) at 0.1 mM, 100% (18/18) and 83% (29/35) for 0.3 mM and 1 mM leptine I, respectively.

Two glycoalkaloids closely related to leptine I, solanine and tomatine, did not elicit responses greater than the control (Figure 2C and D), with bursting of multiple neurons (spikes of two or three amplitudes) observed only with the 1 mM treatments (Figure 4A and B). Bursting did occur within 60 sec in 53% (42/79) of hairs tested with 1 mM solanine, with an average delay of 13 sec. For 1 mM tomatine, bursting occurred in 32% (19/60) of hairs, with an average delay of 29 sec.

Although solanine has no stimulatory effect on this chemoreceptor neuron, it does have a depressive effect on this neuron's response to leptine I that lasts for at least 3 min (Figure 5).

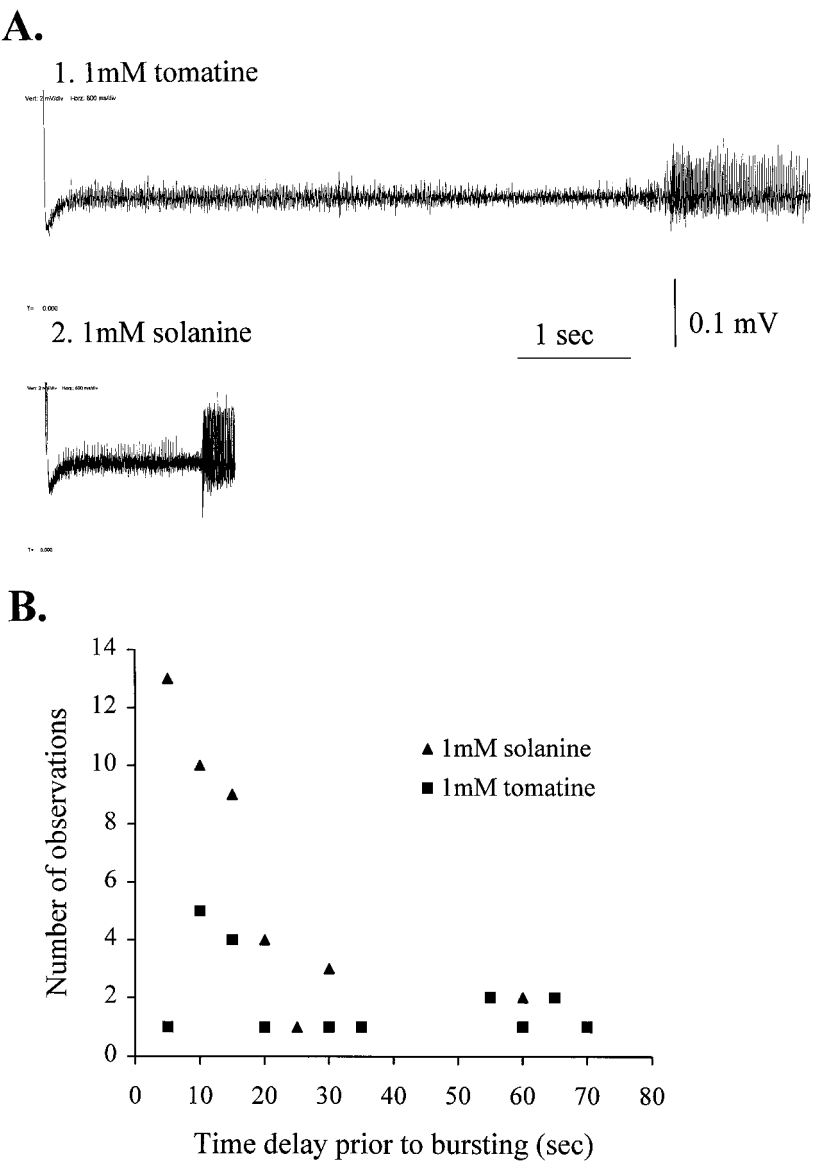


FIG. 4. Bursting response of neurons within adult CPB galeal chemosensory hairs. (A) Neural responses to 1 mM tomatine in 0.01 M NaCl and 1 mM solanine in 0.01 M NaCl illustrating bursts of chemosensory neurons. (B) Plot showing delay times before bursting activity for solanine and tomatine stimuli.

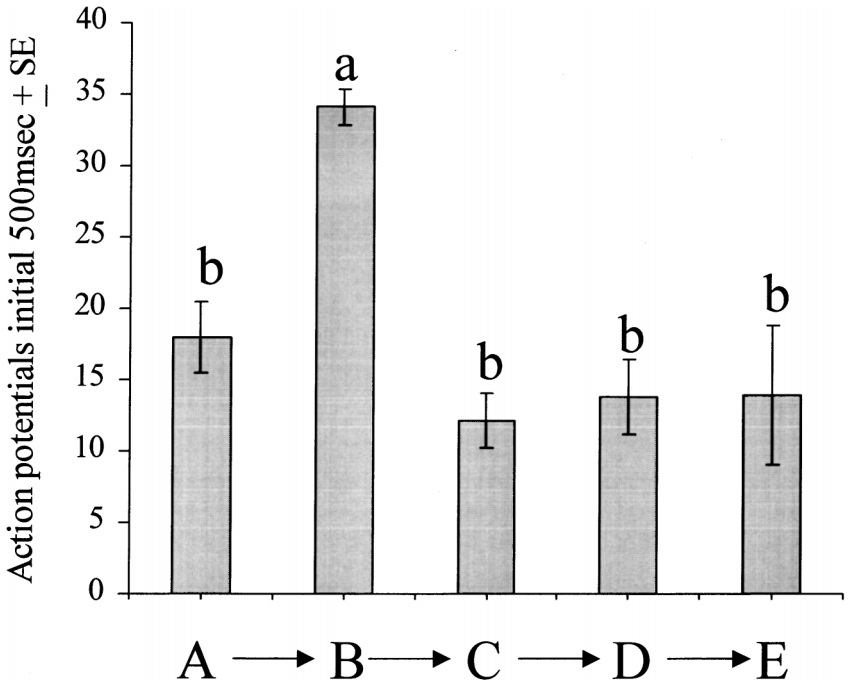


FIG. 5. Mean neural responses from adult female CPB galeal chemosensory hairs to sequential stimuli. Treatments were applied to individual hairs in the following order with three minutes between treatments: (A) 0.01 M NaCl, (B) 0.3 mM leptine I, (C) 1 mM solanine, (D) 0.3 mM leptine I + 1 mM solanine, and (E) 0.3 mM leptine I. $N = 9$ hairs/treatment. Different letters above the bars indicate significant differences between treatment means at the $\alpha = 0.05$ level.

DISCUSSION

We report here the first recordings of a specific chemosensory neural response to a glycoalkaloid in CPB. This slowly adapting response to leptine I is largely of a single neuron and contrasts with the lack of response to two other host plant glycoalkaloids, solanine and tomatine, on adult CPB chemosensory hairs.

The main differences in the three molecules tested here lie in the acetyl group at C-23 of the glycoalkaloid carbon skeleton in leptine I, as well as in the sugar group (Figure 1). Perhaps the differential effect observed is a function of different solubilities of individual glycoalkaloid molecules, with leptine I more readily solubilized and available at the receptor site. The more amphipathic tomatine and solanine may dissolve into and disrupt the dendritic membrane and thereby

elicit sporadic firing of several neurons and bursts of nerve impulse activity in adult CPB galeal taste hairs.

Specialist deterrent chemoreceptors identified for several species of larval Lepidoptera (Schoonhoven, 1982) respond to the alkaloids quinine and strychnine, as well as azadirachtin. In *Pieris* spp., receptors sensitive to cardenolides (threshold of 0.1–0.3 μM) occur in both adults (Stadler et al., 1995) and larvae (van Loon and Schoonhoven, 1999). These compounds are present in cruciferous plants and deter both feeding and oviposition. Such deterrent receptor neurons have only recently been reported for beetles in the family Chrysomelidae. Chyb et al. (1995) showed chemosensory responses in adult western corn rootworm (*Diabrotica virgifera* LeConte) to the feeding deterrents β-hydrastine and strychnine. They also found chemosensory responses to cucurbitacin B isolated from host plants in the family Cucurbitaceae. Cucurbitacins, strong feeding deterrents for most insects, act as potent arrestants and feeding stimulants for western corn rootworm and a small group of closely related species. Messchendorp et al. (1998) reported a deterrent receptor in CPB larvae in epipharyngeal sensilla that respond to the glucosinolate, sinigrin, and a synthetic sesquiterpene analogue, drimane, with threshold concentrations of 0.01 and 0.1 mM, respectively. Maximal numbers of action potentials to drimane were in response to 1 mM. Our observations of the first deterrent receptor neuron in adult CPB reveal a similar response range between 0.03 and 0.3 mM for leptine I. Both sinigrin and the drimane analog are feeding deterrents, but are isolated from non host plant species from the families Cruciferae and Polygonaceae, respectively. Adult CPB response to leptine I is similar to the *Pieris* spp. response to cardenolide in one respect, namely, both are deterrent receptor neuron responses to particular compounds from unacceptable plants in their own host-plant families.

Glycoalkaloids and other deterrents reduce neuronal responses to chemicals that stimulate feeding in CPB. Response to 10 mM γ-aminobutyric acid (GABA) was completely abolished following stimulation of the same hair with 0.5 mM solanine for 20 sec (Mitchell and Harrison, 1984); 1 mM tomatine, papaverine, and sparteine also significantly reduced this response (Mitchell, 1987). Response to 10 mM sucrose was similarly inhibited by 1 mM quinine and papaverine (Mitchell, 1987). Solanine and tomatine suppress neuronal response to (*E*)-2-hexenol, which Mitchell and McCashin (1994) suggest stimulates the same cell as sucrose, alanine, and GABA in some adult CPB galeal chemosensilla. Messchendorp et al. (1998) conclude that drimane inhibits the response of a sucrose cell in larval CPB epipharyngeal sensilla. Schoonhoven (1982) discussed the perception of antifeedants at the sensory level and identifies at least two possible mechanisms: (1) stimulation of specialized receptors by feeding deterrents, or (2) modification of the activity of receptors responding to feeding stimulants. Previous reports provide evidence for the latter mechanism of feeding deterrence. Our observation of sensory neurons

responsive to leptine I provides evidence for both mechanisms. Interestingly, here we also show suppression of a deterrent sensory neuron responsive to submillimolar levels of leptine I by millimolar levels of another glycoalkaloid, solanine, for which no specific taste receptor was identified. Thus, glycoalkaloid effects may be modulated by related compounds, and such interaction effects among the complex mixture of chemicals in host plant foliage must be considered when drawing conclusions about behavioral effects and mechanisms of host-plant selection.

Responses to solanine and tomatine were consistent with those described by Mitchell and Harrison (1985) and Mitchell (1987) for chemosensory neurons within galeal sensilla and by Stürckow (1959) for neurons within sensilla on the palps and tarsi. In both cases, the responses consisted of irregular firing of several neurons and bursting activity after a delay of several seconds. Discovery by us of an identifiable sensory neuron for leptine I provides a mechanism by which glycoalkaloids of the Solanaceae are detected by CPB. Our results not only establish a neural pathway for detection of leptine I but also provide a mechanism of feeding deterrence and resistance to CPB feeding attributed to this glycoalkaloid (Stürckow and Lüw, 1961; Tingey, 1984; Sinden et al., 1986). Previous experiments to determine the effects of leptines on feeding by CPB have either involved correlation of reduced consumption of foliage with levels of leptine, or feeding tests with leptine of partial (60%) purity. Feeding bioassays with purified leptine I coupled with electrophysiological observations will establish input–output relationships to more conclusively relate behavioral effects of leptine I to neural responses of identified chemoreceptor neurons.

Acknowledgments—Critical reviews of the manuscript were kindly provided by Prof. Dr. B. K. Mitchell, Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, and Prof. Dr. F. E. Hanson, Department of Biological Sciences, University of Maryland–B. C., Baltimore, Maryland, USA. J.C.D. gratefully acknowledges Dr. Autar Mattoo and Dr. Barbara Leonhardt for support and encouragement.

REFERENCES

CHYB, S., EICHENSEER, H., HOLLISTER, B., MULLIN, C. A., and FRAZIER, J. L. 1995. Identification of sensilla involved in taste mediation in adult western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *J. Chem. Ecol*. 21:313–329.

DICKENS, J. C. 1999. Predator-prey interactions: Olfactory adaptations of generalist and specialist predators. *Agric. Forest Entomol*. 1:47–54.

DICKENS, J. C. 2000. Orientation of Colorado potato beetle to natural and synthetic blends of volatiles emitted by potato plants. *Agric. Forest Entomol*. 2:167–172.

HARRISON, G. D., and MITCHELL, B. K. 1988. Host plant acceptance by geographic populations of the Colorado potato beetle, *Leptinotarsa decemlineata*: Role of solanaceous alkaloids as sensory deterrents. *J. Chem. Ecol*. 14:777–788.

HODGSON, E. S., LETTVIN, J. Y., and ROEDER, K. D. 1955. Physiology of a primary chemoreceptor unit. *Science* 122:417–418.

KOWALSKI, S. P., DOMEK, J. M., DEAHL, K. L., and SANFORD, L. L. 1999. Performance of Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say), reared on synthetic diets supplemented with *Solanum* glycoalkaloids. *Am. J. Potato Res*. 76:305–312.

KOWALSKI, S. P., PEREZ, F. G., SANFORD, L. L., and DEAHL, K. L. 2000. Partial preparative purification of leptine I from foliage of the wild potato, *Solanum chacoense* (Bitt.). *Prep. Biochem. Biotechnol*. 30:133–144.

LAWSON, D. R., VEILLEUX, R. E., and MILLER, A. R. 1993. Biochemistry and genetics of *Solanum chacoense* steroidal alkaloids: Natural resistance factors to the Colorado potato beetle. *Curr. Top. Bot. Res*. 1:335–352.

MESSCHENDORP, L., SMID, H. M., and VAN LOON, J. J. A. 1998. The role of an epipharyngeal sensillum in the perception of feeding deterrents by *Leptinotarsa decemlineata* larvae. *J. Comp. Physiol*. 183:255–264.

MITCHELL, B. K. 1987. Interactions of alkaloids with galeal chemosensory cells of Colorado potato beetle. *J. Chem. Ecol*. 13:2009–2022.

MITCHELL, B. K. 1994. The chemosensory basis of host-plant recognition in Chrysomelidae, pp. 141–151, *in* P. H. Jolivet, M. L. Cox, and E. Petitpierre (eds.). Novel Aspects of the Biology of Chrysomelidae. Kluwer Academic, Dordrecht, The Netherlands.

MITCHELL, B. K., and HARRISON, G. D. 1984. Characterization of galeal chemosensilla in adult Colorado potato beetle, *Leptinotarsa decemlineata. Physiol. Entomol*. 9:49–56.

MITCHELL, B. K., and HARRISON, G. D. 1985. Effects of *Solanum* glycoalkaloids on chemosensilla in the Colorado potato beetle: A mechanism of feeding deterrence? *J. Chem. Ecol*. 11: 73–83.

MITCHELL, B. K., and MCCASHIN, B. G. 1994. Tasting green leaf volatiles by larvae and adults of Colorado potato beetle, *Leptinotarsa decemlineata. J. Chem. Ecol*. 20:753–769.

MURRAY, K. D., DRUMMOND, F. A., ALFORD, A. R., STORCH, R. H., and BENTLEY, M. D. 1996. Citrus limonoid effects on Colorado potato beetle larval survival and development. *Entomol. Exp. Appl*. 80:503–510.

SCHOONHOVEN, L. M. 1982. Biological aspects of antifeedants. *Entomol. Exp. Appl*. 31:57–69.

SIKINYI, E., HANNAPEL, D. J., IMERMAN, P. M., and STAHR, H. M. 1997. Novel mechanism for resistance to Colorado potato beetle (Coleoptera: Chrysomelidae) in wild *Solanum* species. *J. Econ. Entomol*. 90:689–696.

SINDEN, S. L., SANFORD, L. L., CANTELO, W. M., and DEAHL, K. L. 1986. Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense. Environ. Entomol*. 15:1057–1062.

SINDEN, S. L., SANFORD, L. L., CANTELO, W. W., and DEAHL, K. L. 1988. Bioassays of segregating plants: A strategy for studying chemical defenses. *J. Chem. Ecol*. 14:1941–1950.

SMITH, J. J. B., MITCHELL, B. K., ROLSETH, B. M., WHITHEAD, A. T., and ALBERT, P. J. 1990. SAPID Tools: Micro-computer programs for analysis of multi-unit nerve recordings. *Chem. Senses* 15: 253–270.

SNEDECOR, G. W., and COCHRAN, W. G. 1967. Statistical Methods. The Iowa State University Press, Ames, Iowa, pp. 258–275.

STADLER, E., RENWICK, J. A. A., RADKE, C. D., and SACHDEV-GUPTA, K. 1995. Tarsal contact chemoreceptor response to glucosinolates and cardenolides mediating oviposition in *Pieris rapae. Physiol. Entomol*. 20:175–187.

STÜRCKOW, B. 1959. Über den geschmackssinn und den tastsinn *von Leptinotarsa decemlineata* Say (Chrysomelidae). *Z. Vergl. Physiol*. 42:255–302.

STÜRCKOW, B., and LÖW, I. 1961. Die wirkung einiger *Solanum*-alkaloidglykoside auf den kartoffelkäfer, *Leptinotarsa decemlineata* Say. *Entomol. Exp. Appl*. 4:133–142.

TINGEY, W. M. 1984. Glycoalkaloids as pest resistance factors. *Am. Potato J*. 61:157–167.

TINGEY, W. M., and YENCHO, G. C. 1994. Insect resistance in potato: A decade of progress, pp. 405–425, *in* G. W. Zehnder, R. K. Jansson, M. L. Powelson, and K. V. Raman (eds.). Advances in Potato Pest Biology and Management. APS Press, St. Paul, Minnesota.

VAN LOON, J. J. A, and SCHOONHOVEN, L. M. 1999. Specialist deterrent chemoreceptors enable *Pieris* caterpillars to discriminate between chemically different deterrents. *Entomol. Exp. Appl*. 91: 29–35.

YENCHO, G. C., KOWALSKI, S. P., KENNEDY, G. C., and SANFORD, L. L. 2000. Segregation of leptine glycoalkaloids and resistance to Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) in F2 *Solanum tuberosum* (4×) × *S. chacoense* (4×) potato progenies. *Am. J. Potato Res*. 77:167–178.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

ELECTROPHYSIOLOGICAL AND BEHAVIORAL RESPONSES OF FEMALE *Helicoverpa armigera* TO COMPOUNDS IDENTIFIED IN FLOWERS OF AFRICAN MARIGOLD, *Tagetes erecta*

TOBY J. BRUCE* and ALAN CORK

Natural Resources Institute, University of Greenwich
Central Avenue, Chatham Maritime
Kent ME4 4TB, UK

(Received December 12, 1998; accepted February 7, 2001)

Abstract—Seven electrophysiologically active compounds were detected in air-entrained headspace samples of live flowers of *Tagetes erecta* analyzed by gas chromatography (GC) linked to a female *Helicoverpa armigera* electroantennograph (EAG) using polar and nonpolar capillary columns. These compounds were subsequently identified using GC linked to mass spectrometry as benzaldehyde, (*S*)-(−)-limonene, (*R*,*S*)-(±)-linalool, (*E*)-myroxide, (*Z*)-β-ocimene, phenylacetaldehyde, and (*R*)-(−)-piperitone. Electrophysiological activity was confirmed by EAG with a 1-μg dose of each compound on filter paper eliciting EAG responses that were significantly greater than the solvent control response from female moths. Wind-tunnel bioassays with *T. erecta* headspace samples, equivalent to 0.4 flower/hr emission from a live flower, elicited a significant increase in the number of upwind approaches from female *H. armigera* relative to a solvent control. Similarly, a seven-component synthetic blend of EAG-active compounds identified from *T. erecta* presented in the same ratio (1.0 : 1.6 : 0.7 : 1.4 : 0.4 : 5.0 : 2.7, respectively) and concentration (7.2 μg) as found in the natural sample elicited a significant increase in the number of upwind approaches relative to a solvent control during a 12-min bioassay that was equivalent to that elicited by the natural *T. erecta* floral volatiles.

Key Words—*Helicoverpa armigera*, *Tagetes erecta*, floral attractant, kairomone.

INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), is a serious insect pest throughout much of its distribution in tropical and subtropical

* To whom correspondence should be addressed.

0098-0331/01/0600-1119$19.50/0

regions of the Old World. Its economic importance has increased in recent years because of its propensity for developing resistance to insecticides. Additional means of controlling *H. armigera* are urgently needed because even well—organized resistance management programs, such as the one developed in Australia, have only reduced the rate at which insecticide resistance has evolved rather than stopping it (Forrester et al., 1993).

Improved understanding of *H. armigera*–host plant interactions could increase options available for innovative pest management. One possibility is to develop attractants for female moths that could be used in a lure-and-kill control program, such as the one described by Landolt et al. (1991) for the cabbage looper moth, *Trichoplusia ni*. Female *H. armigera* can produce 1000–1500 individually laid eggs during their lives (Fitt, 1989). To be effective, control strategies based on lures developed from plant odors do not have to be as attractive as Lepidopterous female sex pheromones because killing one female moth before she can oviposit is equivalent to killing many larvae. However, for practical usage in population suppression, floral baited traps would still have to capture or kill a sufficient proportion of the female moths entering a control area to prevent the subsequent larval population exceeding the economic threshold.

Night observations of *H. armigera* have shown that oviposition is interspersed with nectar foraging (Roome, 1975) suggesting that female moths are attracted to flowers while ovipositing. Traps attracting female *H. armigera* could be used for monitoring populations and, for this purpose, lower levels of attraction might be sufficient provided that enough moths were captured to give a reliable indication of the potential threat to the crop.

The role played by olfactory cues in host-plant location by polyphagous moth species, such as *H. armigera*, is uncertain. Some authors (e.g., Ramaswamy, 1988) have suggested that for flight, orientation, and landing no specific olfactory cues are involved and that host location and acceptance are mostly a random process in which phototaxis and anemotaxis play a greater role than chemoanemotaxis, whereas others (e.g., Rembold et al., 1991) have hypothesized that polyphagous insects utilize volatile olfactory cues for host recognition and location. The presence of flowers has been shown to be important for host-plant selection by *H. armigera* (Roome, 1975; Fitt, 1989), and oviposition has been shown to occur on flowering nonhost plants in preference to nonflowering host plants (Firempong and Zalucki, 1990). Thus, if volatile olfactory cues are important in host location, flowers would appear to be the most likely source of attractants.

African marigold, *Tagetes erecta*, is a favored host for oviposition by *H. armigera* and has been successfully used as a trap crop (Patel and Yadav, 1992; Srinivasan et al., 1994). Given the apparent uncertainty about the role of plant odors in host selection by female *H. armigera*, the present study was undertaken to identify electrophysiologically active compounds from headspace samples of *T. erecta* flowers and to elucidate their behavioral activity in a wind-tunnel bioassay.

METHODS AND MATERIALS

Plant Material. An Indian strain of *T. erecta* was grown from seed in glasshouses at the Natural Resources Institute (NRI), Chatham Maritime, UK. Samples of floral volatiles were collected by air entrainment of individual freshly cut flowers or individual flowers still attached to growing plants, referred to here as a live flower. Acetone-washed and air-dried three-necked Quickfit flange lids and flasks (500 ml) were clamped together and a live flower inserted through a B52 neck. The gap between the stem and the flask was packed with charcoal (ca. 20 g, 10–18 mesh, from BDH) and held in place with aluminum foil. Incoming air was filtered through a charcoal filter (20 g, 10–18 mesh) and drawn over the flower at 2 liters/min for 10–14 hr by a diaphragm pump (Charles Austin Pumps Ltd., Weybridge, UK) connected to a Porapak Q filter (200 mg, 50–80 mesh, Phase Separations) that had previously been conditioned by Soxhlet extraction with chloroform for 8 hr, then air dried. Entrained volatiles were subsequently eluted from the Porapak Q filters with dichloromethane (2 ml) and stored at -20°C. A similar setup was used to entrain volatiles from freshly cut flowers except that the flowers were held in fully enclosed flasks.

Experimental Insects. A laboratory strain of *H. armigera* originating from the International Center for Research in the Semi-Arid Tropics, India, was maintained on a semisynthetic chickpea diet at NRI, by using rearing procedures similar to those described by Armes et al. (1992). Groups of 12 male and 12 female moths were kept in Perspex containers (20 cm diam. $\times$ 36 cm high) with metal gauze lids and provided with a 10% sucrose solution with vitamin supplement. The culture was maintained at 25°C, 50% relative humidity, and 14L:10D photoperiod.

Electroantennography (EAG). EAG recordings were carried out using 1- to 3-day-old adult female *H. armigera*. Whole insects were used, held down on a plasticine block by a strip of expanded polystyrene. A glass recording microelectrode filled with saline (0.5 M NaCl in distilled water) was mounted on a micromanipulator (Leitz) and inserted into the distal end of one antenna and a similar reference microelectrode inserted into the proximal end of the other antenna. The microelectrodes were connected via Ag–AgCl junctions to a high-impedance AC/DC amplifier (model UN06, Syntech). EAG data were recorded, stored, and processed on a PC using Turbochrom 4.0 software (Perkin Elmer Ltd., Beaconsfield, UK).

The EAG activity of entrained headspace samples was tested by placing an aliquot of test solution (2–20 μl) onto a Whatman No. 4 filter paper strip (5×15 mm) placed inside a Pasteur pipet and nitrogen pulsed (100 ml/min) through the pipet for 3 sec over the EAG preparation. The EAG preparation was exposed to pulses at 1-min intervals as initial tests had shown that the EAG preparation had fully recovered in that time. EAG responses were measured and compared with those generated by the same volume of solvent on filter paper. When an entrainment

sample was found to elicit an EAG response at least 30% greater than that of the solvent alone, linked GC-EAG analyses were carried out.

The EAG activity of synthetic compounds was measured at a single dose (1 μg on filter paper). Samples were prepared as above by using solutions in dichloromethane (1 mg/ml). Each compound was tested five times on each of nine 1- to 9-day-old female *H. armigera*. The order of exposure of compounds to the EAG preparations was randomized, and only the last four of the five EAG responses elicited per compound were used, because these were found to be more reproducible. Control stimuli (1 μl dichloromethane) were presented at the beginning and end of each EAG analysis. EAG responses elicited by each compound were averaged for each insect tested and compared with the averaged responses for the solvent control with the Student's *t* test.

Gas Chromatography (GC) and Linked GC-EAG Analyses. Linked GC-EAG analyses were conducted as described by Cork et al. (1990) using a modified Carlo Erba 5300 Mega series gas chromatograph equipped with two fused silica capillary columns coated with polar CP-Wax 52CB (30 m $\times$ 0.32 mm ID, Carbowax equivalent, Chrompack) and nonpolar CP-Sil 5CB (25 m $\times$ 0.32 mm ID, methylsilicone equivalent, Chrompack). The GC carrier gas was helium (40 kPa). Samples were introduced onto a GC column in splitless mode using a Grob split/splitless injector at 200°C with the flame ionization detector (FID) at 250°C. The GC oven was held for 2 min at 50°C, then temperature programmed at 20°C/min to 80°C and then at 5°C/min to 240°C. GC column effluent was split 50 : 50 between the FID and EAG outlets. GC column effluent to the EAG preparation was stored in a silanized glass reservoir inside the GC oven and expelled over the EAG preparation for 3 sec at 17-sec intervals in a stream of nitrogen (250 ml/min).

Linked GC-EAG analyses of entrained flower volatiles were replicated four times using different insects and carried out on four different headspace collections. GC retention times of components associated with EAG activity were converted into retention indices (RI) by comparison with the retention times of saturated, straight-chain hydrocarbons; thus, *n*-tetradecane = 1400. The quantity of each compound in an aliquot was calculated by comparison of peak area with that of a known quantity of an external standard, 20 ng tetradecyl acetate, and not corrected for FID response factor.

The enantiomeric composition of natural and synthetic compounds was determined by GC analysis on a β-cyclodextrin column (50 m $\times$ 0.25 mm ID, Scientific Glass Engineering Ltd., Milton Keynes, UK) in a Varian 3700 GC using split injection with helium carrier gas (150 kPa) and oven temperature held at 90°C for 2 min and then programmed at 2°C/min to 190°C.

Analysis by Linked Gas Chromatography–Mass Spectrometry (GC-MS). Headspace collections were analyzed by GC-MS using a Carlo Erba 5160 Mega Series GC linked directly to an Ion Trap Detector 700 mass spectrometer (70 eV, 200°C) (Finnigan MAT, Hemel Hempstead, UK) in electron impact (EI) mode.

GC conditions were as described above. Compounds were identified by comparing their mass spectra with library spectra (Adams, 1995) and confirmed by comparing GC RI and EI-MS with synthetic standards.

Synthetic Chemicals. Benzaldehyde, (*S*)-(−)-limonene [(*S*)-(−)-4-isopropenyl-1-methylcyclohexene], (*R*)-(+)-limonene, (*R,S*)-(±)-linalool [(*R,S*)-3,7-dimethyl-1,6-octadien-3-ol], (*R*)-(−)-linalool, and phenylacetaldehyde were purchased from Sigma-Aldrich Chemical Company Ltd., Gillingham, UK. Myroxide [2,2-dimethyl-3-(3-methylpenta-2,4-dienyl) oxirane] was provided by Quest International, Ashford, UK, as a 50 : 50 mixture of *E*- and *Z*-isomers. The isomers were separated on a flash silica gel column (silica gel 60, 0.040- to 0.063-mm particle size, Merck, Darmstadt, Germany) eluted with aliquots (100 ml) of 40–60 petroleum spirit in which 1%, 2%, 3%, and 5% diethyl ether was added sequentially. The isomeric purity of collected fractions was subsequently determined by GC analysis, and those containing >95% (*E*)-myroxide were combined and used without further purification. β-Ocimene (3,7-dimethyl-1,3,6-octatriene), purchased from Fluka (Gillingham, UK), contained 59% (*Z*)-β-ocimene, 1% (*E*)-β-ocimene, and 40% limonene. (*Z*)-β-Ocimene was separated from this mixture using flash silica gel column chromatography as described for isomers of myroxide. (*R*)-(−)-Piperitone (6-isopropyl-3-methyl-2-cyclohexen-1-one) was a gift from H.E. Daniel Ltd., Cleveland, UK. In order to obtain GC RI and ^{1}H NMR data on the (+)-enantiomer, an aliquot of the (−)-enantiomer was racemized in methanolic potassium hydroxide at room temperature for 24 hr and analyzed both by GC on the β-cyclodextrin column and by ^{1}H NMR in the presence of a chiral shift reagent, (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)-ethanol.

Wind-Tunnel Bioassay. A wind tunnel without a moving floor, but otherwise similar to that described by Miller and Roelofs (1978), was used to investigate the behavioral responses of female *H. armigera* to natural and synthetic blends of the putative kairomone components. The wind tunnel was made from a polyethylene sheet placed over a metal frame (225 × 60 × 60 cm). Airspeed, as measured by a smoke test, was 50 cm/sec. All bioassays were carried out under reduced lighting (0.8 lux) provided by a series of three bulbs (60 W) fitted with red filters and a variable power output positioned at 40-cm intervals 1.0 m above the wind tunnel and angled to provide even coverage over the whole apparatus. Bioassays were conducted at 25°C and 50% relative humidity during the first 2–3 hr of the scotophase, which corresponded to the natural time of nectar foraging and oviposition by female *H. armigera* (Roome, 1975).

Test samples (60 μl) were applied to Whatman No. 1 filter papers (7 cm diam.) from stock solutions. This was done in a separate room to avoid contamination. Filter papers were immediately clipped to a vertical support (30 cm height) positioned at the midpoint in the upwind end of the wind tunnel. Behavioral responses to solvent (dichloromethane) control, entrained floral volatiles from live flowers of *T. erecta*, and blends of synthetic compounds identified

from *T. erecta* in the same ratio and concentration as natural material were investigated.

Individual test moths were carefully released into the odor plume (as confirmed by smoke tests) from a holding container placed 200 cm downwind of the source immediately after introduction of a freshly prepared odor source. Each test moth was used only once. Maximum distance flown upwind and number of approaches to within 20 cm of the odor source were scored during a 12-min bioassay period. The control treatment was tested first to ensure there was no contamination from previous sessions. Test treatments were then bioassayed in a randomized order.

Wind-tunnel data on number of upwind approaches and furthest distance flown upwind were analyzed by the Kruskal-Wallis test (Statistical Package for Social Scientists for Windows Release 9.0.0) to determine if there was a significant difference between treatments. If significant, then test treatment medians were compared with control treatment medians using the Mann-Whitney U test.

RESULTS

GC-EAG Analyses. In GC analyses on a polar column with FID, 45 compounds were detected in volatiles from *T. erecta* flowers collected on Porapak Q. Of these compounds, seven consistently elicited EAG responses above background in linked GC-EAG analyses with female *H. armigera* on both polar and nonpolar columns. The EAG responses were typically −0.2 to −0.6 mV above a background of −0.8 mV. Retention indices of EAG-active compounds and abundance expressed as the quantity, relative to an external standard, entrained from a live flower per hour are given in Table 1. There was no apparent correlation between

TABLE 1. ELECTROPHYSIOLOGICALLY ACTIVE COMPOUNDS IDENTIFIED IN VOLATILES FROM LIVE *T. erecta* FLOWERS COLLECTED ON PORAPAK Q

Compound	Ratio with respect to benzaldehyde	Concentration (μg/live flower/hr)	Retention index			
			Polar column		Nonpolar column	
			Natural	Synthetic	Natural	Synthetic
(*S*)-(−)-Limonene	1.62	1.72	1200	1200	1017	1019
(*Z*)-β-Ocimene	0.36	0.38	1234	1232	1027	1028
(*E*)-Myroxide	1.39	1.47	1485	1483	1123	1126
Benzaldehyde	1.0	1.06	1522	1522	925	926
(*R,S*)-(±)-Linalool	0.68	0.72	1539	1541	1085	1088
Phenylacetaldehyde	4.36	4.61	1642	1642	1007	1011
(*R*)-(−)-Piperitone	2.7	2.86	1725	1723	1226	1225

the EAG activity of natural compounds eluting from the GC and their relative abundance.

GC-MS Analyses. Floral volatiles were analyzed by GC-MS to obtain EI-MS of compounds that eluted at the RI where EAG activity was observed in linked GC-EAG analyses. Tentative structural assignments were made on the basis of a comparison of EI-MS with library spectra (Adams, 1995). The assignments were confirmed by comparison of EI-MS and GC RI on polar and nonpolar columns of natural compounds with those of authentic synthetic compounds. Thus, the EAG-active compounds were identified as benzaldehyde, limonene, linalool, (*E*)-myroxide, (*Z*)-β-ocimene, phenylacetaldehyde, and piperitone. The configurations of the natural limonene, linalool, and piperitone were established by comparison of GC retention times with those of authentic synthetic compounds on a β-cyclodextrin column. Synthetic (*R*)-(+)- and (*S*)-(−)-limonene, (*R*)-(−)- and (*S*)-(+)-linalool, and (*R*)-(−)- and (*S*)-(+)-piperitone eluted at 18.25, 18.39, 27.83, 27.96, 40.22, and 40.37 min, respectively. Retention times of compounds in the natural sample were 18.42, 27.87, 27.99, and 40.26 min confirming the presence of (*S*)-(−)-limonene and (*R*)-(−)- and (*S*)-(+)-linalool (König et al., 1990) in a 1 : 2 ratio, and (*R*)-(−)-piperitone.

Comparison of Headspace Volatiles from Live and Cut Flowers. The relative proportions of each of the seven EAG-active compounds in headspace entrainments of individual live and cut *T. erecta* flowers were determined by GC analysis and compared by Student's *t* test. The proportions of aromatic compounds, benzaldehyde ($P = 0.035$) and phenylacetaldehyde ($P = 0.001$), were found to be lower in volatiles from cut flowers compared to those from live flowers while the proportion of (*Z*)-β-ocimene was higher ($P = 0.01$) (Table 2).

TABLE 2. COMPARISON OF RATIOS OF COMPOUNDS EMITTED FROM LIVE AND CUT *T. erecta* FLOWERS

Chemical	Cut flowers (%, mean ± SE)[a]	Live flowers (%, mean ± SE)[a]	P^b
(*S*)-(−)-Limonene	28.0 ± 5.7	16.5 ± 4.6	0.12
(*Z*)-β-Ocimene	14.9 ± 3.5	3.8 ± 1.0	0.01
(*E*)-Myroxide	8.5 ± 1.2	5.0 ± 2.0	0.13
Benzaldehyde	5.3 ± 2.9	9.0 ± 2.2	0.04
(*R*,*S*)-(±)-Linalool	1.2 ± 0.3	2.0 ± 1.3	0.52
Phenylacetaldehyde	8.5 ± 5.9	48.2 ± 8.3	0.01
(*R*)-(−)-Piperitone	33.7 ± 8.2	15.4 ± 4.2	0.06

[a] Amounts are expressed as a percentage of the total area under the GC peaks for the seven compounds.
[b] Unpaired *t* test comparing percentage means from cut and live flowers, $N = 5$.

TABLE 3. EAG RESPONSES ELICITED FROM FEMALE *H. armigera* BY SYNTHETIC COMPOUNDS[a]

Chemical	EAG responses (−mV, mean ± SE)	P^b
1 μl CH_2Cl_2	0.35 ± 0.05	
(*E*)-Myroxide	0.69 ± 0.05	<0.001
Benzaldehyde	0.61 ± 0.07	0.001
(*R*,*S*)-(±)-Linalool	0.59 ± 0.11	0.032
Phenylacetaldehyde	0.75 ± 0.08	<0.001
(*R*)-(−)-Piperitone	0.85 ± 0.16	0.001
(*Z*)-β-Ocimene	0.51 ± 0.06	0.003
(*S*)-(−)-Limonene	0.39 ± 0.03	0.012

[a](1 μg in 1 μl CH_2Cl_2, $N = 9$).
[b]Paired Student's t test between treatments and dichloromethane control means.

EAG Activity of Synthetic Compounds. At a 1-μg dose, each of the synthetic compounds identified from linked GC-EAG analyses elicited an EAG response that was significantly above that of the solvent control (Table 3). EAG responses ranged from −0.39 mV for (*S*)-(−)-limonene to −0.85 mV for (*R*)-(−)-piperitone at the 1-μg dose. The highest EAG responses were elicited by (*E*)-myroxide, (*R*)-(−)-piperitone, and phenylacetaldehyde, although differences in molecular weight and volatility of the compounds tested meant that data were not strictly comparable.

Wind-Tunnel Bioassay. An aliquot of *T. erecta* floral headspace collection from live flowers equivalent to 0.4 flower/hr was tested against a seven-component blend of synthetic compounds containing the same ratio and concentration of EAG-active compounds: 7.2 μg of benzaldehyde, (*S*)-(−)-limonene, (*R*,*S*)-(±)-linalool, (*E*)-myroxide, (*Z*)-β-ocimene, phenylacetaldehyde, and (*R*)-(−)-piperitone in a 1.0 : 1.6 : 0.7 : 1.4 : 0.4 : 5.0 : 2.7 ratio. An equivalent six-component blend in which (*E*)-myroxide was omitted was also tested. Flight responses were compared with those of insects tested when only the control treatment (dichloromethane) was present.

The number of upwind approaches to within 20 cm of the lure increased significantly when natural *T. erecta* floral odors were presented to female *H. armigera* compared to when moths were presented with a solvent control (Mann-Whitney U test, $P = 0.036$) (Figure 1). The six- and seven-component synthetic blends of EAG-active compounds elicited similar increases in number of upwind approaches (Mann-Whitney U test, $P = 0.015$ and $P = 0.006$, respectively). In addition, there was no significant difference between the number of upwind approaches elicited by the natural floral collections and the six- and seven-component synthetic blends. Mean furthest distances flown upwind to the natural floral volatile collection, six- and seven-component blends (171, 171 and 173 cm, respectively) from that to the

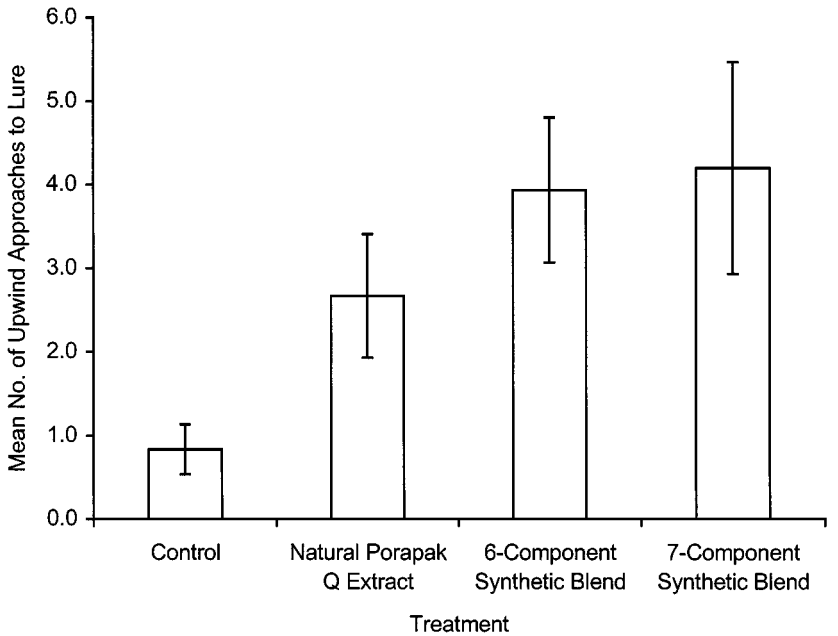


FIG. 1. Mean number of upwind approaches by female *H. armigera* to within 20 cm of air-entrained natural and synthetic blends of *T. erecta* floral odors in a wind tunnel ($N = 30$).

solvent control (143 cm), (Kruskal-Wallis test, $P = 0.25$) differences were not significant.

Replacement of (*E*)-myroxide with a 50 : 50 mixture of *Z*- and (*E*)-isomers in the seven-component synthetic blend resulted in a reduction in the number of upwind approaches to a point where it was not different from that of the solvent control (Mann-Whitney U test, $P = 0.70$). However, a five-component blend, omitting (*E*)-myroxide and (*Z*)-β-ocimene and in which (*R*)-(+)-limonene was used instead of (*S*)-(−)-limonene, was found to elicit a increase in the number of upwind approaches compared to the solvent control (Mann-Whitney U test, $P = 0.003$).

DISCUSSION

The present findings demonstrate that the polyphagous moth, *H. armigera*, can recognize and behaviorally respond to olfactory cues from a host plant in a no-choice situation. Significant increases in the number of upwind flight approaches by female *H. armigera* were observed in a wind-tunnel bioassay in response to floral volatiles collected from *T. erecta* and to synthetic blends of electrophysiologically

active compounds identified from that source (Figure 1). However, this does not preclude the possibility that other cues are involved in host recognition and selection by female *H. armigera*, such as the contrast of a flower silhouette or contact chemoreception upon alighting on a plant, as suggested by Kevan and Baker (1983).

Linked GC-EAG analysis, as originally conceived by Moorhouse et al. (1969), has proven to be of great utility in identifying components of insect sex pheromones and kairomones (Cork et al., 1990) even though the magnitude of EAG responses elicited by natural compounds was not necessarily related to their behavioral activity (Suckling et al., 1996). In the current study, the amplitude of EAG responses elicited by some compounds in linked GC-EAG analyses of floral volatiles, e.g., (*S*)-(−)-limonene, were difficult to discern above the high background responses (on average −0.78 mV) recorded from mechano- and thermoreceptors stimulated by the pulses of nitrogen-containing GC effluent. Nevertheless, the EAG activity of the compounds identified in *T. erecta* floral headspace samples by this procedure were subsequently confirmed by EAG tests using 1-μg doses of synthetic compounds on filter papers (Table 3). EAG activity of floral extracts and compounds identified in the extracts demonstrated that female *H. armigera* possessed chemoreceptors capable of detecting floral volatiles, and this was subsequently confirmed by the wind-tunnel bioassay results.

Two synthetic blends of EAG-active compounds identified from floral headspace samples of *T. erecta* elicited similar levels of upwind flight activity to the natural headspace samples collected from live flowers in the wind-tunnel bioassay. The dose used was approximately equivalent to that emitted by two live flowers over a 12-min period. Repeated upwind approaches observed in the wind-tunnel bioassays to synthetic and natural odor sources were typical of the "seeking flight" reported by Brantjes (1978) in a description of the sensory responses of night-flying moths to flowers. This implied that the presence of volatile host plant cues can induce upwind flight and sustained searching behavior in *H. armigera* and is of particular relevance to our understanding of the mechanisms involved in host location.

Five of the seven EAG-active compounds characterized from the volatiles produced by *T. erecta* have been implicated in the host location of related moth species: phenylacetaldehyde as an attractant for *H. armigera* and other spp. (Landolt et al., 1991); benzaldehyde, in blends containing phenylacetaldehyde, as a floral kairomone for the cabbage looper moth, *Trichoplusia ni* (Haynes et al., 1991); linalool (enantiomer unspecified) as an oviposition attractant for the light-brown apple moth, *Epiphyas postvittana* (Suckling et al., 1996), limonene (enantiomer unspecified) as an oviposition stimulant for *H. armigera* (Blaney and Simmonds, 1990), and piperitone (enantiomer unspecified) as an attractant of the European grapevine moth, *Lobesia botrana* (Gabel et al., 1992). As far as we are aware, there are no published reports of the electrophysiological or behavioral activity of benzaldehyde, (*R,S*)-(±)-linalool, (*E*)-myroxide; and (*R*)-(−)-piperitone specifically

to *H. armigera*, although Burguière et al. (2001) found benzaldehyde and (*R,S*)-(±)-linalool did elicit significant EAG responses from female *H. armigera*.

Linalool, myroxide, and piperitone are known constituents of the essential oil prepared from steam distillates of air-dried *T. erecta* (Gupta and Bhandari, 1974), but benzaldehyde and phenylacetaldehyde have not previously been described. Benzaldehyde and phenylacetaldehyde are prone to oxidation and thus might not occur in samples collected by steam distillation. Indeed, larger quantities of these two compounds were collected from air entrainments with live flowers than in entrainments with cut flowers. Because *H. armigera* is highly polyphagous, it is of interest to note that the behaviorally active volatiles identified from flowers of *T. erecta* also occur in some of its other host plants, although this does not necessarily mean that they have a significant role in the location of those plants. Thus, limonene was the only monoterpene found by Flath et al. (1978) in maize silks, although Buttery et al. (1980) subsequently found benzaldehyde and phenylacetaldehyde in maize silks and Buttery and Ling (1984) identified limonene and linalool in maize leaf volatiles. Limonene, benzaldehyde, and phenylacetaldehyde were identified in tobacco (Loughrin et al., 1990); benzaldehyde and (*R*)-(+)-limonene were identified in chickpea seed volatiles (Rembold et al., 1989). Limonene has been found in the flowers of sunflower (Eteviant et al., 1984) and as a tomato leaf volatile (Buttery et al., 1987).

In preliminary field testing in Israel, traps baited with a four-component synthetic blend of benzaldehyde, (*S*)-(−)-limonene, (*R,S*)-(±)-linalool, and phenylacetaldehyde caught more moths than unbaited control traps ($P = 0.002$), although the actual numbers of moths captured were low (Bruce et al., 2001). It is anticipated that by addition of other behaviorally active compounds and a better understanding of the role of these chemicals in the behavior of *H. armigera* a useful floral bait can be developed for use in monitoring female populations of this economically damaging pest. Floral odors might also be worthy of consideration in breeding programs where the objective is to breed out traits attractive to *H. armigera*.

Acknowledgments—The authors thank Dr. K. Srinivasan, Nagarjuna Agricultural Research and Development Institute, India, for suggesting this work on African marigold and provision of seed material; Quest International Ltd., for a sample of synthetic myroxide; H.E. Daniel Ltd., Cleveland, UK, for a sample of (*R*)-(−)-piperitone; and ICRISAT, Patancheru, India, for supplying the pupae used to start the culture of *H. armigera*. The authors are particularly grateful to Prof. D. R. Hall for constructive comments and advice on the manuscript. T.J.B was funded by a NRI Research Studentship.

REFERENCES

ADAMS, R. P. 1995. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing, Carol Stream, Illinois.

ARMES, N. J., BOND, G. S., and COOTER, R. J. 1992. The laboratory culture and development of *Helicoverpa armigera*. Natural Resources Inst. Bull. 57. Natural Resources Institute, Chatham, UK.

BLANEY, W. M., and SIMMONDS, M. S. J. 1990. The role of chemicals from legumes in mediating host selection by adults and larvae of *Helicoverpa armigera*: A behavioural and electrophysiological study, *in* Host Selection Behaviour of *Helicoverpa armigera*. Summary Proceedings of the First Consultative Group Meeting, March 5–7, 1990, ICRISAT Center. ICRISAT, Patancheru, India.

BRANTJES, N. B. M. 1978. Sensory responses to flowers in night-flying moths. *In* A. J. Richards (ed.). The Pollination of Flowers by Insects. Academic Press, London.

BRUCE, T. J., CORK, A., HALL, D. R., and DUNKELBLUM, E. 2001. Laboratory and field evaluation of floral odours from African marigold, *Tagetes erecta*, and sweet pea, *Lathyrus odoratus*, as kairomones for the cotton bollworm *Helicoverpa armigera*. IOBC Bulletin Vol. 24. Proceedings of a conference, "Use of Pheromones and Other Semiochemicals in Integrated Control." September 2000.

BURGUIÈRE, L., MARION-POLL, F., and CORK, A. 2001. Electrophysiological responses of female *Helicoverpa armigera* (Hubner) (Lepidoptera, Noctuidae) to synthetic odours. *J. Insect Physiol.* 47:509–514.

BUTTERY, R. G., and LING, L. C. 1984. Corn leaf volatiles: identification using tenax trapping for possible insect attractants. *J. Agric. Food. Chem.* 32:1104–1106.

BUTTERY, R. G., LING , L. C., and TERANISHI, R. 1980. Volatiles of corn tassels: Possible corn ear worm attractants. *J. Agric. Food Chem.* 28:771–774.

BUTTERY, R. G., LING, L. C., and LIGHT, D. M. 1987. Tomato leaf volatile aroma components. *J. Agric. Food Chem.* 35:1039–1042.

CORK, A., BEEVOR, P. S., GOUGH, A. J. E., and HALL, D. R. 1990. Gas chromatography linked to electroantennography: A versatile technique for identifying insect semiochemicals, pp. 271–279, *in* A. R. McCaffery and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, London.

ETIEVANT, P. X., AZAR, M., PHAM-DELEGUE, M. H., and MASSON, C. J. 1984. Isolation and identification of volatile constituents of sunflowers (*Helianthus annus* L.) *J. Agric. Food Chem.* 32:503–509.

FIREMPONG, S., and ZALUKI, M. P. 1990. Host plant selection by *Helicoverpa armigera* : Role of certain plant attributes *Aust. J. Zool*. 37:675–683.

FITT, G. P. 1989. The ecology of *Heliothis* species in relation to agroecosystems *Annu. Rev. Entomol*. 34:17–52.

FLATH, R. A., FORREY, R. R., JOHN, J. O., and CHAN, B. G. 1978. Volatile components of corn silk (*Zea mays* L.): possible *Heliothis zea* (Boddie) attractants. *J. Agric. Food. Chem.* 26:1290–1293.

FORRESTER, N. W., CAHILL, M., BIRD, L. J., and LAYLAND, J. K. 1993. Management of pyrethroid and endosulphan resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Australia. *Bull. Entomol. Res.* Suppl. 1.

GABEL, B., THIÉRY, D., SUCHY, V., MARION-POLL, F., HRADSKY, P., and FARKAS, P. 1992. Floral volatiles of *Tanacetum vulgare* L. attractive to *Lobesia botrana* Den. and Schiff. females. *J. Chem. Ecol.* 16:701–711.

GUPTA, Y. N., and BHANDARI, K. S. 1974. Chemical examination of essential oil from the flowering tops of *Tagetes erecta. Indian Perfumer* 18:29–33.

HAYNES, K. F., ZHAO, J. Z., and LATIF, A. 1991. Identification of floral compounds from *Abelia grandiflora* that stimulate upwind flight in cabbage looper moths. *J. Chem. Ecol.* 17:637–646.

KEVAN, P. G., and BAKER, H. G. 1983. Insects as flower visitors and pollinators. *Annu. Rev. Entomol.* 28:407–453.

KÖNIG, W. A., KREBBER, R., EVERS, P., and BRUHN, G. 1990. Stereochemical analysis of constituents of essential oils and flavour compounds by enantioselective capillary gas chromatography. *J. High Resolut. Chromatogr*. 13:328–332.

LANDOLT, P. J., LENCZEWSKI, B., and HEATH, R. R. 1991. Lure and toxicant system for the cabbage-looper (Lepidoptera, Noctuidae). *J. Econ. Entomol.* 84:1344–1347.

LOUGHRIN, J. H., HAMILTON-KEMP, T. R., ANDERSEN, R. A., and HILDEBRAND, D. F. 1990. Headspace compounds from flowers of *Nicotiana tabacum* and related species. *J. Agric. Food Chem.* 38:455–460.

MILLER, J. R., and ROELOFS, W. L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

MOORHOUSE, J. E., YEADON, R., BEEVOR, P. S., and NESBITT, B. F. 1969. Method for use in studies of insect chemical communication. *Nature* 223:1174–1175.

PATEL, R. K., and YADAV, D. N. 1992. Impact of intercropping marigold on *Heliothis armigera* Hübner and its natural enemies in seed crop of tobacco. *Tob. Res.* 18:65–72.

RAMASWAMY, S. B. 1988. Host finding by moths: Sensory modalities and behaviors. *J. Insect Physiol.* 34:235–249.

REMBOLD, H., WALLNER, P., NITZ, S., KOLLMANNSBERGER, H., and DRAWERT, F. 1989. Volatile components of chickpea (*Cicer arietinum*) seed. *J. Agric. Food Chem.* 37:659–662.

REMBOLD H., KÖHNE A. C., and SCHROTH, A. 1991. Behavioral response of *Heliothis armigera* Hb. (Lep., Noctuidae) moths on a synthetic chickpea (*Cicer arietinum*) kairomone. *J. Appl. Entomol.* 112:254–262.

ROOME, R. E. 1975. Activity of adult *Heliothis armigera* (Hb) (Lep. Noctuidae) with reference to flowering of sorghum and maize in Botswana. *Bull. Entomol. Res.* 65:523–530.

SRINIVASAN, K., KRISHNA MOORTHY, P. N., and RAVIPRASAD, T. N. 1994. African marigold as a trap crop for the management of the fruit borer *Helicoverpa armigera* on tomato. *Int. J. Pest Manage.* 40:56–63.

SUCKLING, D. M., KARG, G., GIBB, A. R., and BRADLEY, S. J. 1996. Electroantennogram and oviposition responses of *Epiphyas postvittana* (Lepidoptera: Tortricidae) to plant volatiles. *N.Z. J. Crop Hortic. Sci.* 24:323–333.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

CLOSE-RANGE ATTRACTION IN *Lygocoris pabulinus* (L.)

FALKO P. DRIJFHOUT[1,*] and ASTRID T. GROOT[2]

[1]*Laboratory of Organic Chemistry, Phytochemical Section*
Wageningen University
Dreijenplein 8, 6703 HB Wageningen, The Netherlands

[2]*Plant Research International, Wageningen UR*
P.O. Box 16, 6700 AA Wageningen, The Netherlands

(Received May 1, 2000; accepted February 10, 2001)

Abstract—Males of the green capsid bug, *Lygocoris pabulinus*, exhibit a specific courtship behavior, i.e., a vibration of the abdomen. When both live and dead females were offered to males, this vibration behavior was elicited in most of the males tested. When females were dissected into separate body parts, heads, wings, and legs elicited equal responses, while thorax plus abdomen elicited a much lower response. When separate body parts were extracted, the leg extracts elicited significantly stronger responses than any other extract. This suggests that female *L. pabulinus* legs are either the source of a close-range sex pheromone or that pheromone is accumulated on the legs due to grooming behavior. The leg extracts contained several hydrocarbons such as *n*-alkenes, *n*-alkanes, and some methylalkanes. Female extracts contained more (*Z*)-9-pentacosene and male extracts contained more (*Z*)-9-heptacosene. Substrates on which females had walked elicited similar responses as female legs, indicating that the pheromone is deposited on the substrate. This enlarges the functional range of low-volatility compounds, which are thought to function only when sexes are in close vicinity or in contact.

Key Words—Heteroptera, Miridae, contact sex pheromone, male vibration, (*Z*)-9-pentacosene, (*Z*)-9-heptacosene, cuticular hydrocarbons, close-range pheromone, SPME.

INTRODUCTION

Sex pheromones are commonly used by insects to locate mates at long range and to stimulate mating at close range (Carlson et al., 1971; Cardé et al., 1975; Muhammed et al., 1975). Long-range sex pheromones were first described and chemically identified in moths (Butenandt et al., 1959) and are now widely used for

*To whom correspondence should be addressed. e-mail: Falko.Drijfhout@bio.oc.wau.nl

0098-0331/01/0600-1133$19.50/0

monitoring of lepidopterous pests (e.g., Minks and Van Deventer, 1992; Cardé and Minks, 1997). In recent years identification of nonlepidopteran sex pheromones has received growing attention (Hardie and Minks, 1999). In mirids (Heteroptera: Miridae), whose virgin females attract males, long-range sex pheromones have been identified for three species so far (Smith et al., 1991; Millar et al., 1997; Millar and Rice, 1998; McBrien and Millar, 1999).

Close-range sex pheromones initiate courtship behavior. Such pheromones are usually less volatile than long-range pheromones (Blomquist et al., 1993). Despite their low volatility, close-range pheromones may play an important role in the decision of an insect to land at a certain spot (Carlson et al., 1971). Without the addition of such pheromones, arriving males may not enter a trap (Cardé et al., 1975; Kennedy, 1977). In mirids close-range sex pheromones have not yet been reported. Major focus has been on attractive and alarm compounds from the metathoracic and accessory scent glands (e.g., Carayon, 1971; Staddon, 1979; Aldrich, 1988). Compounds identified from these glands have carbon chain lengths of 2–15 and are most commonly acids, aldehydes, ketones, alcohols, and esters (Staddon, 1979; Aldrich, 1988). Close-range pheromones may have carbon chain lengths of 20–30 or even more (Blomquist et al., 1993). The source of long-range pheromones in mirids has been suggested to be the metathoracic scent gland (Aldrich, 1988), or at least the thoracic region (Millar et al., 1997), although Graham (1988) identified the ovipositor region as source of attraction. Since the chemical nature of close-range pheromones may differ completely from long-range pheromones, their sources probably differ as well.

To identify close-range sex pheromones, a specific arousal or courtship behavior of one of the sexes should be distinguished. Males of the green capsid bug [*Lygocoris pabulinus* (L.), Heteroptera: Miridae] exhibit a characteristic sex-specific courtship behavior, a repeated vibration of the abdomen; only males vibrate in the presence of females and only when they are sexually mature (Groot et al., 1998). We used this vibration behavior of male *L. pabulinus* to determine the source of attraction in females at close range. Additionally, we attempted to identify compounds involved in this close-range attraction.

METHODS AND MATERIALS

Insects. *Lygocoris pabulinus* was reared on potted potato plants, (cultivar Bintje) in wooden cages in a greenhouse at $22 \pm 2^{\circ}$C, 65 ± 5 % relative humidity, and 18L : 6D photoregime, following the procedure of Blommers et al. (1997). Every two to three days newly emerged adults were collected from the rearing cages, after which the sexes were isolated in separate rearing cages. In this way, virgin males and females of known age were continuously available for the experiments (Groot et al., 1998).

Bioassays. One to two hours before each test, virgin males 6–9 days old were collected from the separate rearing cages and isolated in small glass tubes. Glass Petri dishes 5 cm diam. were cleaned with acetone, and the bottoms were covered with white filter paper disks of the same diameter. The stimuli to be tested were placed in the Petri dishes, after which one male per dish was introduced. Stimuli consisted of one bug equivalent per Petri dish, and originated from bugs that were virgin and 6–9 days old. All males were observed for 15 min. If a male in a dish started to vibrate within this period, that dish was set aside and counted as a positive response. The number of Petri dishes with positive responses was calculated as a fraction of the total number of Petri dishes in which the stimulus had been applied. Different stimuli were tested at the same time, and stimuli were tested on several different days. All experiments were carried out at 19–23°C between 10:00 and 14:00 hr C.E.T.

In a second experiment, a solid-phase microextraction (SPME) needle (100 μm polydimethylsiloxane coating from Supelco, Bellefonte, Pennsylvania) was positioned on the bottom of the Petri dish through a hole in the side. A metal strip was placed in a V-shape around the needle to reduce the amount of space the bug had, thus increasing the chance of bug–needle contact. One or two bugs were then placed in the Petri dish for 2–3 hr.

Stimuli Tested. First, a series of live females, dead females, live males, and dead males were tested. Bugs were anesthetized with CO_2, after which the heads were clipped off. In a following series freshly anesthetized females were dissected into heads, wings, legs, and thorax plus abdomen. Thorax and abdomen were not subdivided, since clipping would mean cutting through several organs and glands that run from thorax to abdomen, which may then release a variety of chemicals. Third, extracts were made of the different body parts of females, *i.e.*, heads, wings, legs, and thorax plus abdomen. After anesthetizing fresh females with CO_2, the body parts were dissected and placed in 1.8- or 4-ml vials. After dissecting all available females, 15–50 μl of either dichloromethane, pentane, pentane–ether (2 : 1), or water per female was added, the amount of which was set as one female equivalent of the particular extract. The extracts were stored in a freezer (-20 ± 2°C) until used. All extracts were used 1–14 days after the initial dissections. In a fourth series, one female equivalent of a synthetic mixture (2.5 μg in total) was tested. The synthetic mixture consisted of 1-hexanol (40 ng), hexyl butyrate (500 ng), (*E*)-2-hexenyl butyrate (25 ng), (*Z*)-9-tricosene (150 ng), (*Z*)-7-pentacosene (200 ng), (*Z*)-9-pentacosene (1000 ng), (*Z*)-9-heptacosene (200 ng), tricosane (80 ng), and pentacosane (80 ng) (Table 1 below).

Chemical Analysis. Extracts were analyzed with a dual-column GC (HP 6890) equipped with an apolar DB-1 column (J&W Scientific, Folsom, California; 60 m $\times$ 0.25 mm; 0.25-μm) and a polar Stabilwax column (Restek, Bellefonte, Pennsylvania; 60 m $\times$ 0.25 mm; 0.25-μm) and two flame ionization detectors. Oven program: 30°C (2 min hold) to 238°C (25 min hold) at 4°C/min. Hydrogen

TABLE 1. AVERAGE COMPOSITION (%) OF EXTRACTS OF LEGS FROM FEMALE AND MALE *L. pabulinus*

No.[a]	Compound	Composition(%, mean ± SD)	
		Females	Males
1	Hexan-1-ol[b]	1.7 ± 1.8	0.6 ± 0.4
2	Hexyl butyrate[b]	19.2 ± 13.7	22.3 ± 23.4
3	(*E*)-2-Hexenyl butyrate[b]	0.8 ± 0.5	0.8 ± 0.9
4	Tricosane[b]	6.3 ± 1.9	0.4 ± 0.7
5	(*Z*)-9-Tricosene[b]	3.1 ± 1.7	0.1 ± 0.1
	(*Z*)-9-Tetracosene	≤0.2	≤0.2
	Tetracosane	≤0.2	≤0.2
8	2-Methyltetracosane	3.3 ± 1.5	1.8 ± 0.2
9	Pentacosane[b]	8.6 ± 2.8	10.6 ± 4.9
10	(*Z*)-9-Pentacosene[b]	40.8 ± 9.2	3.4 ± 2.3
11	(*Z*)-7-Pentacosene[b]	8.7 ± 1.9	20.5 ± 4.8
	9-Hexacosene	≤0.2	≤0.2
	2-Methylhexacosane	≤0.2	≤0.2
14	(*Z*)-9-Heptacosene[b]	7.6 ± 4.6	39.6 ± 13.2
	(*Z*)-7-Heptacosene	≤0.2	≤0.2
	Heptacosane	≤0.2	≤0.2
	(*Z*)-9-Nonacosene	≤0.2	≤0.2

[a] Numbers according to Figures 3 and 4.
[b] Compounds used in the synthetic mixture.

was used as the carrier gas (constant flow of 2.4 ml/min, linear velocity: 48 cm/sec). GC-MS analyses were carried out on Varian 3400 GC connected to a Finnigan MAT95 mass spectrometer. The BP5 column (SGE; 25 m × 0.25 mm; 0.25-μm) was programmed from 50°C to 270°C (4 min. hold) at 5°C/min. The mass spectrometer was operated in EI mode (at 70 eV) and scanning was done from mass 24 to 500 at 0.7 sec/dec. ^{1}H NMR (200 MHz) spectra were recorded on a Bruker AC200 spectrometer. FTIR spectra were recorded on a Perkin-Elmer 1725X spectrometer. Identification of compounds in extracts was carried out with GC-MS and by comparison of retention times of reference compounds with GC. The position of the double bond in the alkenes was determined by derivatization with DMDS according to Carlson et al. (1989).

Chemicals. Hexanol, tricosane, pentacosane, 1-bromotetradecane, 1-bromohexadecane, 1-bromooctadecane, triphenylphosphine, nonanal, heptanal, *n*-butyllithium in hexane, DMDS, and urea were all purchased from Acros Organics (Geel, Belgium) and hexyl butyrate from Roth (Karlsruhe, Germany). (*E*)-2-Hexenyl butyrate was synthesized according to Drijfhout et al. (2000). (*Z*)-9-Tricosene, (*Z*)-9-pentacosene, (*Z*)-7-pentacosene, and (*Z*)-9-heptacosene were all synthesized as described below. All the chemicals used were >98% pure. All solvents used were distilled twice before use.

Synthesis of Alkyltriphenylphosphonium Bromide. A mixture of 0.3 mmol of the alkylbromide (1-bromotetradecane, 1-bromohexadecane, or 1-bromooctadecane) and 0.3 mmol of triphenylphosphine was heated to 140°C under a nitrogen atmosphere for 5 hr. The reaction mixture formed a solid when cooled down. Dry acetone (5 ml) and dry diethyl ether (12 ml) were added to the solid and cooled to −20°C (overnight). After filtration, the alkyltriphenylphosphonium bromide was obtained as white crystals.

Synthesis of Alkenes. A slurry of 5 mmol of powdered alkyltriphenylphosphonium bromide in 10 ml of THF was prepared under nitrogen. The mixture was cooled in an ice bath, 5 ml of DMSO was added after which 5 mmol of *n*-butyllithium in hexane was injected. The butyllithium was added at such a rate that the temperature of the mixture remained at 10–15°C. After 5 min, 5 mmol of the aldehyde (nonanal or heptanal) was injected, and the resulting mixture was stirred for 30 min at ambient temperature. The mixture was diluted with water and extracted with petroleum ether 40/60. The dried ($MgSO_4$) extract was filtered and concentrated to give a 90% yield of the alkene. The alkene was further purified with column chromatography on silica gel and eluted with hexane to give a mixture of *Z* and *E* isomers in a ratio of 85 : 15.

Separation of Z and E Isomers. The two isomers of the alkenes obtained during the synthesis were separated making use of their different complexation with urea (Leadbetter and Plimmer, 1979). One part of alkene and five parts of urea were dissolved in 20 parts of methanol. This was left to crystallize at room temperature. The white crystals were separated by filtration. The methanol fraction was evaporated to obtain the (*Z*)-isomer in 97% purity. The procedure was repeated to improve the purity of the *Z* isomer. If the alkene could not be dissolved in methanol, isopropanol was added.

(Z)-9-Tricosene. ^{1}H NMR δ ($CDCl_3$) 0.83–0.89 (t, 6 H, $-CH_3$), 1.2–1.6 (m, 34 H, $-CH_2CH_2-$), 2.0 (m, 4 H, $=CH-CH_2$), 5.3–5.35 (m, 2 H, CH=CH). MS: $m/z = 322$ (M^+); Kovats indices: 2320 on Stabilwax and 2271 on DB-1. IR (film): $\nu_{HC=CH(cis)}$ 722 (m) cm^{-1}

(Z)-7-Pentacosene. ^{1}H NMR δ ($CDCl_3$) 0.83–0.89 (t, 6 H, CH_3), 1.2–1.4, (m, 38 H, $-CH_2CH_2-$), 1.9–2.0 (m, 4 H, $=CH-CH_2$), 5.3, (m, 2 H, CH=CH). MS: $m/z = 350$ (M^+); Kovats indices: 2526 on Stabilwax and 2477 on DB-1; IR (film): $\nu_{HC=CH\ (cis)}$ 722 cm^{-1}

(Z)-9-Pentacosene. ^{1}H NMR δ ($CDCl_3$) 0.83–0.89 (t, 6 H, $-CH_3$), 1.2 (m, 38 H, $-CH_2CH_2-$), 1.9–2.2 (m, 4 H, $=CH-CH_2$), 5.3–5.4 (m, 2 H, CH=CH). MS: $m/z = 350$ (M^+); Kovats indices: 2519 on Stabilwax and 2470 on DB-1; IR (film): $\nu_{HC=CH\ (cis)}$ 722 cm^{-1}

(Z)-9-Heptacosene. ^{1}H NMR δ ($CDCl_3$): 0.9 (t, 6 H, CH_3), 1.1–1.5 (m, 40 H, $-CH_2CH_2-$), 2.2 (m, 4 H, $=CH-CH_2$), 5.3 (m, 2 H, CH=CH). MS: $m/z = 378$ (M^+); Kovats indices: 2721 on Stabilwax and 2671 on DB-1; IR (film): $\nu_{HC=CH\ (cis)}$ 722 cm^{-1}

Statistical Analysis. If males responded to a source, differences in responses towards the different sources were statistically analyzed by fitting a logit regression model with overdispersion to the daily observed counts of responses of a test (McCullagh and Nelder, 1989), using the computer program Genstat 5 (release 4.1, PC/Windows NT, 1997). In the model, source was taken as the explanatory variable and the variance was assumed to be proportional to the binomial variance. First, a chi-square test for the residual deviance was conducted to determine overdispersion. The overall effect of treatments was determined by performing an F test for the ratio of the mean deviance for treatment and the mean deviance of the rest. If the overall test was significant ($P < 0.05$), pairwise comparisons by t test between treatment means on the logit scale were conducted.

RESULTS

Live and dead females elicited similar responses, the fractions of males vibrating being 0.88 ± 0.08 and 0.74 ± 0.09 (mean $\pm$ SE), respectively (Figure 1A). Live and dead males elicited vibration responses in few males. When the bodies of females were dissected, the head, wings, and legs were equally attractive and as attractive as dead females, while the thorax plus abdomen of females were significantly less attractive (Figure 1B). Responses to freshly dissected wings and heads may be due to grooming, which spreads attractive compounds over the body surface. For confirmation of presence of attractive compounds on the whole body surface, small pieces of filter paper were rubbed over female bodies (after anesthetizing and clipping off heads). When these pieces of paper were offered in clean Petri dishes, almost half of the males tested (0.41 ± 0.07, mean $\pm$ SE) started vibrating ($N = 46$).

After extraction of the separate body parts of females, leg extracts elicited significantly more vibrational response than all other extracts (Figure 1C). Extracts from thorax plus abdomen did not elicit a response from males, which may be due to defensive compounds in the metathoracic gland. Therefore, extracts were also made of female thorax plus abdomen, from which the metathoracic gland was removed by gently cutting the cuticle with two sharp tweezers, trying to destroy as little tissue as possible. Few males responded to this extract (Figure 1C). When differences in responses between freshly dissected body parts and their corresponding extracts were statistically compared, male responses to the leg extracts were not different from responses to freshly dissected legs, while wing and head extracts elicited lower responses ($P < 0.05$) than freshly dissected wings and heads.

Organic solvent extracts stimulated significantly more males to vibrate than water extracts (Figure 2). The graph indicates that "water extracts" caused only minimal vibration in males. Legs from female bugs, used for these "water extracts,"

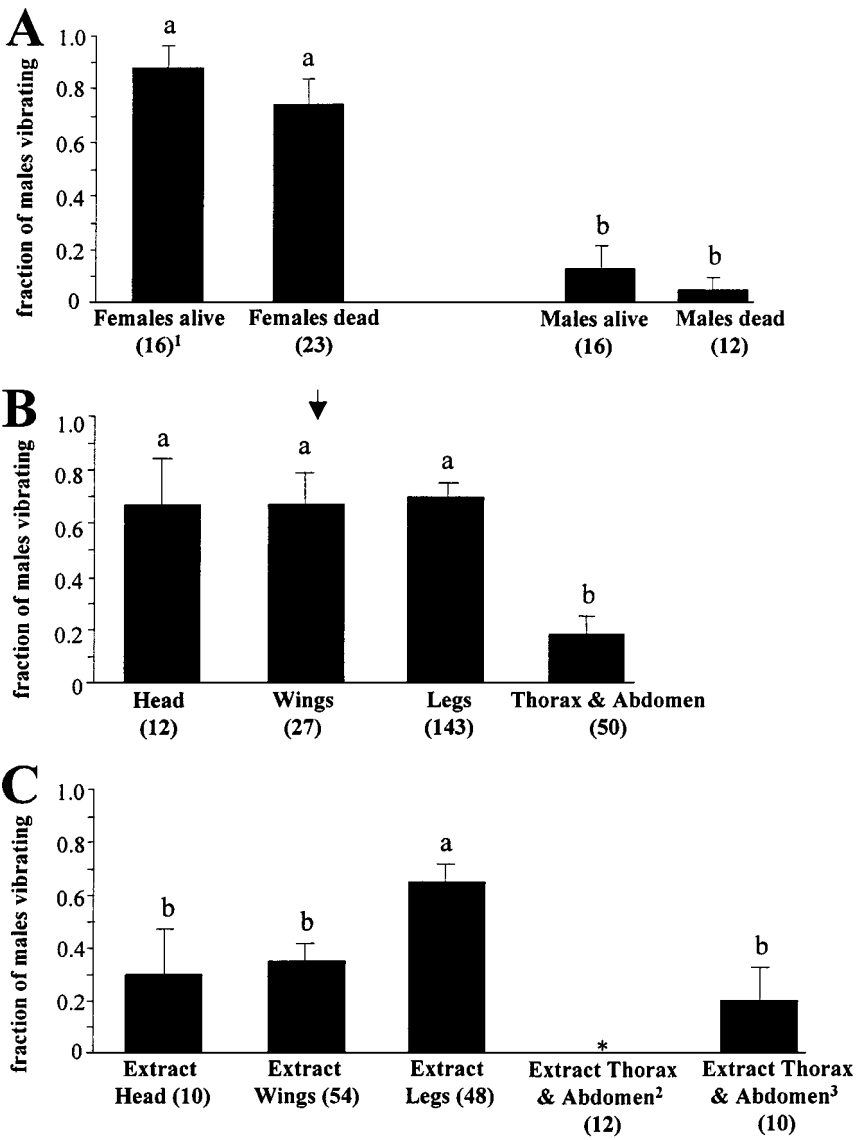


FIG. 1. Male *L. pabulinus* responses (mean ± SE) to different stimuli: (A) whole insects, (B) body parts of females, (C) extracts of female body parts. [1]Total number of males tested; [2]Metathoracic gland left in thorax; [3]Metathoracic gland removed from thorax. Significant differences were determined between sources within one group (A, B, C). Different letters above the bars indicate significant differences in each group at the 5% level. See text for statistical methods used. *Not included in the statistical analyses, as no males had responded.

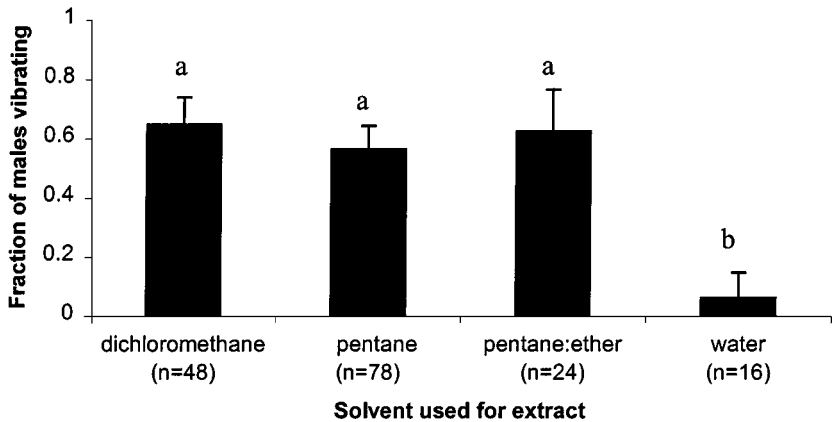


FIG. 2. Response of male *L. pabulinus* to extracts of female legs prepared in different solvents (mean ± SE; n = number of males tested). Different letters above bars indicate significant difference at $P \leq 0.05$ (two-tailed).

still elicited vibratory behavior in males. No significant difference in male vibration occurred using dichloromethane, pentane or the mixture of pentane and ether (Figure 2).

In both male and female leg extracts, the major part of the compounds consisted of hydrocarbons (Figure 3A). These hydrocarbons consisted of alkenes (75%), alkanes (20%), and some monoalkanes (5%). (*Z*)-9-Pentacosene (10) was the most abundant alkene in females, while (*Z*)-9-heptacosene (14) was the most abundant in males. The ratio of (*Z*)-9-pentacosene to (*Z*)-7-pentacosene (11) was opposite in males and females. Furthermore, sometimes the female extracts contained more (*Z*)-9-tricosene than male extracts. (Figures 3B and C). Both male and female extracts contained three oxygen containing compounds: hexyl butyrate (2) and (*E*)-2-hexenyl butyrate (3) and sometimes 1-hexanol (1). Other minor compounds identified in the leg extracts of male and female *L. pabulinus* are listed in Table 1. Table 2 lists some characteristics used to identify the most abundant alkenes present in the extracts.

The vibrational bioassays suggest that female legs are the source of close-range sex pheromone. To determine whether the source of attraction could be defined more precisely, female legs were subdivided into: (A) forelegs, middle legs, and hind legs; or (B) coxae plus femorae, and tibiae plus tarsi. In series A, one pair of forelegs, middle legs, or hind legs of three females was placed in one Petri dish, so that six legs per dish were offered. In series B, the six coxae plus femorae of one female were placed in one Petri dish, and the six tibiae plus tarsi in another. Table 3 shows that all parts of the legs were equally attractive (no significant differences were found between any pair). However, when the overall

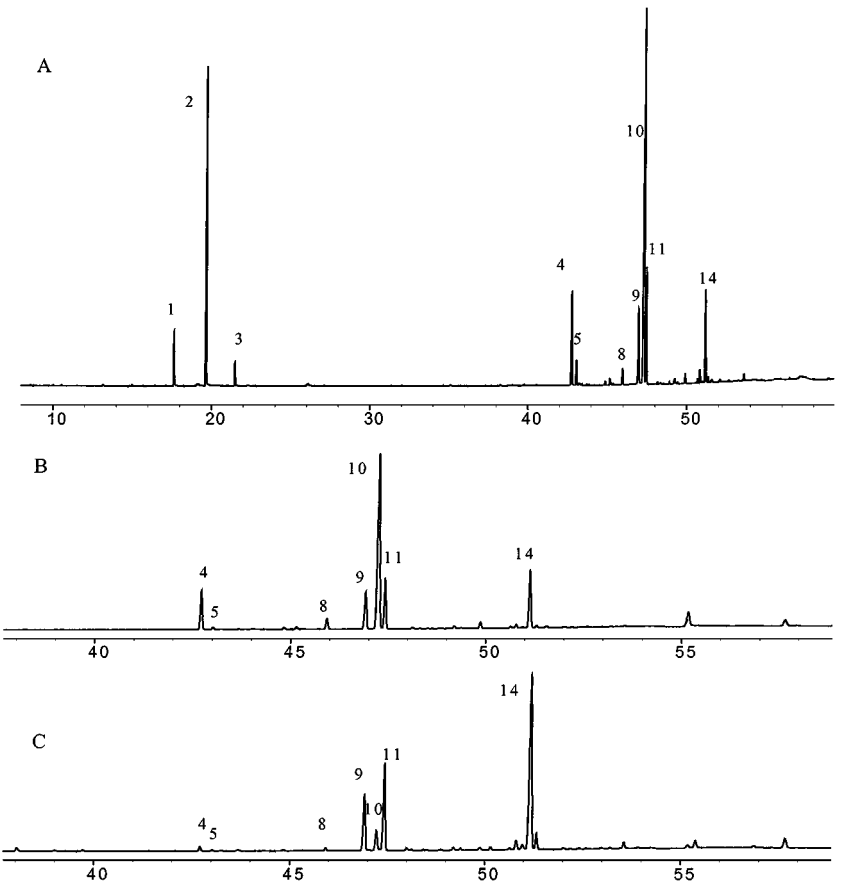


FIG. 3. Gas chromatograms of *L. pabulinus* leg extracts: (A) female leg extract; enlargement of the cuticular hydrocarbon part of female (B) and male (C) extracts. DB-Wax column; FID detector. For explanation of the numbers see Table 1.

response to fore-, middle and hind legs was compared to the overall response to coxae plus femorae and tibiae plus tarsi, responses to entire legs were significantly stronger than to parts.

When legs contain attractive compounds, these compounds may be deposited on the substrate on which female *L. pabulinus* walk. To determine possible deposition of attractive compounds, we tested three different substrates: a piece of potato leaf (cultivar Bintje), a piece of green bean leaf (*Phaseolus vulgaris*, cultivar Miracle), and the glass of an empty Petri dish. One *L. pabulinus* female was allowed to walk in each dish for 75–140 min. As a control, we tested pieces of

TABLE 2. CHARACTERISTICS USED IN IDENTIFICATION OF FOUR MAJOR ALKENES PRESENT IN LEG EXTRACTS OF *L. pabulinus*

	Kovats indices		MS characteristics	
Compound	DBI	Stabilwax	M^+	m/z after DMDS derivatization
5	2271	2319	322	173 (C_1–C_9), 243 (C_{10}–C_{23}), 416 (M^+)
10	2470	2518	350	173 (C_1–C_9), 271 (C_{10}–C_{25}), 444 (M^+)
11	2477	2526	350	145 (C_1–C_7), 299 (C_8–C_{25}), 444 (M^+)
14	2670	2719	378	173 (C_1–C_9), 299 (C_{10}–C_{27}), 472 (M^+)

potato leaf or empty dishes on which males had walked for 60–120 min, as well as pieces of potato leaves on which no bug had walked. Table 4 shows that males responded to substrates on which females had walked, while no males showed vibration behavior in any of the control dishes. During the 75–140 min that females walked around in the dishes, a characteristic pheromone-laying behavior was not observed. In addition, desorption of the SPME needle (250°C) revealed the same hydrocarbon profile as found in the leg extracts of the concerning sex. From Figure 4 it is clear that when females walked on or near the needle, a high concentration of (*Z*)-9-pentacosene (10) was deposited on the needle. The volatile compounds 1-hexanol (1), hexyl butyrate (2), and (*E*)-2-hexenyl butyrate (3) were not always present.

When the synthetic mixture, containing compounds derived from female leg extracts, was tested in the vibration bioassay, no males started to vibrate. A few males showed vibratory behavior when male legs, loaded with 5 μg (*Z*)-9-pentacosene (instead of 1 μg), were offered (Table 5). Adding (*Z*)-9-tricosene to these legs did not result in more males vibrating. As no biological active compound was found, the possibility of (*Z*)-9-heptacosene acting as a repellent was investigated. Female legs were therefore loaded with a high dose of

TABLE 3. MALE VIBRATION RESPONSE TO DIFFERENT PARTS OF FEMALE LEGS

Source	Fraction of males responding (±SE)	*N*		
Female legs	0.70 ± 0.05	143	a[a]	A[b]
Forelegs	0.76 ± 0.14	17	a	
Middle legs	0.71 ± 0.15	17	a	A
Hind legs	0.88 ± 0.10	17	a	
Coxae + femorae	0.45 ± 0.14	22	a	
				B
Tibiae + tarsi	0.41 ± 0.14	22	a	

[a] Different letters indicate signifant differences between pairs ($P < 0.05$).
[b] Different letters indicate significant differences between groups ($P < 0.05$).
See text for statistical methods used.

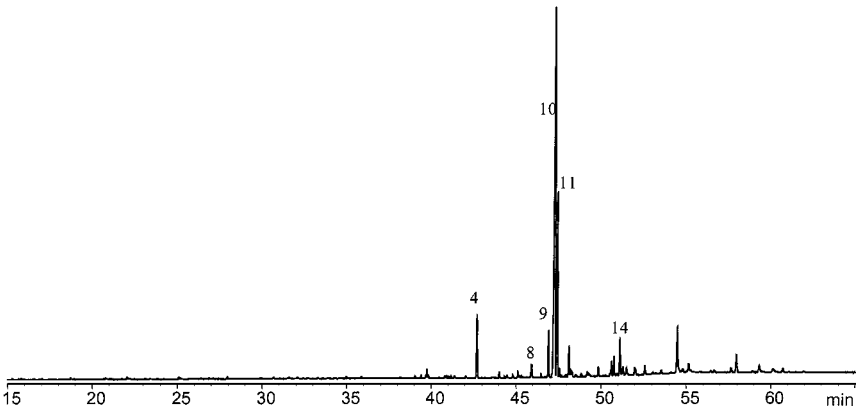


FIG. 4. Gas chromatographic analysis of an SPME needle on which a female bug had walked. DB-Wax column; FID detector. For explanation of the numbers see Table 1.

(*Z*)-9-heptacosene (5–8 μg). Yet, a similar amount of males showed vibration behavior to these legs as when untreated female legs were offered (Table 5).

DISCUSSION

The legs of *L. pabulinus* females most consistently elicited vibration behavior in males, suggesting that legs are the source of close-range attraction in this species. Legs have been recognized as the site of sex pheromone release in the aphid *Megoura viciae* (Marsh, 1972), the mosquito *Culiseta inornata* (Lang, 1977), the tsetse fly *Glossina morsitans morsitans* (Carlson et al., 1978), the housefly *Musca domestica* (Schlein et al., 1980), and the parasitoid braconid *Ascogaster reticulatus* (Kainoh and Oishi, 1993). In some species, specific glands in the legs have been

TABLE 4. MALE VIBRATION RESPONSE TO SUBSTRATE ON WHICH FEMALES HAD WALKED

Source	Fraction of males responding (±SE)	*N*	
Potato leaf on which female walked for 75–140 min	0.68 ± 0.10	39	a[a]
Bean leaf on which female walked for 75–140 min	0.64 ± 0.13	22	a
Empty Petri dish on which female walked for 75–140 min	0.37 ± 0.09	46	a
Potato leaf on which male walked for 60–120 min	0	15	*b*
Empty Petri dish on which male walked for 60–120 min	0	10	*b*
Potato leaf	0	10	*b*

[a]Different letters indicate signifant differences ($P < 0.05$). See text for statistical methods used.
[b]Not statistically analysed, as no male had responded.

TABLE 5. MALE VIBRATORY RESPONSE TO FRESHLY DISSECTED LEGS WITH OR WITHOUT ALKENE ADDED

Source	Fractions of males responding (±SE)	Males (N)	
Synthetic mixture	0	32	a[a]
Male legs	0	5	a
Male legs + 5 μg (Z)-9-pentacosene	0.19 ± 0.11	29	a
Male legs + 5 μg (Z)-9-pentacosene + 1 μg (Z)-9-tricosene	0.05 ± 0.06	10	a
Female legs + 5–8 μg (Z)-9-heptacosene	0.76 ± 0.13	21	b
Female legs	0.70 ± 0.06	143	b

[a] Different letters indicate significant difference at $P \leq 0.05$ (two-tailed).

identified as the site of sex pheromone excretion (Marsh, 1972; Schlein et al., 1980). In *L. pabulinus*, response to fore-, middle, and hind legs was similarly strong. The lower response to parts of the legs compared to entire legs may be due to the lower amount of leg biomass per Petri dish in the latter group. From these experiments no specific site of possible glands in legs became apparent.

Specific glands in the legs may not synthesize contact sex pheromones. Cuticular hydrocarbons are probably synthesized by oenocytes, large cells that are rich in smooth endoplasmatic reticulum and mitochondria, which appear to be restricted to epidermal tissue in the thorax and abdomen (Gu et al., 1995; Schal et al., 1998). After synthesis, attractive hydrocarbons may be deposited at specific target sites, as in the German cockroach *Blattella germanica*, where the wings accumulate large amounts of pheromone (Gu et al., 1995). The cuticle of the legs may thus be the specific target deposition site of the attractive compounds.

The presence of a close-range sex pheromone on the legs may also be due to grooming. Grooming may either accumulate pheromone from other body parts on the legs (Howard and Blomquist, 1982), or it may spread the pheromone from leg glands over the whole body surface, as in polistine wasps (Beani and Calloni, 1991), whose territorial marking pheromones from leg glands function as sex attractants as well. *L. pabulinus* males and females groom frequently (Groot et al., 1998) and the attractive compounds are not only present on female legs, but also on other body parts as demonstrated by the male response to female wings and heads and to pieces of filter paper rubbed over female bodies. In short, the site of sex pheromone production does not have to be the site of pheromone release, specific glands are not necessarily involved, and grooming may enhance chemical dispersion or accumulation at specific sites.

In mirids, close-range or contact pheromones have not yet been studied, and to our knowledge this is the first study on cuticular hydrocarbons of a mirid species. In *L. pabulinus* the major part of the hydrocarbons consisted of alkenes, followed by alkanes and some methyl alkanes. All compounds were C_{23}–C_{29}

hydrocarbons. This is also the first time that a clear-cut difference was found in *L. pabulinus* between male- and female-derived compounds, i.e., females produce a high amount of (*Z*)-9-pentacosene, whereas males produce high amounts of (*Z*)-9-heptacosene. Because of the low amount of (*Z*)-9-pentacosene present in males, the ratio of (*Z*)-9-pentacosene and (*Z*)-7-pentacosene is opposite in males and females. To our knowledge, there are only two other studies on cuticular hydrocarbons in heteropteran species, the milkweed bug, *Oncopeltus fasciatus* (Lygaeidae) (Jackson, 1983), and *Triatoma infestans* and *T. mazzotti* (Reduvidae) (Juárez and Blomquist, 1993). In all these species mostly *n*-alkanes, branched monoalkanes, and dimethylalkanes were found. Furthermore hydrocarbons of up to 41–43 carbon atoms were found. More importantly, male and female *O. fasciatus*, *T. infestans*, and *T. mazzotti* had similar profiles, whereas *L. pabulinus* males and females produce a different blend of hydrocarbons.

The hydrocarbons of *L. pabulinus* females appear to elicit vibratory behavior in males, as extracts in water (in which no hydrocarbons are present) were not active. Furthermore, female legs used for water extraction remained active, indicating that the compounds were still present on the legs. Fractionation of the extracts on silica into two fractions (hydrocarbons and oxygenated compounds) suggested that both fractions were needed to elicit vibration behavior in males. The composition of the oxygenated compound fraction of males and females did not show any difference. The similarity of this fraction between males and females was supported by the finding that this fraction from males combined with the hydrocarbon fraction of females did cause vibratory behavior in males (unpublished results).

In various species cuticular hydrocarbons have been identified as contact pheromones (e.g., Muhammed et al., 1975; Carlson et al., 1978; Bolton et al., 1980; Dillwith et al., 1981; Blomquist et al., 1993; Gu et al., 1995; Fukaya et al., 1996; Doi et al., 1997). Alkenes seem to be involved in sexual communication between sexes (Howard and Blomquist, 1982). More precisely, (*Z*)-9-pentacosene is often the main hydrocarbon present in beetles (Baker et al., 1979), ants (Morgan et al., 1992), and bees (Paulmier et al., 1999). In *L. pabulinus*, although (*Z*)-9-pentacosene is the only compound obviously present in much lower amounts in males than in females, the activity of this compound on its own could not be proven in bioassays. Given that the synthetic mixture did not elicit vibration behavior in males, one cannot rule out the possibility of other compounds being part of the contact pheromone. The most abundant male alkene, (*Z*)-9-heptacosene, did not act as a repellent, because female legs sprayed with a high dose of this alkene still elicited vibratory behavior in males (Table 5). More experiments are needed to determine if there are other compounds present in the extracts that act as a vibration elicitor.

The attractive compounds on female legs may be deposited on the substrate. Male *L. pabulinus* showed strong responses to substrates on which females had

walked. The fraction of males responding to potato leaves was even similar to responses to female legs. When females were allowed to walk on a SPME needle, a similar chemical profile was found as in attractive female leg extracts. These results support the hypothesis that these hydrocarbons are deposited on the substrate. As a characteristic pheromone-laying behavior was not observed, we suspect that deposition on the substrate occurs passively or that pheromone is adsorbed to the substrate. Adsorption or deposition of attractive compounds on the substrate increases the probability of sex encounters, as it elicits intensive search by males in these areas (Colwell et al., 1978; Fauvergue et al., 1995). Depending on their volatility, these pheromones are active at some distance, as in *M. viciae* (Pickett et al., 1992), or they elicit response at close range or upon contact, as in the other species mentioned. Adsorption of pheromone to a substrate also increases the surface area from which pheromone evaporates, thereby increasing both the rate of volatilization and the possible communication distance (Colwell et al., 1978). Males may follow a gradient of intensity, created by the decay of the compounds over time, to orient their movements towards females (Fauvergue et al., 1995). In this way, the functional range of low-volatility cuticular hydrocarbons would be greatly enlarged.

Identification of the sex pheromones of mirid species is a challenging task. This study indicates that the sex-specific cuticular hydrocarbons, probably in addition to esters, may play a role in the sexual communication of these bugs. Evidence is accumulating that such hydrocarbons not only function as contact pheromones, but are also involved in attraction at a (short) distance. Uebel et al. (1978) demonstrated that field catches of male *Fannia canicularis* and *F. pusio* increased slightly when alkenes were loaded on the lures. Connor et al. (1980) reported that the pheromone of the arctiid moth *Utetheisa ornatrix* (*Z,Z,Z*-3,6,9-heneicosatriene plus a small quantity of an unidentified C_{21} tetraene), perhaps serves as a close-range orientation cue for locating the female. Recently, Schiestl et al. (1999, 2000) reported that flowers of *Ophrys* orchids also mimic the odor profile of bees by using this class of alkenes in order to attract these bees. Successful pheromone trapping of mirids might also have to take these close-range cues into account.

Acknowledgments—We thank Anneke Heijboer for rearing *L. pabulinus*, Dr. M. A. Posthumus for carrying out all the GS-MS experiments, and Pieter Vereijken for conducting the statistical analyses. Marcel Dicke, Teris van Beek, and Hans Visser are greatly acknowledged for critical comments on earlier versions of the manuscript. This study was funded by the Dutch Technology Foundation (WBI44.3383).

REFERENCES

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol*. 33:211–238.

BAKER, J. E., NELSON, D. R., and FATLAND, C. L. 1979. Developmental changes in cuticular lipids of the black carpet beetle, *Attagenus megatoma. Insect Biochem*. 9:335–339.

BEANI, L., and CALLONI, C. 1991. Leg tegumental glands and male rubbing behavior at leks in *Polistes dominilus* (Hymenoptera: Vespidae). *J. Insect Behav*. 4:449–461.

BLOMMERS, L. H. M., VAAL, F. W. N. M., and HELSEN, H. H. M. 1997. Life history, seasonal adaptations, and monitoring of common green capsid *Lygocoris pabulinus* (L.) (Hemiptera: Miridae). *J. Appl. Entomol*. 121:389–398.

BLOMQUIST, G. J., TILLMAN-WALL, J. A., GUO, L., QUILICI, D. R., GU, P., and SCHAL, C. 1993. Hydrocarbon and hydrocarbon derived sex pheromones in insects: Biochemistry and endocrine regulation, pp. 317–351, *in* D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry & Biology. University of Nebraska Press, Lincoln, Nebraska.

BOLTON, H. T., BUTLER, J. F., and CARLSON, D. A. 1980. A mating stimulant pheromone of the horn fly, *Haematobia irritans* (L.): demonstration of biological activity in separated cuticular components. *J. Chem. Ecol.* 6:951–964.

BUTENANDT, A., BECKMANN, R., STAMM, D., and HECKER, E. 1959. Über den Sexuallockstof des Seidenspinner, *Bombyx mori*. Reindarstellung und Kostitution. *Z. Naturforsch*. 14B:283–284.

CARAYON, J. 1971. Notes et documents sur l'appareil odorant métathoraciques des Hémiptères. *Ann. Soc. Entomol. Fr.* 7:737–770.

CARDÉ, R. T., and MINKS, A. K. 1997. Insect Pheromone Research: New Directions. Chapman and Hall, New York, 684 pp.

CARDÉ, R. T., BAKER, T. C., and ROELOFS, W. L. 1975. Behavioral role of individual components of a multichemical attractant system in the Oriental fruit moth. *Nature* 253:348–349.

CARLSON, D. A., MAYER, M. S., SILHACEK, D. L., JAMES, J. D., BEROZA, M., and BIERL, B. A. 1971. Sex attraction pheromone of the house fly: Isolation, identification and synthesis. *Science* 174:76–77.

CARLSON, D. A., LANGLEY, P. A., and HUYTON, P. 1978. Sex pheromone of the tsetse fly: Isolation, identification, and synthesis of contact aphrodisiacs. *Science* 201:750–753.

CARLSON, D. A., ROAN, C., YOST, R. A., and HECTOR, J. 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* 61:1564–1571.

COLWELL, A. E., SHOREY, H. H., BAUMER, P., and VAN VORHIS KEY, S. E. 1978. Sex pheromone scent marking by females of *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 4:717–721.

CONNOR, W. E., EISNER, T., VANDER MEER, R. K., GUERRO, A., GHIRINGELLI, D., and MEINWALD, J. 1980. Sex attractant of an arctiid moth (*Utetheisa orntrix*): A pulse chemical signal. *Behav. Ecol. Sociobiol*. 7:35–63.

DILLWITH, J. W., BLOMQUIST, G. J., and NELSON, D. R. 1981. Biosynthesis of the hydrocarbon components of the sex pheromone of the housefly, *Musca domestica* L. *Insect Biochem*. 11:247–253.

DOI, M., NEMOTO, T., NAKANISHI, H., KUWAHARA, Y., and OGUMA, Y. 1997. Behavioral response of males to major sex pheromone component, *(Z,Z)*-5,25-hentriacontadiene, of *Drosophila ananassae* females. *J. Chem. Ecol.* 23:2067–2078.

DRIJFHOUT, F. P., VAN BEEK, T. A., VISSER, J. H., and DE GROOT, Æ. 2000. On-line thermal desorption-gas chromatography of intact living insects for pheromone analysis. *J. Chem. Ecol.* 26:1383–1392.

FAUVERGUE, X., HOPPER, K. R., and ANTOLIN, M. F. 1995. Mate finding via a trail sex pheromone by a parasitoid wasp. *Proc. Natl. Acad. Sci. U.S.A.* 92:900–904.

FUKAYA, M., YASUDA, T., WAKAMURA, S., and HONDA, H. 1996. Reproductive biology of the yellow-spotted longicorn beetle, *Psacotheca hilaris* (Pascoe) (Coleoptera: Cerambycidae). III. Identification of contact sex pheromone on female body surface. *J. Chem. Ecol.* 22:259–270.

GRAHAM, H. M. 1988. Sexual attraction of *Lygus hesperus* Knight. *Southwest. Entomol*. 13:31–37.

GROOT, A. T., VAN DER WAL, A. J., SCHUURMAN, A., VISSER, J. H., BLOMMERS, L. H. M., and VAN BEEK, T. A. 1998. Copulation behavior of *Lygocoris pabulinus* under laboratory conditions. *Entomol. Exp. Appl.* 88:219–228.

GU, X., QUILICI, D., JUÁREZ, P., BLOMQUIST, G. J., and SCHAL, C. 1995. Biosynthesis of hydrocarbons and contact sex pheromone and their transport by lipophorin in females of the German cockroach (*Blattella germanica*). *J. Insect Physiol.* 41:257–267.

HARDIE, J., and MINKS, A. K. (eds.). 1999. Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Oxon, UK, 466 pp.

HOWARD, R. W., and BLOMQUIST, G. J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol*. 27:149–172.

JACKSON, L. J. 1983. Cuticular hydrocarbons of the milkweed bug *Oncopeltus fasciatus* by age and sex. *Insect Biochem*. 13:19–25.

JUÁREZ, P., and BLOMQUIST, G. J. 1993. Cuticular hydroacorbons of *Triatoma infestans* and *T. mazzotti*. *Comp. Biochem. Physiol*. 106B:667–674.

KAINOH, Y., and OISHI, Y. 1993. Source of sex pheromone of the egg-larval parasitoid *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *J. Chem. Ecol*. 19:963–969.

KENNEDY, J. S. 1977. Behaviorally discriminating assays of attractants and repellents, pp. 215–229, *in* H. H. Shorey and J. J. McKelvey, Jr. (eds.). Chemical Control of Insect Behavior, Theory and Application. John Wiley & Sons, New York.

LANG, J. T. 1977. Contact sex pheromone in the mosquito *Culiseta inornata* (Diptera: Culicidae). *J. Med. Entomol*. 14:448–454.

LEADBETTER, G., and PLIMMER, J. R. 1979. An improved preparation of some insect sex attractants: Synthesis and separation of geometrical isomers by formation of urea complexes. *J. Chem. Ecol*. 5:101–108.

MARSH, D. 1972. Sex pheromone in the aphid *Megoura viciae. Nature* 238:31–32.

MCBRIEN, H. L., and MILLAR, J. C. 1999. Phytophagous bugs, pp. 277–304, *in* J. Hardie and A.K. Minks (eds.). Pheromones of Non-Lepidopterous Insects Associated with Agricultural Plants. CABI Publishing, Oxon, UK.

MCCULLAGH, P., and NELDER, J. A. 1989. Generalized Linear Models, 2nd ed. Chapman and Hall, London.

MILLAR, J. G., and RICE, R. E. 1998. Sex pheromone of the plant bug *Phytocoris californicus* Knight (Heteroptera: Hemiptera: Miridae). *J. Econ. Entomol.* 91:132–137.

MILLAR, J. G., RICE, R. E., and WANG, Q. 1997. Sex pheromone of the mirid bug *Phytocoris relativus* Knight. *J. Chem. Ecol.* 23:1743–1754.

MINKS, A. K., and VAN DEVENTER, P. 1992. Practical applications: The European scene, pp. 9–18, *in* R. L. Ridgway, M. Inscoe, and H. Arn (eds.). Insect Pheromones and Other Behavior-Modifying Chemicals. BCPC Monograph No 51. Farnham, British Crop Protection Council.

MORGAN, E. D., HÖLLDOBLER, B., VAISAR, T., and JACKSON, B. D. 1992. Contents of poison apparatus and their relation to trail-following in the ant *Deceton atmigerum. J. Chem. Ecol.* 18:2161–2168.

MUHAMMED, S., BUTLER, J. F., and CARLSON, D. A. 1975. Stable fly sex attractant and mating pheromones found in female body hydrocarbons. *J. Chem. Ecol.* 1:399–412.

PAULMIER, I., BAGNÈRES, A., AFONSO, C. M. M., DUSTICIER, G., RIVIÈRE, G., and CLÉMENT, J. 1999. Alkenes as a sexual pheromone in the leaf-cutter bee *Megachile rotundata. J. Chem. Ecol.* 25:471–490.

PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

SCHAL, C., SEVALA, V. L., YOUNG, H. P., and BACHMANN, J. A. S. 1998. Sites of synthesis and transport pathways of insect hydrocarbons: Cuticle and ovary as target tissues. *Am. Zool.* 38:382–393.

SCHIESTL, F. P., AYASSE, M., PAULUS, H. F., LÖFSTEDT, C., HANSSON, B. S., IBARRA, F., and FRANCKE, W. 1999. Orchid pollination by sexual swindle. *Nature* 399:421–422.

SCHIESTL, F. P., AYASSE, M., PAULUS, H. F., LÖFSTEDT, C., HANSSON, B. S., IBARRA, F., and FRANCKE, W. 2000. Sex pheromone mimicry in the early spider orchid (*Ophrys sphegodes*): Patterns of

hydrocarbons as the key mechanism for pollination by sexual deception. *J. Comp. Physiol. A* 186:567–574.

SCHLEIN, Y., GALUN, R., and BEN-ELIAHU, M. N. 1980. The legs of *Musca domestica* and *Glossina morsitans* females as the site of sex pheromone release. *Experientia* 38:1174–1176.

SMITH, R. F., PIERCE, H. D., and BORDEN, J. H. 1991. Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *J. Chem. Ecol*. 17:1437–1447.

STADDON, B. W. 1979. The scent glands of Heteroptera. *Adv. Insect Physiol*. 14:351–418.

UEBEL, E. C., SCHWARS, M., SONNET, P. E., MILLER, R. W., and MENZER, R. E. 1978. Evaluation of the mating stimulant pheromone of *Fannia canicularis*, *F. pusio*, and *F. femoralis* as attractants. *Fla. Entomol*. 61:139–143.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

OVIPOSITION DETERRENT BY FEMALE REPRODUCTIVE GLAND SECRETION IN JAPANESE PINE SAWYER, *Monochamus alternatus*

HISASHI ANBUTSU[1] and KATSUMI TOGASHI*

Department of Environmental Studies
Faculty of Integrated Arts and Sciences, Hiroshima University
Hiroshima 739-8521, Japan

(Received June 20, 2000; accepted January 28, 2001)

Abstract—Adult females of *Monochamus alternatus* are known to use palpation to recognize oviposition scars that contain eggs and to be deterred from oviposition. This study investigated the oviposition-deterring activity of a jellylike secretion deposited by the females immediately after oviposition and methanol extracts of female reproductive organs in the laboratory. When females searching for oviposition sites encountered artificial oviposition scars, they stopped walking and drummed the surface and inside of the oviposition scars with their maxillary and labial palpi. When the females encountered the artificial scars plugged with the jellylike secretion, most of them left the scars after palpation. In contrast, when females encountered artificial scars not plugged with the secretion, most of them deposited single eggs through the scars. In another experiment, most females left artificial scars treated with methanol extracts of the spermathecal gland or other reproductive organs after palpation, but most of them oviposited through the scars treated with methanol alone. The results showed that females' recognition of egg-containing scars and departure from such scars were mediated by the chemical(s) produced by their reproductive organs.

Key Words—Coleoptera, Cerambycidae, egg recognition, female reproductive organs, *Monochamus alternatus*, oviposition behavior, oviposition-deterring pheromone, oviposition scar, spermathecal gland.

INTRODUCTION

In many phytophagous insects where larval development is confined to a limited food resource, ovipositing females recognize and avoid hosts already occupied

[1]Current address: National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, 305-8566, Japan.
*To whom correspondence should be addressed. e-mail: togashi@hiroshima-u.ac.jp

0098-0331/01/0600-1151$19.50/0

by conspecific eggs (e.g., Prokopy, 1972, 1975; Prokopy et al., 1976, 1977, 1978). In such cases, certain chemicals mediate these behavioral strategies. They are called oviposition-deterring pheromones (ODPs) or host-marking pheromones (HMPs).

The cerambycid beetle *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) is a primary vector of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, the causative agent of pine wilt disease in Japan (Mamiya and Enda, 1972; Morimoto and Iwasaki, 1972). They oviposit on dying and recently killed trees of *Pinus densiflora* Sieb. et Zucc. and *P. thunbergii* Parl. The larvae feed on the phloem and pupate in xylem (Togashi, 1989).

Prior to oviposition, a female *M. alternatus* searches for a suitable site for oviposition, walking slowly and touching the bark surface with her maxillary and labial palpi and one or both antennae (Anbutsu and Togashi, 2000). On finding a suitable site, the female stops walking and begins to gnaw at the bark surface with her mandibles to make a wound. Then, the female turns 180° to position the ovipositor over the wound, inserts the ovipositor through the center of the wound, and deposits a single egg in the inner bark. Before withdrawing her ovipositor from the wound, the female deposits a jellylike secretion at the bottom of the central hole of the wound. Just after withdrawal, the female rubs the wound with her abdominal tip and then leaves it (Anbutsu and Togashi, 2000). In this paper, wounds with an obvious hole indicating the insertion of the ovipositor are called oviposition scars.

The oviposition scars of *M. alternatus* are distributed uniformly on the bark surface of dead pine trees (Kobayashi, 1975; Shibata, 1984). The females are deterred from ovipositing at all on pine bolts heavily infested with oviposition scars (Anbutsu and Togashi, 1996). When *M. alternatus* females searching for oviposition sites encounter oviposition scars containing eggs, they palpate the scars with their maxillary and labial palpi and then leave the area (Anbutsu and Togashi, 2000).

The deposition of a jellylike secretion and the rubbing of the oviposition scars with the abdominal tip seem to be related to the marking of the oviposition scars. In most cases, however, the departure of females from egg-containing oviposition scars occurs not after the drumming of the bark surface around the oviposition scars rubbed with abdominal tips, but after they insert their maxillary palpi into the central hole of the scars (Anbutsu and Togashi, 2000). Thus, the jellylike secretion is more likely to be related to the female's recognition of the egg-containing oviposition scars and departure from them. Close observation suggests that this secretion comes from the ovipositor of the female.

The objective of this study was to determine the oviposition-deterring activity of the jellylike secretion and of an extract of female reproductive organs.

METHODS AND MATERIALS

Insects. Laboratory cultures of *M. alternatus* were established from collections of dead, infested *P. densiflora* trees at Tokuyama City, Yamaguchi Prefecture, Japan in 1994.

Newly emerged adults were raised individually in plastic containers (9 × 18 × 4.5 cm) on current year to 2-year-old *P. densiflora* twigs at 25°C and under a 12L:12D photoregime. Seven days after emergence, the females and males were paired and provided with *P. densiflora* bolts (see below) for oviposition. Female fertility was confirmed by larval hatching. All adult females used in this study were allowed to oviposit prior to tests.

Pine Bolts. Healthy *P. densiflora* branches were collected in Higashi-Hiroshima City, Hiroshima Prefecture, Japan, and cut into 7-cm-long branch sections referred to as bolts in this paper. The bolts, which were 1.5–2.5 cm in diameter and 5–10 years old, were then placed into polyethylene bags filled with 60–70°C hot water to kill the cells of the inner bark. After cooling the bolts to room temperature over 12–24 hr, they were washed with tap water. After surficial drying at room temperature, both cut ends of each bolt were sealed with paraffin (melting point: 56–58°C) to retard desiccation. The bolts were then placed in polyethylene bags and stored at 25°C until use.

Artificial Oviposition Scars. Artificial oviposition scars were made on 7-cm-long pine bolts. Each artificial scar consisted of one deep, small hole reaching the inner bark and two shallow, smaller wounds running alongside the hole (Anbutsu and Togashi, 2000). The deep hole, ca. 1.0 mm in diameter, was made with the tip of a nail (2.8 mm in diameter) to imitate the trace left by ovipositor insertion. The two shallow wounds were made with sharp-tipped tweezers and imitated marks made with mandibles.

Response of Females to Artificial Scars Plugged with Jellylike Secretion Deposited by Other Females (Experiment 1). Two treatments were designed to test the effect of the secretion on the response of the females to artificial oviposition scars: (1) artificial scars plugged with the jellylike secretion, and (2) artificial scars with no treatment.

To make artificial scars plugged with jellylike secretion, female beetles were placed individually in plastic containers and provided with pine bolts for oviposition. Immediately after the females finished oviposition, an artificial oviposition scar was made at the center of another 7-cm-long bolt and the fresh secretion that was collected from the new, beetle-made oviposition scar was transferred into the central hole of the artificial oviposition scar. The 7-cm-long bolts, each of which contained an artificial scar either with or without the jellylike secretion, were used for tests immediately after making the scars.

A single, 18- to 57-day-old *M. alternatus* female was introduced into a plastic container with a 7-cm-long bolt with one of the two types of the above scars.

The behavior of the female was then recorded for 2–5 hr as she encountered the artificial scar for the first time during her search for an oviposition site. As soon as the beetle departed the scar, the bolt was removed from the container. The scar was then examined, and the number of eggs deposited was determined. All tests were conducted in the laboratory at 25–28°C and under electric light (250–600 lx). Tests for each type of scar were replicated 20 or 21 times. Each female was tested once for each type of scar.

Response of Females to Artificial Scars Treated with Methanol Extract of Female Reproductive Organs (Experiment 2). Since the jellylike secretion deposited into the oviposition scar averaged 1 × 1 × 1 mm, its physiological reservoir was considered to be large and detectable. Dissection revealed that an *M. alternatus* female has a long spermathecal gland (Figure 1). When living females were dissected, the gland contained an almost colorless or slightly yellowish, transparent liquid. However, when females were dissected within a day of their death, the gland was filled with an amber colored, transparent jellylike substance. The substance was very similar in color and viscosity to the jellylike secretion deposited into oviposition scars. Therefore, the spermathecal gland was thought to be the reservoir of the jellylike secretion.

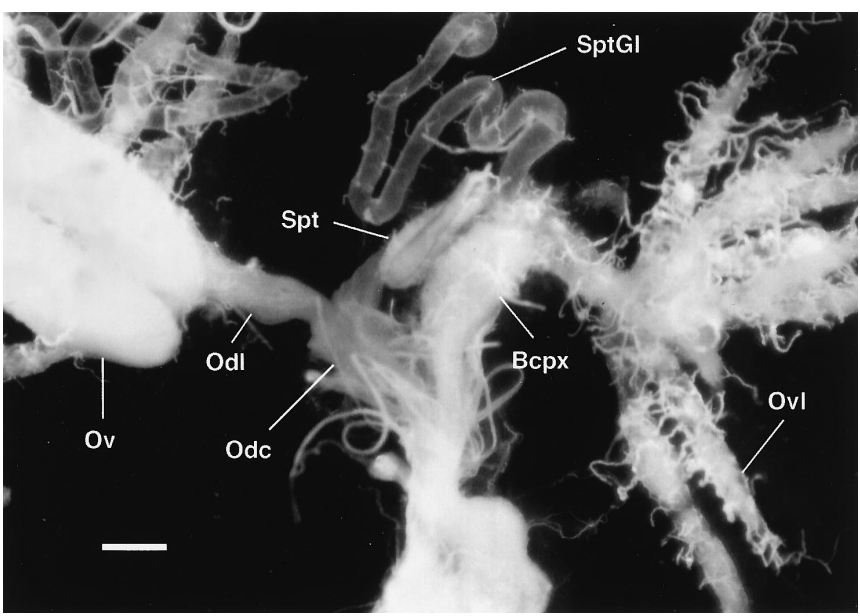


FIG. 1. Reproductive organs of the *Monochamus alternatus* female. Bcpx, bursa copulatrix; Odc, oviductus communis; Odl, oviductus lateralis; Ovl, ovariole; Ov, ovary; Spt, spermatheca; SptGl, spermathecal gland. Scale bar = 1 mm.

Living females anesthetized at 5°C were dissected in a physiological saline solution, the reproductive organs were removed, and the spermathecal gland was separated from the others. The spermathecal gland and other reproductive organs were placed separately in 99.8% methanol, sonicated for 1 min and then allowed to stand at 4°C for 12–24 hr. The methanol containing the spermathecal glands or other reproductive organs was filtered using an injector with a piece of absorbent cotton, the filtrate was then concentrated by evaporation at 25–28°C. The equivalent of four females was placed on a 1 × 10 mm rectangular piece of filter paper (Toyo Roshi Kaisha, Advantec 2) and dried for 30 min in the laboratory. After removing the solvent by evaporation, the paper was cut into 10 square pieces 1 × 1 mm, so that the amount of substances contained in each paper square was 0.4 equivalents of spermathecal glands or other reproductive organs. One microliter of deionized water was put into the central hole of a newly made artificial scar on a 7-cm-long pine bolt, and the filter paper square was then placed in the hole. Immediately after the treatment, the bolt was subjected to testing.

Experiment 2 had three treatments: (1) artificial scars treated with extract of spermathecal glands alone, (2) artificial scars treated with extract of female reproductive organs except the spermathecal glands, and (3) artificial scars treated with methanol alone (control).

The response of females to each type of scar was observed as described for experiment 1. These tests were replicated 20–22 times. A 43- to 74-day-old female was tested once for each type of scar.

Statistical Analyses. The Kolmogorov-Smirnov two-sample test was used to compare the behavioral sequence of *M. alternatus* between treatments. A chi-square test in a contingency table was used to compare the proportion of ovipositing females between treatments.

RESULTS

Response of Females to Artificial Scars Plugged with Jellylike Secretion Deposited by Other Females (Experiment 1). When *M. alternatus* females searching for oviposition sites reached the artificial oviposition scars, they exhibited the same response to the artificial scars as they did to oviposition scars made by the beetles (Anbutsu and Togashi, 2000). They stopped walking and then drummed the surface and central holes of the scars with their maxillary and labial palpi, frequently inserting the former alternately into the central holes. After about 5 sec of palpation, they either left the scars or began to gnaw at them and deposited single eggs.

When the females encountered artificial oviposition scars plugged with the jellylike secretion, 80% left the scars after palpation (Figure 2A). In contrast, when the females encountered artificial scars not plugged with the jellylike secretion,

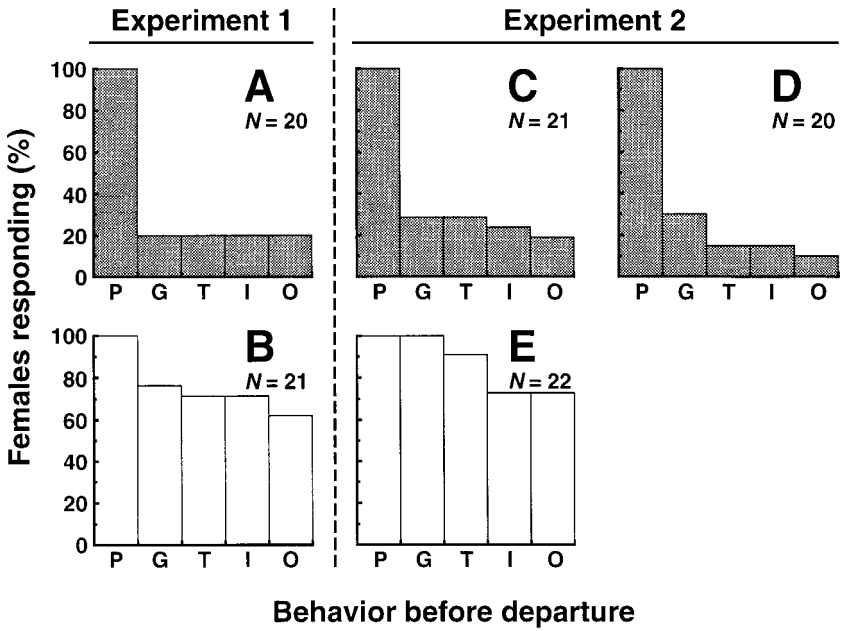


FIG. 2. Response of *Monochamus alternatus* females to the artificial oviposition scars plugged with the jellylike secretion deposited by other females (A); not plugged with the secretion (B); treated with a methanol extract of the spermathecal gland (C); treated with a methanol extract of other reproductive organs including ovariole, oviductus lateralis, oviductus communis, bursa copulatrix, and spermatheca (D); and treated with methanol alone (E). The abscissa shows the behavioral sequence: palpation of the scars (P), gnawing at them (G), 180° body turn (T), insertion of ovipositor (I), and oviposition (O). The ordinate shows the proportions of females exhibiting the particular behavior. N = number of females used in the tests.

76% began to gnaw at the scars after palpation and 62% deposited a single egg each (Figure 2B). The response of females was different between the artificial scars with and without the jellylike secretion (Kolmogorov-Smirnov two sample test, $D = 0.56$, $P < 0.01$) (Figure 2A and B); the proportion of ovipositing females was lower for secretion-containing artificial scars (20%) than for scars without secretion (62%) (contingency table, $\chi^2 = 7.4$, $df = 1$, $P < 0.01$) (Figure 3).

Response of Females to Artificial Scars Treated with Methanol Extract of Female Reproductive Organs (Experiment 2). When females searching for oviposition sites encountered artificial scars treated with methanol extracts of female reproductive organs or methanol alone, they stopped walking and palpated the scars. After palpation, about 70% of the females left the scars treated with methanol

extracts of spermathecal glands or other reproductive organs (Figure 2C and D). In contrast, when the artificial scars were treated with methanol alone, all tested females began to gnaw at the scars and 73% deposited single eggs (Figure 2E). There was no difference in their response between the artificial scars treated with methanol extracts of spermathecal gland and other reproductive organs (Kolmogorov-Smirnov two sample test, $D = 0.14$, $P = 0.98$) (Figure 2C and D). However, the response to artificial scars treated with methanol alone differed from responses to the scars treated with methanol extract of spermathecal gland (Kolmogorov-Smirnov two sample test, $D = 0.71$, $P < 0.001$) (Figure 2C and E) and responses to the scars treated with methanol extract of other reproductive organs (Kolmogorov-Smirnov two sample test, $D = 0.76$, $P < 0.001$) (Figure 2D and E).

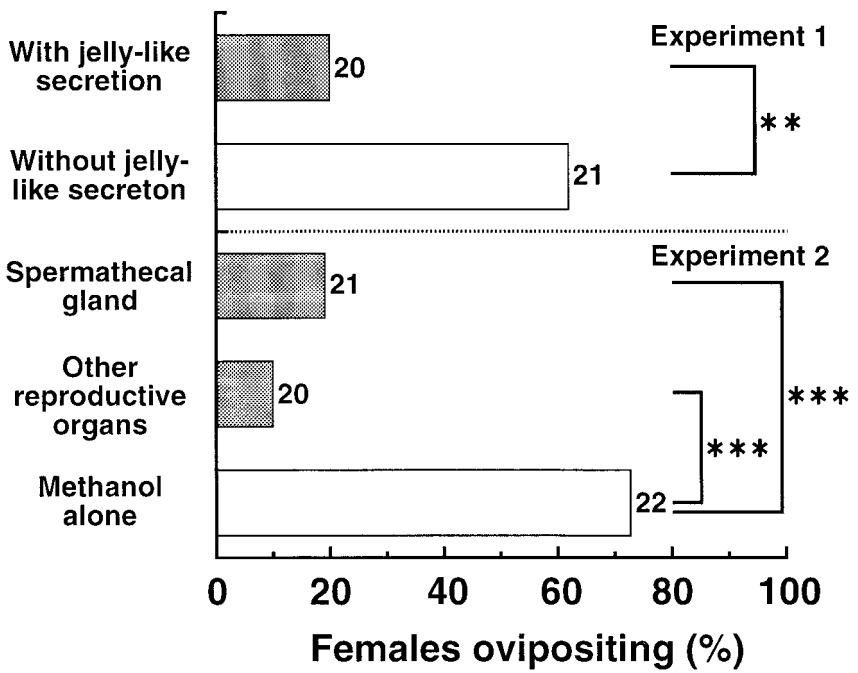


FIG. 3. Ovipositional response of *Monochamus alternatus* females to the artificial oviposition scars plugged with jellylike secretion; not plugged with the secretion; treated with a methanol extract of the spermathecal gland; treated with a methanol extract of other reproductive organs including ovariole, oviductus lateralis, oviductus communis, bursa copulatrix, and spermatheca; and treated with methanol alone. The figures to the right of each column represent the number of females used in the tests. The brackets represent a significant difference by the chi-square test (**$P < 0.01$, ***$P < 0.001$).

The females deposited eggs less frequently through artificial scars treated with methanol extract of spermathecal gland (19%) (contingency table, $\chi^2 = 12.4$, $df = 1$, $P < 0.001$) or other reproductive organs (10%) (contingency table, $\chi^2 = 16.8$, $df = 1$, $P < 0.001$) than through artificial scars treated with methanol alone (73%) (Figure 3).

DISCUSSION

This study showed that *M. alternatus* females were deterred from oviposition both by the jellylike secretion that the conspecific females deposited in the oviposition scars immediately after oviposition and by methanol extract of female reproductive organs (Figures 2 and 3). These results show that females' recognition of the egg-containing scars and departure from such scars were elicited by semiochemical(s) contained in the jellylike secretion they deposited. The semiochemicals were detected in female reproductive organs. Deposition of a jellylike secretion to an oviposition scar after egg laying and avoidance of such a scar also have been reported in the closely related *Monochamus saltuarius* (Gebler) (Anbutsu and Togashi, 1997). The same or similar semiochemical(s) as found in *M. alternatus* may play a role in deterred oviposition in *M. saltuarius.*

When the oviposition scar contains a single egg, the survival rate of the egg is about 0.9 (Anbutsu and Togashi, unpublished), but when an *M. alternatus* female deposits an egg through an oviposition scar already occupied by an egg, the earlier-hatching larva bites and kills the other at the egg or young larval stage. The survival rate of later-deposited eggs was experimentally shown to be less than 0.5 (Anbutsu and Togashi, unpublished).

Sexually mature adults of *M. alternatus* are attracted by a mixture of monoterpenes and ethanol, which are released from *P. densiflora* trees felled by man or inoculated with the pinewood nematode (Ikeda and Oda, 1980; Ikeda et al., 1980, 1986). The concentration of the volatiles is assumed to be higher around the oviposition scars because the bark surface is excavated and the inner bark is exposed. In addition, the oviposition of *M. alternatus* is evoked by nonvolatiles present in the inner bark of *P. densiflora* (Yamasaki et al., 1989). Therefore, if the females could not recognize oviposition scars containing eggs, redundant oviposition might occur more frequently and the fitness of females with secondary oviposition would decrease due to the resulting high mortality of their progeny. The period from egg deposition to larval hatching varies from six to nine days at 25°C in *M. alternatus* (Okuda, 1973). Thus, if the interval between the first and second oviposition events is less than three days, some secondary eggs could hatch before the primary egg and kill it, thus decreasing the fitness of the primary female. Other roles of the jellylike secretion such as maintenance of humidity around eggs and protection from predators are unknown.

The presence of chemical marking to prevent oviposition by other individuals is known in phytophagous insects of the species Coleoptera, Lepidoptera, and Diptera; the Azuki bean weevil, *Callosobruchus chinensis* (L.) (Oshima et al., 1973), the cigarette beetle, *Lasioderma serricorne* (F.) (Kohno et al., 1986; Imai et al., 1990); the cabbage seed weevil, *Ceutorhynchus assimilis* Payk. (Ferguson and Williams, 1991; Mudd et al., 1997); the orange-tip butterfly, *Anthocharis cardamines* (L) (Dempster, 1992); the European grapevine moth, *Lobesia botrana* Den. et Schiff. (Gabel and Thiéry, 1992); and *Hylemya* sp. (Zimmerman, 1979). Many tephritid flies (Diptera) are also known to deposit oviposition-deterring pheromones (Katsoyannos, 1975; Prokopy, 1972, 1975; Prokopy et al., 1976, 1977, 1978; Straw, 1989). In previous studies, the oviposition deterrent was shown to originate from ovaries (Prokopy et al., 1976; Zimmerman, 1979), accessory glands (Schoonhoven, 1990), or the epidermal cells of the seventh urotergite posterior fold (Ferguson et al., 1999). Our study indicated that the spermathecal gland was a reservoir of the jellylike secretion (Figure 2) that contained the deterrent chemical(s). Other reproductive organs also showed oviposition-deterring activity (Figure 2). This suggests that two or more reproductive organs may secrete oviposition-deterring pheromone(s) or that other reproductive organs such as the spermatheca and oviductus communis are contaminated with the secretion from the spermathecal gland (Figure 1).

Oviposition-deterring pheromones are important in pest control. An attempt to prevent infestation of tephritid flies by spraying oviposition-deterring pheromone on its host was successful in the field (Katsoyannos and Boller, 1976, 1980). In a pine forest infested with pine wilt disease, *M. alternatus* females deposit their eggs on pine trees killed recently by the disease. In the following year, the adult beetles carrying pathogenic nematodes emerge from the dead trees, disperse, and transmit the nematodes to healthy trees. The application of oviposition-deterring pheromone(s) of *M. alternatus* or the related chemical(s) to pine trees killed recently by pine wilt disease may prevent *M. alternatus* females from depositing eggs on such trees, resulting in the inhibition of a pine wilt epidemic by the reduced reproduction of such insects. Further investigation is needed to determine the organ that produces the oviposition deterrent and to identify the semiochemical(s).

Acknowledgments—The authors are grateful to the staff of the Tokuyama Forest Experiment Station of Kyoto University for assisting the collection of dead pine trees. They acknowledge the Grant-in-Aid for Scientific Research Fund from the Japan Ministry of Education, Science, Culture and Sports (B2, no. 10556033).

REFERENCES

ANBUTSU, H., and TOGASHI, K. 1996. Deterred oviposition of *Monochamus alternatus* (Coleoptera: Cerambycidae) on *Pinus densiflora* bolts from oviposition scars containing eggs or larvae. *Appl. Entomol. Zool.* 31:481–488.

ANBUTSU, H., and TOGASHI, K. 1997. Oviposition behavior and response to the oviposition scars occupied by eggs in *Monochamus saltuarius* (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* 32:541–549.

ANBUTSU, H., and TOGASHI, K. 2000. Deterred oviposition response of *Monochamus alternatus* (Coleoptera: Cerambycidae) to oviposition scars occupied by eggs. *Agric. For. Entomol.* 2:217–223.

DEMPSTER, J. P. 1992. Evidence of an oviposition-deterring pheromone in the orange-tip butterfly, *Anthocharis cardamines* (L). *Ecol. Entomol.* 17:83–85.

FERGUSON, A. W., and WILLIAMS, I. H. 1991. Deposition and longevity of oviposition-deterring pheromone in the cabbage seed weevil. *Physiol. Entomol.* 16:27–33.

FERGUSON, A. W., SOLINAS, M., ZIESMANN, J., ISIDORO, N., WILLIAMS, I. H., SCUBLA, P., MUDD, A., CLARK, S. J., and WADHAMS, L. J. 1999. Identification of the gland secreting oviposition-deterring pheromone in the cabbage seed weevil, *Ceutorhynchus assimilis*, and the mechanism of pheromone deposition. *J. Insect Physiol.* 45:687–699.

GABEL, B., and THIÉRY, D. 1992. Biological evidence of an oviposition-deterring pheromone in *Lobesia botrana* Den. et Schiff. (Lepidoptera, Tortricidae). *J. Chem. Ecol.* 18:353–358.

IKEDA, T., and ODA, K. 1980. The occurrence of attractiveness for *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) in nematode-infested pine trees. *J. Jpn. For. Soc.* 62:432–434.

IKEDA, T., ENDA, N., YAMANE, A., ODA, K., and TOYODA, T. 1980. Attractants for the Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae). *Appl. Entomol. Zool.* 15:358–361.

IKEDA, T., YAMANE, A., ENDA, N., ODA, K., MAKIHARA, H., ITO, K., and ÔKOCHI, I. 1986. Attractiveness of volatile components of felled pine trees for *Monochamus alternatus* (Coleoptera: Cerambycidae). *J. Jpn. For. Soc.* 68:15–19 (in Japanese with English summary).

IMAI, T., KODAMA, H., CHUMAN, T., and KOHNO, M. 1990. Female-produced oviposition deterrents of the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *J. Chem. Ecol.* 16:1237–1247.

KATSOYANNOS, B. I. 1975. Oviposition-deterring, male-arresting, fruit-marking pheromone in *Rhagoletis cerasi*. *Environ. Entomol.* 4:801–807.

KATSOYANNOS, B. I., and BOLLER, E. F. 1976. First field application of oviposition-deterring marking pheromone of European cherry fruit fly. *Environ. Entomol.* 5:151–152.

KATSOYANNOS, B. I., and BOLLER, E. F. 1980. Second field application of oviposition-deterring pheromone of the European cherry fruit fly, *Rhagoletis cerasi* L. (Diptera: Tephritidae). *Z. Angew. Entomol.* 89:278–281.

KOBAYASHI, F. 1975. Studies on the methods for estimating density and distribution of forest insect populations (I). Distribution of coleopterous borers in pine. *Bull. Gov. For. Exp. Stn.* 274:85–124 (in Japanese with English summary).

KOHNO, M., MOCHIZUKI, K., CHUMAN, T., and OHNISHI, A. 1986. Pheromone-like substances affecting the oviposition behavior of the female cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). *Appl. Entomol. Zool.* 21:15–20.

MAMIYA, Y., and ENDA, N. 1972. Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematologica* 18:159–162.

MORIMOTO, K., and IWASAKI, A. 1972. Rôle of *Monochamus alternatus* (Coleoptera: Cerambycidae) as a vector of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae). *J. Jpn. For. Soc.* 54:177–183 (in Japanese with English summary).

MUDD, A., FERGUSON, A. W., BLIGHT, M. M., WILLIAMS, I. H., SCUBLA, P., SOLINAS, M., and CLARK, S. J. 1997. Extraction, isolation, and composition of oviposition-deterring secretion of cabbage seed weevil *Ceutorhynchus assimilis*. *J. Chem. Ecol.* 23:2227–2240.

OKUDA, M. 1973. Effect of temperature on development of *Monochamus alternatus* (Coleoptera: Cerambycidae). *Transactions, 24th Annual Meeting. Kansai Branch of the Japanese Forestry Society*, pp. 146–149 (in Japanese).

OSHIMA, K., HONDA, H., and YAMAMOTO, I. 1973. Isolation of an oviposition marker from Azuki bean weevil, *Callosobruchus chinensis* (L.). *Agric. Biol. Chem.* 37:2679–2680.

PROKOPY, R. J. 1972. Evidence for a marking pheromone deterring repeated oviposition in apple maggot flies. *Environ. Entomol.* 1:326–332.

PROKOPY, R. J. 1975. Oviposition-deterring fruit marking pheromone in *Rhagoletis fausta*. *Environ. Entomol.* 4:298–300.

PROKOPY, R. J., REISSIG, W. H., and MOERICKE, V. 1976. Marking pheromones deterring repeated oviposition in *Rhagoletis* flies. *Entomol. Exp. Appl.* 20:170–178.

PROKOPY, R. J., GREANY, P. D., and CHAMBERS, D. L. 1977. Oviposition-deterring pheromone in *Anastrepha suspensa*. *Environ. Entomol.* 6:463–465.

PROKOPY, R. J., ZIEGLER, J. R., and WONG, T. T. Y. 1978. Deterrence of repeated oviposition by fruit-marking pheromone in *Ceratitis capitata* (Diptera: Tephritidae). *J. Chem. Ecol.* 4:55–63.

SCHOONHOVEN, L. M. 1990. Host-marking pheromones in Lepidoptera, with special reference to two *Pieris* spp. *J. Chem. Ecol.* 16:3043–3052.

SHIBATA, E. 1984. Spatial distribution pattern of the Japanese pine sawyer, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), on dead pine trees. *Appl. Entomol. Zool.* 19:361–366.

STRAW, N. A. 1989. Evidence for an oviposition-deterring pheromone in *Tephritis bardanae* (Schrank) (Diptera: Tephritidae). *Oecologia* 78:121–130.

TOGASHI, K. 1989. Development of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) in relation to oviposition time. *Jpn. J. Appl. Entomol. Zool.* 33:1–8 (in Japanese with English summary).

YAMASAKI, T., SAKAI, M., and MIYAWAKI, S. 1989. Oviposition stimulants for the beetle, *Monochamus alternatus* Hope, in inner bark of pine. *J. Chem. Ecol.* 15:507–516.

ZIMMERMAN, M. 1979. Oviposition behavior and the existence of an oviposition-deterring pheromone in *Hylemya*. *Environ. Entomol.* 8:277–279.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

REPRODUCTIVE CHARACTER DISPLACEMENT IN *Lymantria monacha* FROM NORTHERN JAPAN?[5]

GERHARD GRIES,[1,*] PAUL W. SCHAEFER,[2] REGINE GRIES,[1] JAN LIŠKA,[3] and TADAO GOTOH[4]

[1]*Center for Environmental Biology*
Department of Biological Sciences, Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

[2]*United States Department of Agriculture, Agricultural Research Service*
Beneficial Insects Introduction Research Laboratory
Newark, Delaware 19713-3814

[3]*Forestry and Game Management Research Institute (VULHM)*
CZ 156 04 Praha 5-Zbraslav, Czech Republic

[4]*Tohoku Research Center, Forestry & Forest Product Research Institute*
Nabeyashiki 72, Shimokuriyagawa, Morioka
Iwate Prefecture 020-01, Japan

(Received March 30, 2000; accepted January 27, 2001)

Abstract—Our objective was to test the hypothesis that the pheromone blend and/or diel periodicity of pheromonal communication differ in populations of the nun moth, *Lymantria monacha* (Lepidoptera: Lymantriidae), from eastern Asia (northern Honshu, Japan) and Central Europe (Bohemia, Czech Republic). Coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of pheromone gland extract of female *L. monacha* from Japan confirmed the presence of compounds previously identified in pheromone extracts of *L. monacha* from Bohemia, as follows: (*Z*)-7-octadecene, 2-methyl-(*Z*)-7-octadecene (2me-*Z*7–18Hy), *cis*-7,8-epoxy-octadecane (monachalure), and *cis*-7,8-epoxy-2-methyloctadecane (disparlure). Field experiments in Honshu suggested that (+)-monachalure is the major pheromone component of *L. monacha*. 2me-*Z*7–18Hy significantly enhanced attractiveness of (+)-monachalure. Addition of (+)-disparlure to (+)-monachalure plus 2me-*Z*7–18Hy in Honshu and Bohemia increased attractiveness of lures by 1.2 and 20 times, respectively, indicating that (+)-disparlure is of least and most significance in the respective *L. monacha* populations. Moreover, capture of male *L. monacha* in pheromone-baited traps between 18:00 and 24:00 hr in Bohemia and 2:00 and 5:00 hr in Honshu revealed a markedly different diel periodicity of pheromonal communication. Pheromonal communication late at night and use of (+)-monachalure, rather

[5]Dedicated to Dr. Hans-Karl Dettmar in honor of his 73rd birthday.
*To whom correspondence should be addressed.

0098-0331/01/0600-1163$19.50/0

than (+)-disparlure, as the major pheromone component by *L. monacha* in Honshu may have resulted from interspecific competition with coseasonal *L. fumida*, which uses the early night for pheromonal communication and (+)-disparlure as major pheromone component. Whether communication channel divergence of *L. monacha* in Honshu indeed constitutes a case of reproductive character displacement is difficult to prove. The evolution of such divergence in *sympatric* populations of *L. fumida* and *L. monacha* would have to be demonstrated.

Key Words—*Lymantria monacha*, *Lymantria fumida*, *Lymantria dispar*, diel periodicity, interspecific competition, reproductive character displacement, strain, (7*R*, 8*S*)-*cis*-7,8-epoxy-2-methyloctadecane, (+)-disparlure, (7*R*, 8*S*)-*cis*-7,8-epoxy-octadecane, (+)-monachalure, 2-methyl-(*Z*)-7-octadecene, (*Z*)-7-octadecene.

INTRODUCTION

The nun moth, *Lymantria monacha* (Lepidoptera: Lymantriidae), is a significant forest defoliator. Polyphagous caterpillars defoliate gymnosperm trees mainly in the genera *Picea* (spruce), *Pinus* (pine), *Larix* (larch), and *Abies* (fir), but also feed on angiosperm trees, including *Fagus* (beech), *Carpinus* (white beech), *Betula* (birch), and *Quercus* (oak) spp. (Wellenstein, 1978).

The first recorded *L. monacha* population outbreak (1853–1864) occurred in European Russia. In this outbreak, 147,000,000 m^3 of timber were killed (Bejer, 1988). Since then, outbreaks have occurred throughout Europe. Five outbreaks are recorded in Poland between 1946 and 1986 (Sliwa and Sierpinski, 1986). During the largest, from 1978 to 1983, over 2 million ha of spruce, pine, and larch forests (one quarter of Poland's forests) were infested and partly defoliated (Schönherr, 1985).

In Europe and western Asia, the northern border of the *L. monacha* range coincides with the September isotherm of 10.5°C, which extends from the southern part of England and Scandinavia to the Ural Mountains (Wellenstein, 1978; Bejer, 1988). The southern extent of its range coincides with the isotherm of >20°C for June, July, and August. This isotherm line crosses central Spain, Corsica, Italy, the Ariatic coast from Slovenia to northern Greek and the Black Sea. The range of *L. monacha* extends, disjunctly, eastward into eastern China, Korea and Japan. Asian populations consist of white color morphs with black spotting. Melanistic forms, as frequently found in Europe (Keena et al., 1998), are absent.

Pheromone blend and diel periodicity of pheromonal communication in *L. monacha* from Central Europe and Japan could differ, because respective populations: (1) occupy different geographic ranges, (2) are reproductively isolated, and (3) are part of lymantriid communities that contrast in numbers of congeners. For example, the July–August lymantriid community of Honshu (northern Japan) comprises *L. monacha*, gypsy moth, *L. dispar*, pink gypsy moth, *L. mathura*, *L. fumida*, *L. lucescens*, and *L. bantaizana*. Presence (Honshu) or absence

(Bohemia, Czech Republic) of *L. mathura, L. fumida, L. lucescens,* and *L. bantaizana* could conceivably affect interspecific competion for communication channels, and thus pheromonal communication in *L. monacha*.

Pheromonal communication of *L. monacha* in Bohemia is mediated by a complex volatile blend. At a 10 : 10 : 1 ratio, the three pheromone components (7*R*, 8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] (Bierl et al., 1975; Klimetzek et al., 1976), (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane [(+)-monachalure] (Gries et al., 1996), and 2-methyl-(*Z*)-7-octadecene (2me-*Z*7–18Hy) (Grant et al., 1996; Gries et al., 1996), synergistically attract male *L. monacha*, whereas (−)-disparlure, (+)- and (−)-monachalure, 2me-*Z*7–18Hy, and (*Z*)-7-octadecene (*Z*7–18Hy) synergistically prevent cross-attraction of male *L. dispar* (Gries et al., 1996).

This study reports contrasting pheromone blends and diel periodicities of pheromonal communication in two geographically remote populations of *L. monacha,* and suggests reproductive character displacement as a mechanism responsible for these differences.

METHODS AND MATERIALS

Experimental Insects and Chemical Analyses. Pupae of *L. monacha* were collected in northern Japan. Abdominal tips with pheromone glands of calling, virgin female moths were removed and placed in redistilled hexane. These extracts were hand-carried, and light-trapped male moths couriered to Simon Fraser University in Canada. Aliquots of one female equivalent (FE) of pheromone gland extract and authentic standards were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), using a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m × 0.25 or 0.32 mm ID) coated with either DB-210, DB-5, or DB-23 (J&W Scientific, Folsom, California). Synthetic chemicals for laboratory and field testing, including (+)- and (−)-disparlure, (+)- and (−)-monachalure, 2-methyl-(*Z*)-7-octadecene (2me-*Z*7–18Hy), and (*Z*)-7-octadecene (*Z*7–18Hy), were available from previous work (Gries et al., 1996). If indicated, compounds were purified (>95%) by high-performance liquid chromatography (HPLC), employing a Waters LC 626 high-performance liquid chromatograph equipped with a Waters 486 variable wavelength UV visible detector, a Waters 746 data module, and a Nova-Pak C_{18} column (3.9 × 300 mm) with 1 ml/min of acetonitrile flow.

Composition of Pheromone Blends. All field experiments employed a complete randomized block design with 9–12 blocks (replicates) each. Experiments were conducted in forests near (<50 km) the cities of Morioka (Honshu) and Zbraslav (Bohemia). Forests were stocked mainly with Japanese larch, *Larix leptolepsis,* and Norway spruce, *Picea abies,* respectively. Delta-like traps were made from 2-liter milk cartons (Gray et al., 1984), coated with Tanglefoot (The

Tanglefoot Company, Grand Rapids, Michigan), and suspended from trees 1.5 m above ground at 15 to 20-m spacing. They were baited with a gray sleeve stopper (identification # 10600275; West Pharmaceutical Services, Lionville, Pennsylvania) impregnated with candidate pheromone components in HPLC-grade hexane.

Experiment 1 compared attraction of male *L. dispar* and *L. monacha* to (+)-disparlure and (+)-monachalure, respectively. With only (+)-monachalure significantly attractive to male *L. monacha* (and thus likely being the major pheromone component of *L. monacha* in Honshu), experiments 2–3 investigated potential synergists, including (+)-disparlure, 2me-*Z*7–18Hy (the hydrocarbon analog of disparlure), and *Z*7–18Hy (the hydrocarbon analog of monachalure). Experiments 4–6 tested the effect of presence and proportion of (+)-monachalure in pheromone blends on attraction of male *L. monacha, L. fumida,* and *L. dispar*. Experiments 7–10 compared the relative importance of (+)-disparlure in pheromone blends for attraction of male *L. monacha* in Honshu and Bohemia.

Various treatments in several experiments did not attract any male moths. Despite transformation, trap catch data were not normally distributed and were thus analyzed by nonparametric analyses of variance (Friedman's test) followed by comparison of means by Bonferroni (Dunn) *t* test (Zar, 1984; SAS/STAT, 1988). In all analyses, $\alpha = 0.05$.

Diel Periodicity of Pheromonal Communication. Diel periodicities of pheromonal communication of *L. monacha* in Bohemia (experiment 10) and of *L. monacha* and *L. fumida* in Honshu (experiment 11) were assessed by hourly recording captures of male moths in pheromone-baited traps. Experiment 10 had already been conducted during nine nights in 1989 and employed two sticky beta type traps (ÚOCHB AVCR, Praha, Czech Republic) baited with rubber septa impregnated with (±)-disparlure (100 μg) (ÚOCHB AVCR). For experiment 11, the 20 sticky Delta traps (see above) were suspended from trees ~2 m above ground at 15 to 20-m intervals. Traps were baited with gray sleeve stoppers impregnated with the three-component blend of (+)-monachalure (50 μg), (+)-disparlure (5 μg), and 2me-*Z*7–18Hy (5 μg) for male *L. monacha* or with the two-component blend of (+)-disparlure (50 μg) and 2me-*Z*7–18Hy (5 μg) for male *L. fumida* (Schaefer et al., 1999). Baits for *L. monacha* and *L. fumida* were alternated between traps.

RESULTS

Comparative GC-EAD analyses of pheromone gland extract and synthetic standards on three fused silica columns confirmed the presence of *Z*7–18Hy (peak **1**), 2me-*Z*7–18Hy (peak **2**), monachalure (peak **3**) and disparlure (peak **4**) in pheromone extracts of female moths (Figure 1). Disparlure appeared to be most

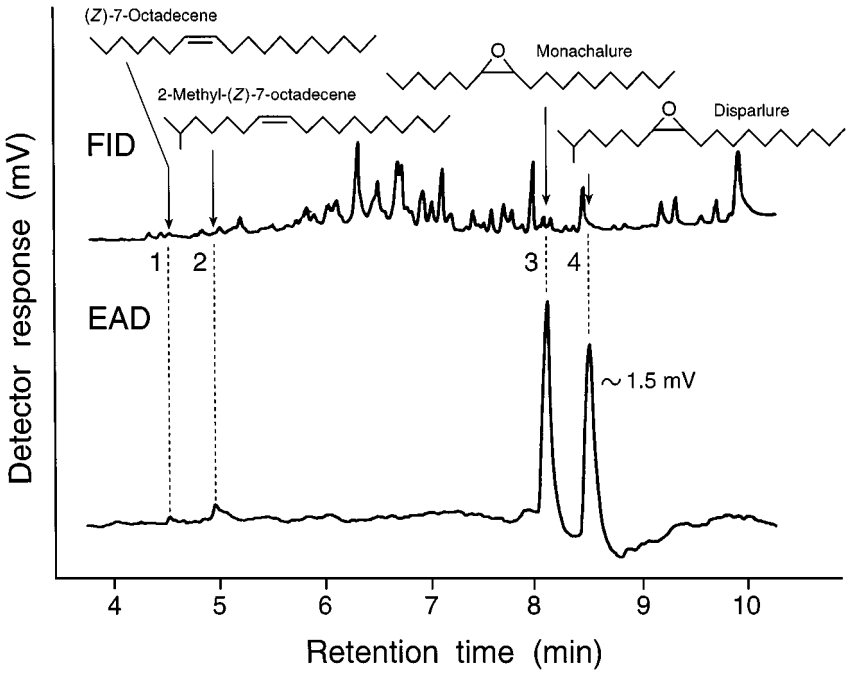


FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: male *L. monacha* antenna) responses to one female (*L. monacha* from Honshu) equivalent of pheromone gland extract. A gas chromatograph was fitted with a fused silica column (30 m × 0.25 mm ID) coated with DB-23; temperature program: 50°C (1 min), then 25°C/min to 100°C then 10°C/min to 220°C (10 min). Compound abbreviations: **1** = (*Z*)-7-octadecene (*Z*7–18Hy); **2** = 2-methyl-(*Z*)-7-octadecene (2me-*Z*7–18Hy); **3** = (7*R*,8*S*)-*cis*-7,8-epoxy-octadecane [(+)-monachalure] or (7*S*, 8*R*)-*cis*-7,8-epoxy-octadecane [(−)-monachalure]; **4** = (7*R*, 8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] or (7*S*, 8*R*)-*cis*-7,8-epoxy-2-methyloctadecane [(−)-disparlure]. Retention indices: *Z*7–18Hy: 1782 (DB-5), 1792 (DB-210), 1825 (DB-23); 2me-*Z*7–18Hy: 1842 (DB-5), 1860 (DB-210), 1877 (DB-23); monachalure: 1971 (DB-5), 2234 (DB-210), 2284 (DB-23); disparlure: 2032 (DB-5), 2306 (DB-210), 2339 (DB-23).

abundant, but GC-mass spectrometry (MS) confirmed that another quantitatively significant compound co-eluted with it. All four EAD-active components occurred at quantities too low to reliably calculate their relative proportion.

In field experiment 1, (+)-monachalure attracted male *L. monacha*, whereas (+)-disparlure attracted male *L. dispar* (Figure 2). The hydrocarbon analog of disparlure, 2me-*Z*7–18Hy, was significantly more effective than (+)-disparlure in enhancing attractiveness of (+)-monachalure (Figure 2, experiment 2). In contrast,

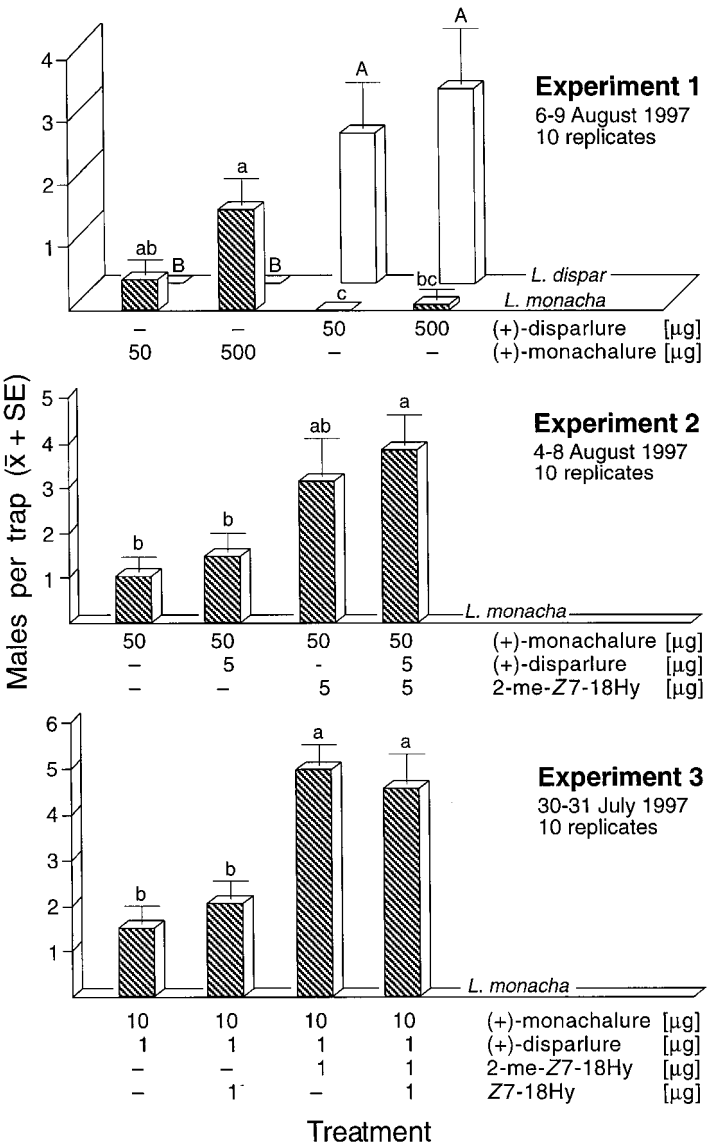


FIG. 2. Mean number of male *L. monacha* or *L. dispar* captured in sticky Delta traps baited with various volatile blends; larch forests near the city of Morioka (northern Honshu, Japan). Compound abbreviations as in Figure 1. Bars within rows of each experiment with the same letter superscript are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) t test, $P < 0.05$] (Zar, 1984; SAS/STAT, 1988).

the hydrocarbon analog of monachalure, *Z*7–18Hy, had no behavioral activity (Figure 2, experiment 3). A low proportion of (+)-monachalure in three-component blends resulted in hardly any captures of males (Figure 3, experiment 4). Conversely, addition of (+)-monachalure at increasing quantities to the two-component blend of (+)-disparlure and 2me-*Z*7–18Hy significantly enhanced trap captures of male *L. monacha,* whereas captures of male *L. fumida* were not affected (Figure 3, experiment 5). Similarly, addition of (+)-monachalure (10 μg) to a blend of (+)-disparlure (10 μg) and 2me-*Z*7–18Hy (1 μg) significantly increased captures of male *L. monacha,* failed to affect captures of *L. fumida,* and suppressed captures of male *L. dispar* (Figure 3, experiment 6). The same three-component blend but with (+)-disparlure at 1 μg attracted only male *L. monacha* (Figure 3, experiment 6). (+)-Disparlure at 5 or 50 μg, but not 500 μg, in admixture with (+)-monachalure (50 μg) and 2me-*Z*7–18Hy (5 μg) doubled trap captures of male *L. monacha* in Honshu (Figure 4, experiments 7 and 8). In Bohemia, addition of 5 and 50 μg of (+)-disparlure to pheromone baits of an equivalent experiment increased trap captures of male *L. monacha* by 10 and 20 times, respectively (Figure 4, experiment 9).

Male *L. monacha* in Bohemia and Honshu were captured in pheromone-baited traps from 19:00 to 24:00 hr and 02:00 to 05:00 hr, respectively (Figure 5).

DISCUSSION

Evidence for Different Strains of L. monacha. Our data support the hypothesis that there are different strains of *L. monacha.* The possibility that we studied different species—rather than strains—was excluded based on comparisons of genitalia (P.W.S., unpublished data) from specimens collected in Honshu and Bohemia. Female *L. monacha* from both strains employ pheromone blends with the same three components but of different relative importance. Moreover, trap captures of males between 19:00 and 24:00 hr in Bohemia and between 02:00 and 05:00 hr in Honshu reflect a marked difference in diel periodicity of pheromonal communication.

In Honshu, (+)-monachalure is the major pheromone component of *L. monacha.* It was more attractive to males than (+)-disparlure (Figure 2, experiment 1), and pheromone blends with no or little (+)-monachalure failed to attract significant numbers of males (Figure 3). The fact that (+)-monachalure suppressed cross-attraction of male *L. dispar* (Figure 3, experiment 6) is supportive evidence that (+)-monachalure is the major component in the *L. monacha* pheromone. Synergistic activity expressed by 2me-*Z*7–18Hy exceeded that of (+)-disparlure (Figure 2, experiment 2), suggesting that the hydrocarbon analog of disparlure is the second most important pheromone component. (+)-Disparlure as

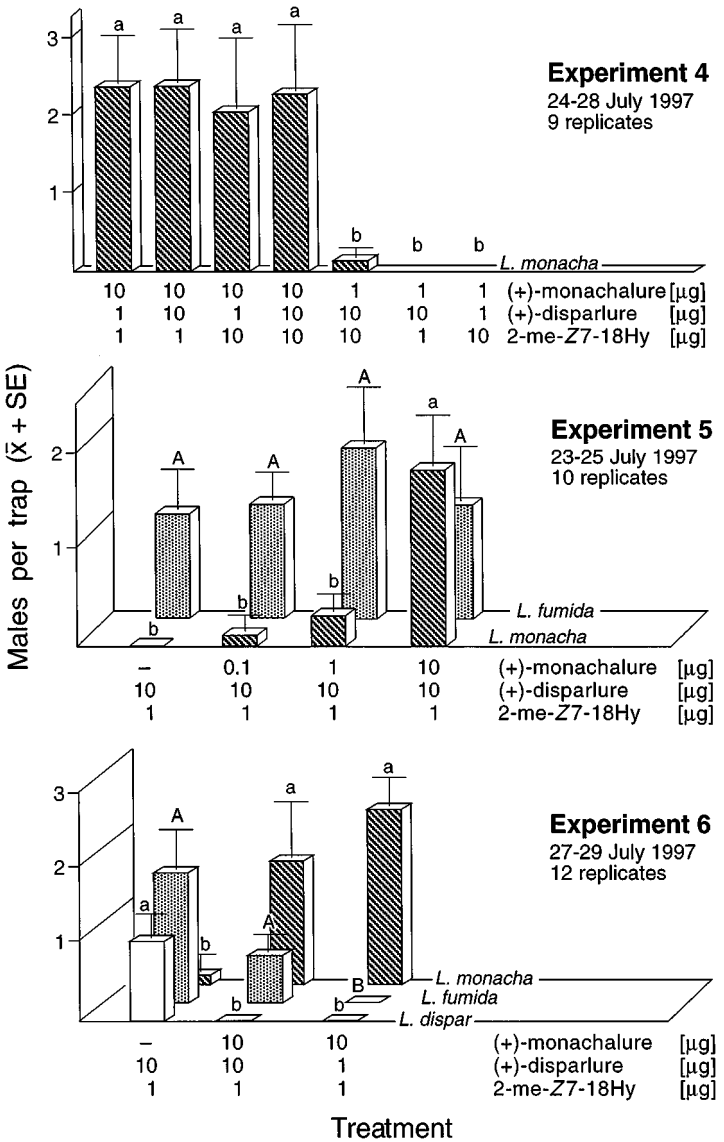


FIG. 3. Mean number of male *L. monacha*, *L. fumida*, or *L. dispar* captured in sticky Delta traps baited with various volatile blends; larch forests near the city of Morioka (northern Honshu, Japan). Compound abbreviations as in Figure 1. Bars within rows of each experiment with the same letter superscript are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) *t* test, $P < 0.05$] (Zar, 1984; SAS/STAT, 1988).

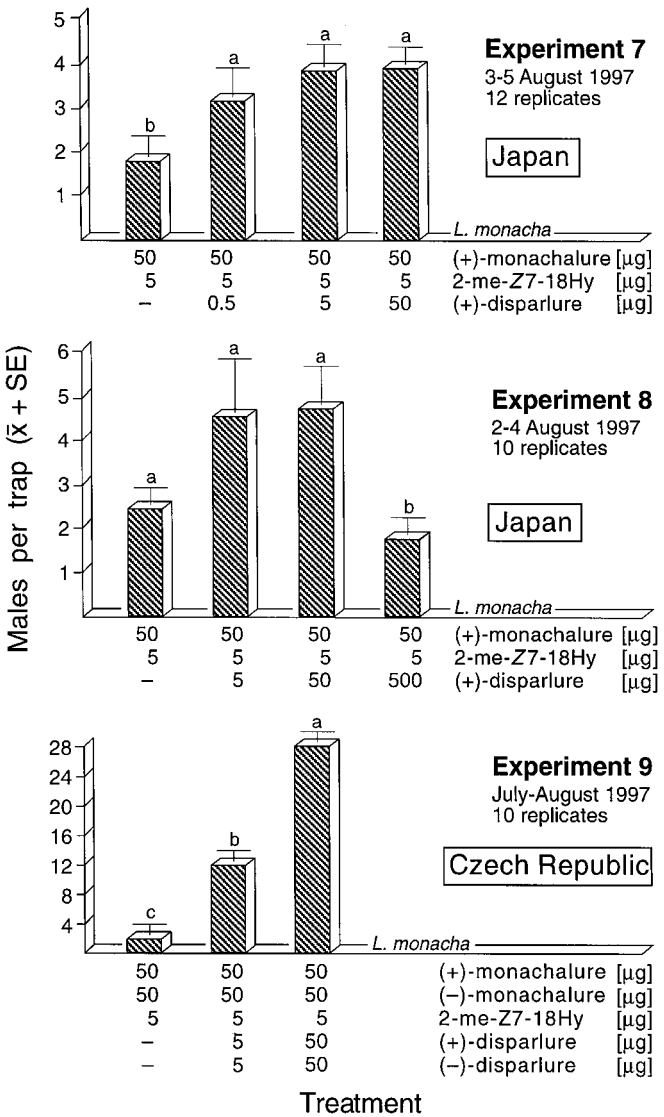


FIG. 4. Mean number of male *L. monacha* captured in sticky Delta traps baited with various volatile blends; larch forests near the city of Morioka (northern Honshu, Japan) (experiments 7 and 8) and spruce forests near the city of Zbraslav (Czech Republic) (experiment 9). Compound abbreviations as in Figure 1. Bars with the same letter superscript in each experiment are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means [Bonferroni (Dunn) t test, $P < 0.05$] (Zar, 1984; SAS/STAT, 1988).

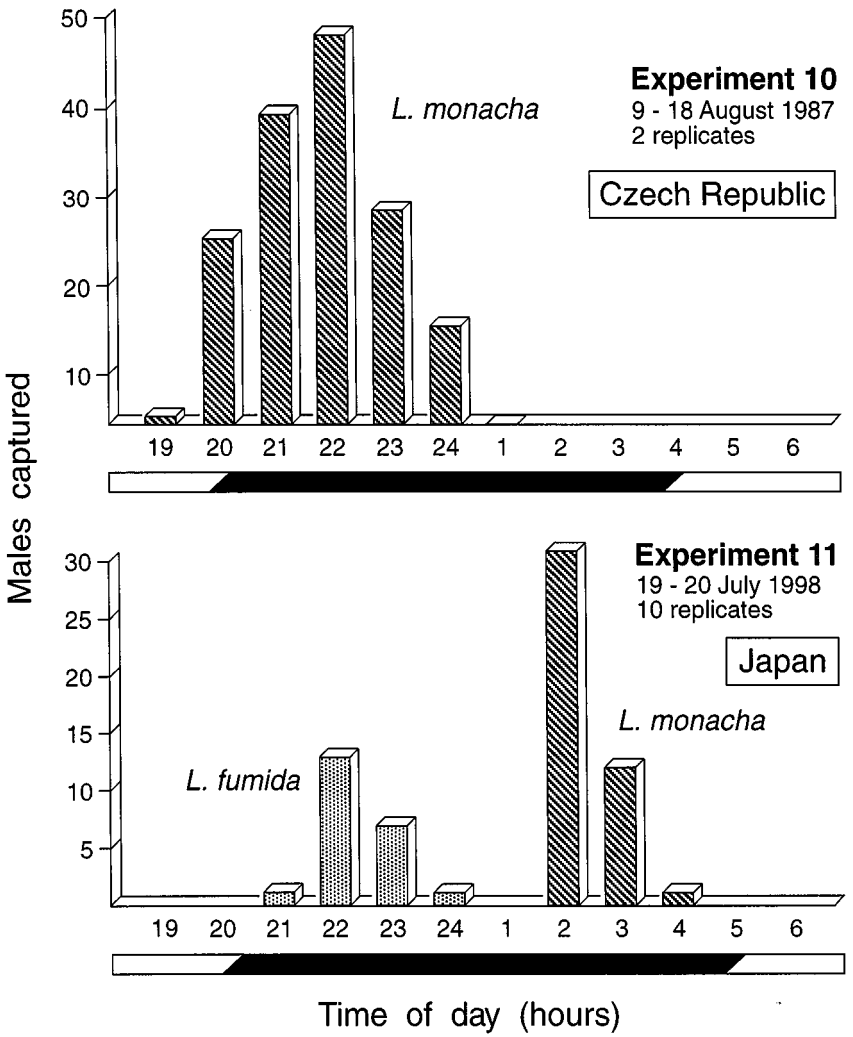


FIG. 5. *Top*: Total numbers of male *L. monacha* captured in sticky Beta traps baited with (±)-disparlure (100 μg). Spruce forests near the city of Zbraslav (Czech Republic). *Bottom* (adapted from Schaefer et al., 1999): Total numbers of male *L. monacha* and *L. fumida* captured in 20 sticky Delta traps baited with (+)-monachalure (50 μg), (+)-disparlure (5 μg), and 2me-*Z*7–18Hy (5 μg) for male *L. monacha* or with (+)-disparlure (50 μg) and 2me-*Z*7–18Hy (5 μg) for male *L. fumida*. Larch forests near the city of Morioka (northern Honshu, Japan). Black horizontal bars indicate scotophase.

a third pheromone component has only weak behavioral activity. Added to a binary blend of (+)-monachalure and 2me-*Z*7–18Hy, it barely increased (Figure 2, experiment 2) or doubled (Figure 4, experiments 7 and 8) attractiveness of the lure. This marginal effect of (+)-disparlure in the pheromone blend of *L. monacha* from Honshu contrasts with superior importance of (+)-disparlure in the pheromone blend of *L. monacha* from Bohemia. In Bohemia, addition of (+)-disparlure at 5 and 50 μg to the pheromone blend increased captures of males by 10 and 20 times, respectively (Figure 4, experiment 9).

There was no significant difference in antennal response pattern in GC-EAD analyses of pheromone extract of female *L. monacha* from Honshu (Figure 1) and Bohemia (Gries et al., 1996). This result does not reflect the contrasting importance of (+)-monachalure and (+)-disparlure in pheromone blends of the two strains, supporting our contention that GC-EAD analyses help determine the presence but not behavioral significance of (candidate) pheromone components.

With (+)-monachalure and (+)-disparlure being the major pheromone component of *L. monacha* in Honshu and Bohemia, respectively, the question remains whether pheromone-based detection surveys for *L. monacha* in North America ought to deploy strain-specific pheromone lures. The three-component blend of (+)-disparlure, (+)-monachalure and 2me-*Z*7–18Hy at a 10 : 10 : 1 ratio was most attractive to males in Bohemia (Gries et al., 1996). The same blend and blend ratio is effective in attracting males in Honshu (Figure 3, experiment 4), although the amount of (+)-disparlure could be reduced 10-fold without any effect. The presence of (−)-disparlure and (−)-monachalure had no effect on attraction of males in Bohemia (Gries et al., 1996) or Honshu (data not shown). Thus, the recently developed pheromone lure for *L. monacha* detection surveys in North America (Morewood et al., 1999), containing (±)-disparlure, (±)-monachalure and 2me-*Z*7–18Hy at a 20 : 20 : 1 blend remains suitable for attraction of *L. monacha* from both Central Europe and Japan (Gries et al., 1998).

Reproductive Character Displacement in L. monacha *Strain from Japan*? There are numerous local populations of *L. monacha* with *g*eno- and phenotypic characteristics shaped by physical and biological factors of the local environment. If we consider the presence of congeners a biological factor (Linn and Roelofs, 1995), five congeners (*L. dispar, L. fumida, L. mathura, L. lucescens,* and *L. bantaizana*) in Honshu should constitute a greater "factor" than the single congener (*L. dispar*) in Bohemia. With five synchronic congeners emitting communication signals, the forest habitat certainly becomes noisy, with selection pressure to improve the signal-to-noise ratio of communication channels (Baker, 1985; Cardé and Baker, 1984). Competition-induced reproductive character displacement *sensu* Butlin (1987) (divergence of traits that are coincidentally shared by sympatric species after speciation has occurred, so that hybridization is impossible) may cause signal divergence to avoid heterospecific matings. We hypothesize that dissimilar communication channels of *L. monacha* in Bohemia and Honshu

are the result of signal divergence caused by *L. fumida* as a competing species in Honshu.

According to our hypothesis, *L. fumida* seem to have affected both communication time and signal (pheromone blend) of *L. monacha*. With *L. fumida* signaling early at night, signaling of *L. monacha* has shifted to the early morning (Figure 5). With (+)-disparlure being most important in the pheromone of *L. fumida* (Schaefer et al., 1999), it has become insignificant in the pheromone of *L. monacha* (Figure 2, experiment 2; Figure 4, experiments 7 and 8). Our hypothesis that *L. monacha* in Honshu has substituted (+)-disparlure with (+)-monachalure as its major pheromone component (in response to competing *L. fumida*) is supported by the fact that the hydrocarbon analog of monachalure (*Z*7–18Hy) has no behavioural activity (Figure 2; experiment 3), whereas the hydrocarbon analog of disparlure (2me-*Z*7–18Hy) remains a synergistic pheromone component (Figure 2, experiments 2 and 3).

The concept of reproductive character displacement was proposed by Löfstedt et al. (1991) to explain pheromone variation in closely related ermine moths and can be considered in the context of studies that demonstrate geographic differences in a species' pheromone in the presence of competing species. For example, male *Archips argyrospilus* from New York respond to a more narrowly defined blend of pheromone components than do conspecifics from British Columbia, likely due to interference by the sibling species *A. mortuanus* in New York (Cardé et al., 1977; Butlin., 1995). Similarly, contrasting pheromone blends of the oblique-banded leafroller, *Choristoneura rosaceana* from New York (Hill and Roelofs, 1979) and British Columbia (Vakenti et al., 1988) may be attributable to the competing species *Archips rosanus* in British Columbia (Thomson et al., 1991). Finally, saturniid female and male *Hemileuca electra mojavensis* may have modified their pheromone-based communication to minimize interference from congeneric *H. burnsi* (McElfresh and Millar, 1999).

The above-cited examples and our own data favor the hypothesis that interspecific competition *can* be a selective force on a species' communication channel. Whether communication channel divergence of *L. monacha* in Honshu indeed constitutes a case of reproductive character displacement is difficult to prove. The evolution of such divergence in *sympatric* populations of *L. fumida* and *L. monacha* would have to be demonstrated. Greater differences, if demonstrated, in communication channels of *L. fumida* and *L. monacha* in sympatric than in allopatric populations in eastern Asia would support our hypothesis that the pheromone biology of *L. monacha* was shaped, in part, by competition with *L. fumida*.

Acknowledgments—We thank two anonymous reviewers for constructive comments. The research was supported, in part, by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to G.G.

REFERENCES

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c:722–725.

BAKER, T. C. 1985. Insect mating and courtship behavior, pp. 621–672, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Press, New York.

BIERL, B. A., BEROZA, M., ADLER, V. E., KASANG, G., SCHRÖTER, H., and SCHNEIDER, D. 1975. The presence of disparlure, the sex pheromone of the gypsy moth, in the female nun moth. *Z. Naturforsch.* 30c:672–675.

BEJER, B. 1988. The nun moth in European spruce forest, pp. 211–231, *in* A. A. Berryman (ed.). Dynamics of Forest Insect Populations: Patterns, Causes, Implications. Plenum Press, New York.

BUTLIN, R. K. 1987. Speciation by reinforcement. *Trends Ecol. Evol*. 2:8–13.

BUTLIN, R. K. 1995. Genetic variation in mating signals and responses, pp. 327–366, *in* D. M. Lambert and H. G. Spencer (eds.). Speciation and Recognition Concept: Theory and Application. John Hopkins University Press, Baltimore, Maryland.

CARDÉ, R. T., and BAKER, T. C. 1984. Sexual communication in insects, pp. 355–386, *in* W. Bell and R. T. Cardé (eds.). Chemical Ecology of Insects. Sinauer, Sunderland, Massachusetts.

CARDÉ, R. T., CARDÉ, A. M., HILL, A. S., and ROELOFS, W. L. 1977. Sex pheromone specificity as a reproductive isolating mechanism among the sibling species *Archips argyrospilus* and *A. mortuans* and other sympatric Tortricine moths (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 3:71–84.

GRANT, G. G, LANGEVIN, D., LIŠKA, J., KAPITOLA, P., and CHONG J. M. 1996. Olefin inhibitor of gypsy moth, *Lymantria dispar,* is a synergistic pheromone component of nun moth, *L. monacha. Naturwissenschaften* 83:328–330.

GRAY, T. G., SLESSOR, K. N., SHEPHERD, R. F., GRANT, G. G., and MANVILLE, J. F. 1984. European pine shoot moth, *Rhyacionia buoliana* (Lepidoptera: Tortricidae): Identification of additional pheromone components resulting in an improved lure. *Can. Entomol.* 116:1525–1532.

GRIES, G., GRIES, R., KHASKIN, G., SLESSOR, K. N., GRANT, G. G., LIŠKA J., and KAPITOLA, P. 1996. Specificity of nun and gypsy moth sexual communication through multiple-component pheromone blends. *Naturwissenschaften* 83:382–385.

GRIES, G., GRIES, R., and SCHAEFER, P. W. 1998. Pheromone blend attracts nun moth, *Lymantria monacha* (Lepidoptera: Lymantiidae) in Japan. *Can. Entomol.* 129:1177–1178.

HILL, A. S., and ROELOFS, W. L. 1979. Sex pheromone components of the oblique-banded leafroller moth *Choristoneura rosaceana. J. Chem. Ecol.* 5:3–11.

KEENA, M., SHIELDS, K., and TORSELLO, M. 1998. Nun moth: potential new pest. United States Department of Agriculture, Forest Service, Northeastern Area; NA-PR-95-98, 2 pp.

KLIMETZEK, D., LOSKANT, G., VITÉ, J. P., and MORI, K. 1976. Disparlure: Differences in pheromone perception between gypsy moth and nun moth. *Naturwissenschaften* 63:581–582.

LINN, C. E., JR., and ROELOFS, W. L. 1995. Pheromone communication in moths and its role in the speciation process, pp. 263–300, *in* D. M. Lambert and H. G. Spencer (eds.). Speciation and the Recognition Concept. John Hopkins University Press, Baltimore, Maryland.

LÖFSTEDT, C., HERREBOUT, W. M., and MENKEN, S. B. J. 1991. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology* 2:20–28.

MCELFRESH, J. S., and MILLAR, J. G. 1999. Geographic variation in sex pheromone blend of *Hemileuca electra* from southern California. *J. Chem. Ecol.* 25:2505–2525.

MOREWOOD, P., GRIES, G., HÄUSSLER, D., MÖLLER, K., LIŠKA, J., KAPITOLA, P., and BOGENSCHÜTZ, H. 1999. Towards pheromone-based detection survey of nun moth, *Lymantria monacha*, in North America. *Can. Entomol.* 131:687–694.

SAS/STAT. 1988. User's guide, release 6.03 edition, SAS Institute, Cary, North Carolina.

SCHAEFER, P. W., GRIES, G., GRIES, R., and HOLDEN, D. 1999. Pheromone components and diel periodicity of pheromonal communication in *Lymantria fumida*. *J. Chem. Ecol.* 25:2305–2312.

SCHÖNHERR, J. 1985. Nun moth outbreak in Poland 1978–1984. *Z. Angew. Entomol.* 99:73–76.

SLIWA, E., and SIERPINSKI, Z. 1986. Gradation der Nonne (*Lymantria monacha* L.) in Polen von 1978–1984. *Anz. Schaedlingskde. Pflanzen. Umweltschutz* 59:81–86.

THOMSON, D. R., ANGERILLI, N. P. D., VINCENT, C., and GAUNCE, A. P. 1991. Evidence for regional differences in the response of obliquebanded leafroller [*Choristoneura rosaceana* (Lepidoptera: Trotricidae)] to sex pheromone blends. *Environ. Entomol.* 20:935–938.

WELLENSTEIN, G. 1978. *Lymantria monacha* L., Nonnenspinner, Nonne, pp. 207–278, *in* W. Schwenke (ed.). Die Forstschädlinge Europas, Paul Parey, Hamburg.

VAKENTI, J. M., GAUNCE, A. P., SLESSOR, K. N., KING, G. G. S., ALLAN, S. A., MADSEN, H. F., and BORDEN, J. H. 1988. Sex pheromone components of the oblique-banded leafroller moth, *Choristoneura rosaceana*, in the Okanagan Valley of British Columbia. *J. Chem. Ecol.* 14:605–621.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey, 718 pp.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

IDENTIFICATION AND SYNTHESIS OF A MALE-PRODUCED SEX PHEROMONE FROM THE STINK BUG *Chlorochroa sayi*

HSIAO-YUNG HO[1,2] AND JOCELYN G. MILLAR[1,*]

[1]*Department of Entomology*
University of California
Riverside California, 92521

[2]*Institute of Zoology*
Academia Sinica
Taipei, Taiwan

(Received October 16, 2000; accepted February 13, 2001)

Abstract—The reproductive behavior of the stink bug *Chlorochroa sayi* was studied in the laboratory. There was a sexual maturation period of about 10 days before bugs began mating. Sexually mature adult bugs engaged in courtship consisting of antennation and head-butting of the female by the male, before the female adopted a receptive posture and copulation occurred. Both sexes mated multiple times during their life-spans, with the mean duration of copulations of virgin bugs (42.3 ± 19.6 min) and experienced bugs (37.3 ± 28.4 min) being similar. Most matings were initiated in the late afternoon or evening, when pheromone production by males was greatest. Males transferred sperm and nutrients constituting about 17% of their body weight to females during mating. Three male-specific components, methyl geranate, methyl citronellate, and methyl (*E*)-6-2,3-dihydrofarnesoate in a ratio of 100 : 0.45 : 1.6, were first detected in volatiles collected from male bugs on green beans about 9–12 days after the final molt to the adult stage. In vertical Y-tube bioassays, females were attracted to odors from mature male bugs, and to a blend of the three male-produced components. Low numbers of females also were attracted in field trials with the three-component blend. The relatively weak attraction may be a result of other, as yet unknown cues being required in addition to the pheromone, such as visual or substrate-borne vibrational cues.

Key Words—*Chlorochroa sayi*, Pentatomidae, pheromone, methyl geranate, methyl citronellate, methyl (*E*)-6-2,3-dihydrofarnesoate, Y-tube bioassay, reproductive behavior.

*To whom correspondence should be addressed. e-mail: jocelyn.millar@ucr.edu

0098-0331/01/0600-1177$19.50/0

INTRODUCTION

Phytophagous stink bugs (Heteroptera: Pentatomidae) are found in all types of crops, including grain, forage and fiber crops, vegetables, and perennial crops such as tree fruits and nuts (Metcalf and Metcalf, 1993; McPherson and McPherson, 2000). Damage is caused by both immatures and adults, but only adults are winged and capable of long-distance movement. Injury to young seeds, fruits, or nuts produces necrotic lesions and often results in premature abortion, while attacked leaves may wilt and die. Stink bugs are also known or implicated as vectors of plant pathogens such as yeast, fungi, and bacteria (Hoffmann et al., 1987; Panizzi, 1997).

Many stink bug species are polyphagous, and the adults are highly mobile, which exacerbates problems with their monitoring and control. Some crops, such as cotton, alfalfa, and legumes, have chronic problems with bugs, whereas damage to tree crops is more sporadic and difficult to predict, because significant damage only occurs when large numbers of bugs move into a crop from the surrounding habitat (LaRue and Johnson, 1989). These migrations occur in response to natural events, such as the senescence of native vegetation in the habitat, or in response to mowing or harvesting of nearby crops harboring large bug populations (Toscano and Stern, 1976). Effective bug management hinges on the rapid detection of these invasions, so that appropriate control measures can be implemented before serious crop damage occurs. However, monitoring and sampling methods for most bug species are still relatively primitive, consisting mainly of sweep-net or beating tray sampling, or visual inspection of fruits for feeding damage or excrement. Effective monitoring methods based on pheromones or other attractants have not yet been developed for economically important stink bug species.

The identification and development of applications for stink bug pheromones has been complicated by several factors (McBrien and Millar, 1999). First, stink bugs produce voluminous quantities of volatile defensive secretions in response to disturbance, so it can be difficult to obtain extracts uncontaminated with these defensive secretions, which may mask or overwhelm the pheromone components. Second, for the few phytophagous stink bug species that have been studied, pheromone components were produced by mature males from patches of unicellular glands (Borges et al., 1987; Evans et al., 1990) rather than from discrete, macroscopic glands that can be dissected for analysis. Third, bugs must be in an appropriate physiological state to produce or respond to pheromones. After the final molt, stink bugs take a number of days to reach sexual maturity and begin producing and responding to pheromones (e.g., Wang and Millar, 1997). Fourth, adults enter reproductive diapause in response to deteriorating conditions (short daylength, poor food) and stop producing or responding to pheromones (e.g., Strong et al., 1970; Aldrich et al., 1991). Finally, the roles of sex and aggregation pheromones are poorly understood in stink bugs. Sex-specific compounds have

been identified from several phytophagous species, including *Nezara viridula* (L.) (Baker et al., 1987; Brézot et al., 1994), *Euschistus* spp. (Aldrich et al., 1991, 1994; Borges et al., 1998a), *Plautia stali* Scott (Sugie et al., 1996), *Thyanta pallidovirens* Stål (Millar, 1997), *Piezodorus hybneri* Gmelin (Leal et al., 1998), *P. guildinii* (Westwood) (Borges et al., 1998b), and *Biprorulus bibax* Breddin (James et al., 1994). However, the function of many of the compounds that have been identified to date remains unclear. There is some evidence that the major male-produced components function as sex or aggregation pheromones, but in general, the role of minor components in sex-specific odors remains obscure.

The stink bug *Chlorochroa sayi* (Stål) is distributed throughout the western United States, where it damages wheat (Caffrey and Barber, 1919), tomato (Ohlendorf, 1990), cotton (Morrill, 1910), and alfalfa (Russell, 1952). In California, it can have several generations per year, and details of its life history have been reported by Morrill (1910) and Russell (1952). We report here the results of studies on the reproductive behavior and semiochemistry of *C. sayi*. Our overall goal was to identify bug-produced semiochemicals that might be of use for developing better bug monitoring methods. Specific objectives were: (1) to describe the reproductive behavior of *C. sayi*; (2) to identify volatile chemicals produced by *C. sayi*, focusing particularly on sex-specific compounds that might be sex pheromone components; and (3) to test reconstructed blends of sex-specific compounds as stink bug attractants in laboratory and field bioassays.

METHODS AND MATERIALS

Insects. A colony of *C. sayi* was started from bugs collected by sweep-netting at the University of California Agricultural Operations site in Riverside, California. Additional bugs were collected from Russian thistle (*Salsola iberica* Sennen) in Riverside County in the summers of 1998 and 1999. Bugs were reared on a diet of organically grown green-bean pods (*Phaseolus vulgaris* L.), raw shelled peanuts (*Arachis hypogaes* L.), and raw sunflower seeds (*Helianthus annuus* L.). The diet was supplemented with bouquets of alfalfa (*Medicago sativa* L.) or seasonal weeds including mustard [*Brassica campestris* (L.)], London rocket (*Sisymbrium irio* L.), Russian thistle, shepherd's purse (*Capsella bursa-pastoris* L.), and cheeseweed (*Malva parviflora* L.), depending on availability. Bugs were reared in a controlled environment chamber, on a 16L : 8D cycle (lights on 06:00–22:00 hr), with lighting provided by banks of eight fluorescent light tubes (Sylvania Octron, 32W, F032/T35), at $23 \pm 2^{\circ}$C, and $>50\%$ relative humidity. Eggs were collected from the colony every other day and were held in covered Petri dishes through to the second nymphal stage. After the second molt, nymphs were transferred to 1.9-liter cardboard ice-cream containers with muslin lids. Nymphs were fed as described above, with food changed every other day. After the final molt,

adults were collected and sexed, and cohorts of virgin adults were maintained with food in clean ice-cream cartons (5–7 bugs per 0.95-liter container, 10–20 bugs per 1.9 liter container) until used for collection of volatiles or bioassays.

Observations of Reproductive Behavior. Eight pairs of mature (at least 14 days old) virgin laboratory-reared female and male *C. sayi* were put in clear plastic cylinders (4 cm ID $\times$ 15 cm long with small holes in the tube walls for ventilation), with segments of green beans provided for food. A fluorescent light (GE wide-spectrum light for plants and aquariums, F20T12-PL/AQ) provided light during photophase (06:00–22:00 hr), and a red incandescent light bulb (40 W) on a rheostat was used during the scotophase, with light intensity just sufficient for observation with a video camera (about 6 lux). The bugs were video-taped continuously with a video cassette recorder (Panasonic AG-6730) in time-lapse mode (1.6 frames/sec; clock time recorded on each frame), using a desktop video camera (model VCM7310, RockVision). The tubes and the camera were set up in a box (75 $\times$ 60 $\times$ 50 cm) with a removable front panel, which was only open when changing food or bugs, so that the bugs were not disturbed. The tubes were set up in a row so that all were in the camera's field of view simultaneously. Pairs of bugs were put into tubes at 10:00 hr, and video taping was started immediately. The bugs were examined and food was changed every morning at 10:00 hr. If either bug of any pair died, that tube was replaced with a new pair of bugs in a fresh tube. Data recorded included the time each copulation started, duration of each copulation, and the total number of copulations/per pair. To determine diurnal mating rhythms, the numbers of matings initiated per hour were plotted versus the time period. A total of 32 matings from 13 pairs in nine consecutive days were observed.

Weight Change During Mating. Sexually mature ($>$12 days old) virgin adults were weighed to the nearest 0.1 mg, then put into clear plastic cylinders as described above, with one pair per cylinder, without food, between 17:00 and 18:00 hr. Pairs were observed in the bug rearing room (lighting as above, temperature 24 $\pm$ 2°C, humidity 40 $\pm$ 20%) for 3 hr. For those pairs that copulated, immediately after mating, each bug was weighed, and the experienced individuals then were held in same-sex cohorts in a cardboard ice-cream carton with a screen lid, with food provided. Pairs that did not mate within 3 hr were used as controls, and the individuals were weighed after 3 hr and then held in same-sex virgin cohorts for a repeat trial the next evening. Thirty virgin pairs and 39 experienced pairs were observed between March 8 and 16, 2000, with a total of 13 copulations between virgin pairs and 15 copulations between experienced pairs being observed.

Collection of Insect-Produced Compounds by Aeration of Live Bugs. Two to five sexed, virgin adult bugs and two green beans were put into a 300-ml glass aeration chamber lined with hardware cloth screen for the bugs to perch on. Humidified, charcoal-filtered air (6–14 mesh activated charcoal granules) was drawn through the chamber (300 ml/min). Entrained bug volatiles were collected on activated charcoal traps made from glass tubes (4 mm ID.) loaded with a 0.4-cm bed

of 80–100 mesh activated charcoal (Fisher Scientific, Pittsburgh, Pennsylvania), precleaned by heating at 200°C under a flow of clean N_2 (~100 ml/min) overnight. Aerations were conducted continuously for two to three weeks at ~25°C with cohorts of bugs of known age, changing the food and the collectors every other day. Collectors were eluted with pentane (500 μl), and extracts were stored in glass vials with Teflon-lined screw caps at about −20°C until needed. Aeration chambers were set up near the window so that bugs had natural light, and supplementary fluorescent light was provided with a light bank directly overhead to provide long day conditions (lights on from 06:00 to 22:00 hr). Bugs that died were replaced with virgin individuals from a cohort of the same age. Dead bugs were removed as soon as discovered to minimize contamination; as muscles relaxed in dead bugs, the contents of the defensive metathoracic glands were released (Ho, personal observation). As a control, five green beans were aerated for 17 hr, collecting the volatiles as described above.

Dynamics of Pheromone Release. Cohorts of virgin male bugs of known age were aerated to determine the onset of production of male-specific compounds. Cohorts were aerated in 300-ml (300 ml/min; 3–5 bugs) or 1-liter chambers (1 liter/min; 10–15 bugs), respectively. Aerations were continued for two weeks, with the collectors changed daily.

To determine the diurnal pattern of pheromone release, 10–20 sexed, virgin adult male bugs and 5 green beans were aerated in a 1-liter chamber as described above, changing the collectors daily. Once a cohort of bugs was confirmed to be producing male-specific compounds, the collectors were changed every 2 hr between 7:00 and 23:00 hr, for periods of 24 hr. A single collector was used to collect the volatiles produced between 23:00 and 07:00 hr. The 24-hr aeration periods were replicated with five cohorts of males. Internal standard (10-methylnonadecane, 77 ng) was added to each extract so that the amounts of bug-produced compounds could be estimated from GC analyses. The total amounts of the major male-specific compounds produced during each 24-hr aeration period were calculated, and then the amounts produced during each 2-hr interval were calculated as a percentage of the total. For the 8-hr period between 23:00 and 07:00 hr, the average amount per 2 hr was used. Data were analyzed by ANOVA, and differences between means were determined by application of the Student-Newman-Keuls test.

Analysis of Extracts. Crude extracts prepared as described above were concentrated by evaporation under a gentle stream of nitrogen, as required, and analyzed by splitless gas chromatography on a DB-17 column (30 m × 0.25 mm, J&W Scientific, Folsom, California), with a temperature program of 50°C for 1 min and then 10°C/min to 250°C. Injector and detector temperatures were 250°C and 280°C, respectively, with helium carrier gas. Extracts were also analyzed by splitless coupled gas chromatography–mass spectrometry (GC-MS) with a Hewlett Packard 5890 GC fitted with a DB-5MS column (20 m × 0.2 mm ID), and interfaced to an Hewlett Packard 5970B mass selective detector (electron impact ionization,

70 eV). The GC was programmed from 40°C at 1 min, then 10°C/min to 250°C, with injector and transfer line temperatures of 250 and 280°C, respectively. Compounds were tentatively identified from interpretation of the mass spectra and from matches with the NBS-NIH mass spectral data base. Identifications were confirmed by comparison of retention times and spectra with those of authentic standards.

Fractionation of Bug Aeration Extracts. A 2.8-ml solid-phase extraction cartridge (500 mg silica gel, Alltech, Deerfield Illinois; stock # 209250) was flushed with 4 ml of 25% ether in pentane and 4 ml pentane. A concentrated (∼250 μl) bug extract in pentane, prepared by combining 10 extracts prepared from June 1997 to March 1998, was loaded onto the cartridge, rinsing with 2–3 drops of pentane. The cartridge was then eluted with 4 × 1 ml pentane, 3 × 1 ml of 10% ether in pentane, 3 × 1 ml 25% ether in pentane, and 1 × 3 ml ether, collecting each as a separate fraction.

Laboratory Bioassays. Laboratory bioassays were carried out with a vertical glass Y-tube olfactometer (4.5 cm ID, arms 14 cm long, center tube 18 cm long). Each arm of the Y terminated in a female ground glass fitting, with matching male fittings terminating in hose nipples. Teflon tubing connected the Y tube to stimulus flasks, and vinyl tubing connected the bottom outlet to a vacuum source.

Test bugs were reared under long day conditions as described above. Lighting was provided by a light bank fitted with a daylight fluorescent lamp and a wide-spectrum grow-light fluorescent lamp [Sylvania Octron 32W, to mimic natural daylight, as suggested by Shields (1989)] suspended 30 cm above the olfactometer. The light level at the upper end of the Y-tube was ∼600 lux and at the lower end ∼300 lux. Bioassays were conducted at ambient temperature and humidity conditions in the laboratory (26°C ± 3°C and 50 ± 15% humidity). Because preliminary studies had indicated that most reproductive activity took place in early evening, bioassays were started at around 18:00 hr, and continued until ∼22:00 hr.

Bioassays Using Odors from Live Male Bugs as Test Stimulant. Individual sexually mature virgin male bugs were put in a 125-ml Erlenmeyer flask with half a green bean and a piece of screen (7 × 7 cm) for the bugs to perch on. Flasks with male bugs were placed in the bioassay room at about 16:30 hr to allow the bugs to acclimate to the bioassay room conditions. To begin a bioassay, a flask containing a male bug was connected to one arm of the Y-tube olfactometer by Teflon tubing, with the flask placed close to the light bank (∼1000 lux at the flask position). The other arm of the Y tube was connected to a control flask with half a green bean and a screen only. Visual cues were blocked by placing a piece of paper towel in the bottom of the stimulus and control flasks. Room air purified by passage through an activated charcoal trap was pulled through the stimulus and control flasks and then into the Y tube at a total flow rate of 2 liters/min.

Individual sexually mature female bugs were transferred carefully into the bottom end fitting of the Y tube, with a piece of translucent weighing paper placed

on top to prevent escape. The fittings with females were set up in the bioassay room about 1 hr before starting bioassays to allow the females time to settle down after being transferred to the fittings. Female bugs were not provided with food. To begin a bioassay, a flask containing a male bug and a control flask were connected to the Y tube, and air was pulled through the system for 5 min. Then an end fitting with a female test bug was gently maneuvered into place, and the vinyl tubing was attached to the hose nipple to restart flow through the olfactometer. The criterion for a response was that the female test bug crossed the midpoint of either the stimulus or control arm. If the female did not cross the midpoint of either arm after 5 min, she was considered a nonresponder. To avoid any positional bias, the positions of the stimulus and control flasks were switched every two assays. For each bioassay, a new virgin male in a clean flask was used, with a new female bug as a responder, and the Y tube was cleaned after each assay by washing with soapy water, rinsing with distilled water and acetone and oven drying for at least 20 min at 140°C. The Teflon inlet tubing was not changed between replicates, but at the end of each day's bioassays, the tubing was rinsed with acetone and air dried. Using a series of six identical Y tubes, a maximum of 15–20 bioassays could be run per 4-hr session.

Laboratory Bioassays of Extracts of Bug Volatiles, and Synthetic Chemicals. The Y tube olfactometer was used to test the responses of sexually mature, virgin adult female and male bugs to extracts of male volatiles and to single or reconstructed blends of synthesized components. The bioassay was as described above with the following modifications. The test material [an aliquot of a bug extract or a solution of the synthetic chemical(s)] was assayed versus a control consisting of the same amount of clean solvent (pentane) as was used in the dose of the treatment being tested. Pentane solutions of extracts or synthetic chemical(s) were loaded on a filter paper disk (1.5 cm diam.), the solvent was allowed to evaporate for 1 min, and the disk was mounted on a coiled wire hanger placed inside the ground glass end piece. After the aeration extract and pentane controls were set up in the Y tube, bioassays were conducted as described above.

Field Bioassays. The reconstructed blend of the male-specific compounds was used in field bioassays. Lures consisting of 10-cm lengths of amber natural rubber latex tubing (0.15 cm OD, 0.08 cm wall thickness, Fisher Scientific #14-178-5A) were impregnated with methyl geranate (20 mg), methyl citronellate (0.1 mg) and racemic methyl (*E*)-6-2,3-dihydrofarnesoate (0.4 mg). The compounds were dissolved in 1 ml of dichloromethane and added to a Petri dish (9 cm ID) containing a length of tubing. The dish was covered until the solution had been absorbed into the tubing. Lures were hung in the upper portion of Russian thistle plants (one per plant). Control lures consisted of rubber tubes soaked in 1 ml of dichloromethane. Each plant with a treatment or control lure was marked with red flag tape. The height of each plant was approximately 100 cm, and lures were spaced about 10 m apart. Bioassays were carried out in vacant lots overgrown with Russian thistle and wild mustard in or near Moreno Valley, Redlands, Ontario, and

Riverside, California. Lures were put out in the morning around 09:00–10:00 hr. Bioassays were evaluated every evening at around 17:00–19:00 hr, by collecting all the bugs within ~20 cm of the lures by hand and recording the sex and numbers on each plant. In the first set of trials (August 3–16, 1999, different blocks in Ontario, Moreno Valley, Redlands, and Riverside, with three replicates of each treatment in each block), the bugs were collected, counted, and released. In the second trial (August 19–24, 1999, six experimental blocks in Moreno Valley and Riverside, with each treatment and control replicated three times at each site), all responding bugs were collected and taken back to the laboratory to augment laboratory colonies.

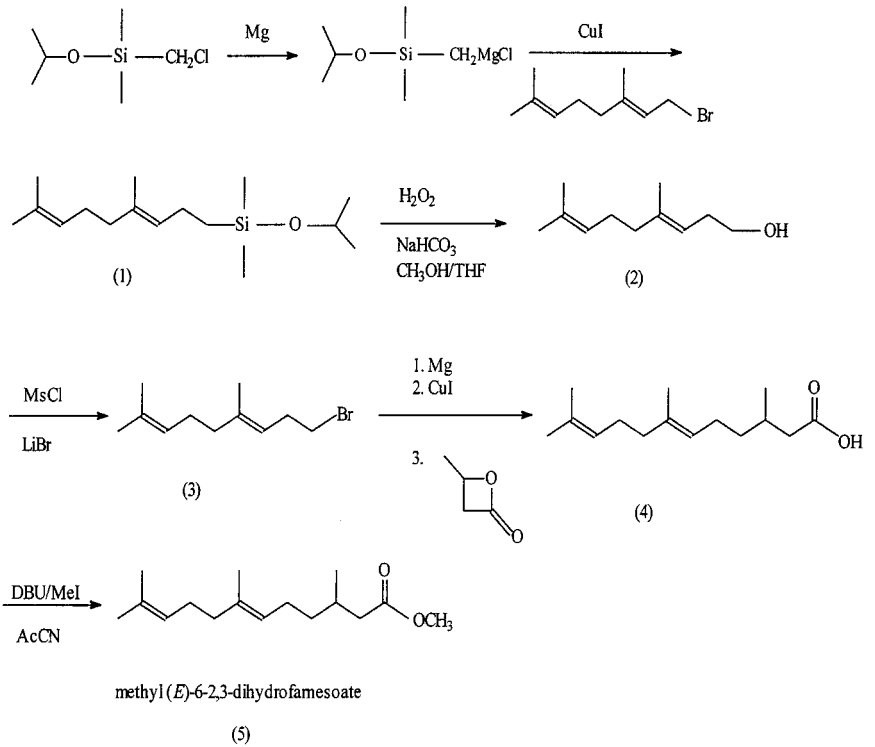
For both trials, where appropriate, the numbers of responding bugs were transformed, using the $\sqrt{(x + 0.5)}$ transformation, and the transformed data were subjected to two-way ANOVA, with differences between treatments and controls determined by Dunnett's method. For those data sets that did not satisfy the assumptions of ANOVA, differences between treatments and controls were determined with the nonparametric multi-response permutation procedure (Biondini et al., 1985; McCune and Mefford, 1999).

Chemicals. Dodecane, (*E*)-2-hexenal, and (*E*)-2-octenal were purchased from Aldrich Chemical (Milwaukee Wisconsin), (*E*)-2-octenyl acetate was obtained from Bedoukian Research (Danbury, Connecticut), and tridecane was obtained from ICN/K&K Laboratories (Irvine, California). (*Z*)-2-Octenyl acetate was prepared by acetylation of (*Z*)-2-octenol by standard methods (acetyl chloride and triethylamine in ether). Commercial methyl geranate (2.5 g) containing ~25% methyl nerate (Bedoukian Research) was purified by flash chromatography on silica gel (5-cm × 25-cm column, 40–63 μm particles), eluting with 1 liter each of 1%, 1.5%, and 2% ether in hexane (Lee et al., 1991), yielding 1.18 g of methyl geranate (97.7% pure) and 0.4 g methyl nerate (100% pure) after Kugelrohr distillation (oven temperature 53°C, 0.2 mm Hg). The ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) of methyl geranate matched literature data (Erman et al., 1969).

Synthesized Compounds. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 and 75.48 MHz, respectively, using a General Electric QE 300 instrument. GC-MS analyses (electron impact ionization, 70 eV) were performed on a Hewlett Packard 5890 gas chromatograph interfaced to an HP 5970B mass selective detector (Palo Alto, California). A DB-5MS column was used (20 m × 0.2 mm, J&W Scientific). Routine work-up of reactions included drying solutions over anhydrous Na_2SO_4 and concentrating by rotary evaporation under an partial vacuum (~80 mm Hg). All reactions were carried out under an argon atmosphere in oven-dried glassware unless otherwise specified.

Methyl Farnesoate. A reference sample of methyl farnesoate was prepared as described elsewhere (Ho, 2000).

Synthesis of Racemic Methyl (*E*)*-6-2,3-dihydrofarnesoate* **5**. This is shown in Scheme 1.



SCHEME 1. Preparation of racemic methyl (*E*)-6-2,3-dihydrofarnesoate.

Isopropoxy-dimethylsilyl-4,8-dimethyl-3,7-nonadiene (**1**). The synthesis was modified from previous syntheses of similar compounds (McMurry and Bosch, 1987; Nagano et al. 1997). Thus, a solution of (chloromethyl)isopropoxy-dimethylsilane (24.9 g, 150 mmol; Aldrich) in THF (150 ml) was added to magnesium turnings (4.8 g, 200 mmol; briefly ground in a mortar and pestle to expose clean Mg surfaces) over ~2 hr. The reaction was initiated by addition of 15 ml of the solution to the Mg turnings in the flask, with several crystals of iodine and 10 drops of dibromoethane, and stirring until the iodine color disappeared. The rest of the chloride solution was added over 2 hr, maintaining the temperature of the mixture about 30°C. When the addition was complete, the solution was heated at 40°C for 1 hr. After cooling to 0°C, the solution was transferred with a large syringe to a precooled (0°C) flask containing 3 g of CuI. The resulting mixture was stirred and cooled to −78°C, and geranyl bromide (22 g; 100 mmol; Aldrich) was added dropwise with stirring over ~30 min. Stirring was continued (~30 min) at −78°C until all the geranyl bromide had been consumed (checking by GC). When the

reaction was complete, the mixture was warmed to 0°C, and quenched with 10 ml ice water. The mixture was poured into an ice-cold mixture of 250 ml saturated aq. NH_4Cl and 25 ml conc. ammonium hydroxide, and extracted with hexane (1×250 ml). The hexane layer was washed with 0.1 M HCl, sat. aq. $NaHCO_3$, and brine and then dried and concentrated. The crude material was used in the next step without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.9–5.1 (m, 2H, H3 and H7), 3.88 [septet, 1H, $J = 6$ Hz, O–C$H(CH_3)_2$], 1.88–2.0 (m, 6H, H2, H5 and H6), 1.58 (s, 3H, H9), 1.49 (d, 6H, $J = 2$ Hz, H4′, H9′), 1.05 (d, 6H, $J = 6$ Hz, O-CH$(CH_3)_2$], 0.5 (m, 2H, m, H1), 0 [6H, s, Si$(CH_3)_2$]. ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 133.50, 131.31, 127.60, 124.53, 39.81, 34.37, 26.83, 25.80, 25.39, 21.63, 18.85, 17.77, 15.93, 0.59. EI-MS m/z: 268 (M^+, 0.1), 225 (2.3), 208 (2), 167 (1), 147 (4), 117 (63), 75 (100), 41 (53).

Homogeraniol (**2**). The synthesis was adapted from those previously reported by McMurry and Bosch (1987) and Tamao and Ishida (1984). Thus, MeOH (200 ml), $NaHCO_3$ (30 g), silane **1** (31 g, purity 85%, ~100 mmol) and THF (200 ml) were added to a three-neck 1-liter flask equipped with a stir bar and condenser. Aqueous H_2O_2 (30%, 80 ml; 784 mmol) was added over 10 min. When the addition was complete, the mixture was refluxed overnight. The mixture was then cooled to room temperature and filtered through a 2.5-cm bed of Cellite in a sintered glass funnel, with suction, rinsing the filter pad with 4×50 ml ether. The solution was concentrated to ~100 ml by rotary evaporation, poured into 400 ml water, and extracted with hexane (1×200 ml, 2×75 ml). The combined hexane layers were washed with water and brine, dried, concentrated, and Kugelrohr distilled, oven temperature 80–100°C, 0.2 mm Hg, yielding 13.5 g of homogeraniol **2**. ^{1}H and ^{13}C NMR spectra agreed with those previously reported (Kocieñski and Wadman, 1989).

Homogeranyl Bromide (**3**). Homogeraniol **2** was converted to the corresponding mesylate by standard methods (mesyl chloride and triethylamine;) (e.g., Kocieñski and Wadman, 1989). The crude mesylate was taken up in THF (150 ml) and LiBr (24.4 g, 289 mmol; dried at 100°C under vacuum for 4 hr) was added in one portion. The mixture was stirred at room temperature until the mesylate was consumed (20 hr), then poured into 100 ml water and extracted three times with hexane. The hexane layer was washed with water and brine, dried, and concentrated. The residue was purified by flash chromatography (5×22 cm, 2% ether in hexane), then Kugelrohr distillation (oven temperature 65–80°C, 0.3 mm Hg) yielding 11.74 g of homogeranyl bromide **3** (64% yield, 95% pure by GC). NMR and mass spectra agreed with those previously reported (Oehlschlager et al., 1991).

(*E*)*-6-2,3-Dihydrofarnesoic Acid* (**4**). Racemic acid **4** (84% yield, purity >95%) was prepared by conversion of homogeranyl bromide **3** to homogeranyl magnesium bromide, followed by a CuI-catalyzed reaction with 2-methyl-β-propiolactone (Aldrich), as previously described by Fujisawa et al. (1980) and

Sato et al. (1980). Spectral data agreed with those previously reported (Yamatsu et al., 1981).

Methyl (*E*)*-6-2,3-Dihydrofarnesoate* (**5**). (*E*)-6-2,3-Dihydrofarnesoic acid **4** was converted to the methyl ester by treatment with methyl iodide and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) in acetonitrile (Rao, 1980). ^{1}H NMR and mass spectra matched those previously reported (Ahlquist and Ställberg-Stenhagen, 1971;Yamatsu et al., 1981). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 173.83, 135.24, 131.39, 124.42, 124.24, 51.46, 41.69, 39.82, 36.84, 30.15, 26.79, 25.79, 25.42, 19.74, 17.78, 16.05.

Racemic Methyl Citronellate and Methyl (*R*)*-3-Citronellate*. Racemic and (*R*)-3-citronellic acids (Aldrich) were converted to their methyl esters as described above for methyl dihydrofarnesoate (Rao, 1980). After Kugelrohr distillation (oven temperature $\sim$80°C, 1 mm Hg) the esters were obtained as colorless oils, $>$99% pure by GC. Spectral data agreed with that reported by Yamatsu et al. (1981).

RESULTS

Reproductive Behavior. Adult *C. sayi* began to mate about 10–14 days after the final molt. Mating was preceded by courtship, consisting of antennation of the female by the male, followed by the male head-butting the female's abdomen. If the female was receptive, she adopted a posture with the head lowered and the abdomen raised, allowing the male to turn around and couple his genitalia with those of the female, copulating in an end to end position. During mating, both bugs remained in an inverted V position with the abdomen higher than the head. When copulation was complete, one of the bugs swung from side to side to disengage from the other.

If the female was not receptive, she walked away. Sometimes, even though the female bug stopped and raised her abdomen in the receptive position, the male would still head-butt for some time before he turned around and mated with her. In cases where the female assumed the receptive position with her abdomen raised, and the male failed to couple with her genitalia, the male resumed antennation and head-butting. In some cases, males were observed to pursue females with periodic bouts of head-butting for $>$1 h before the female acquiesced and copulated with the male. In other cases, the female mated with the male after a relatively brief courtship of a few minutes.

Diurnal Pattern in Mating Behavior. A total of 13 pairs were videotaped from October 20 to 28, 1999, during which 32 matings were observed. One pair died before any mating occurred. Nine pairs mated more than once in this period, with a mean interval of 42 $\pm$ 24 hr (mean $\pm$ SD) between matings (range 8–77 hr). Eggs were laid between matings. The duration of mating ranged from 2 to 125 min, with a mean of 42.3 $\pm$ 19.6 min for virgin bugs ($N = 12$) and 37.3 $\pm$ 28.4 min ($N = 20$)

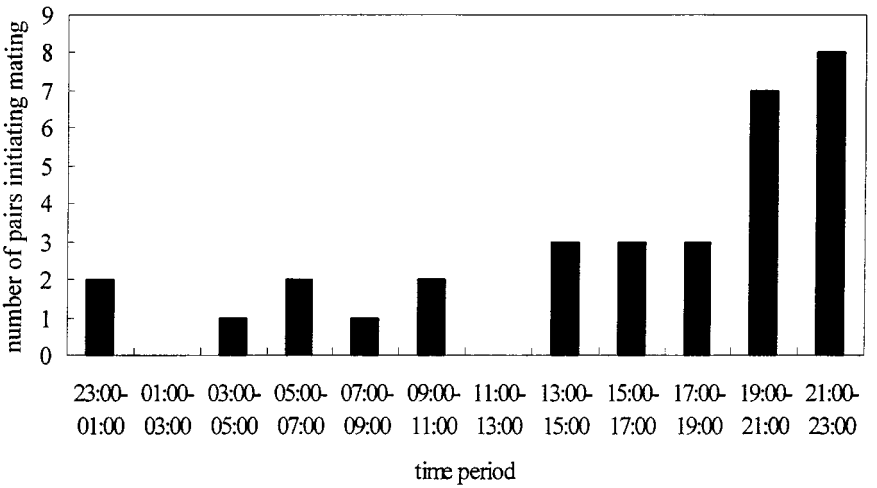


FIG. 1. Diurnal pattern of mating behavior of *C. sayi*. Number of *C. sayi* pairs that initiated a copulation during successive 2-hr time periods. Lights were on from 06:00–22:00 hr each day. The trial was run with 13 individual pairs of insects for nine consecutive days, with 32 matings observed.

for experienced bugs (two-tailed t test, $df = 30$, $P = 0.56$). Most copulations were initiated during the latter part of the photophase (Figure 1).

Weight Change after Mating. Copulations were observed for a total of 13 virgin pairs and 15 experienced pairs of *C. sayi*, and weight changes are plotted in Figures 2 and 3. For virgin pairs, female weights increased 24 ± 8 mg (16.3 ± 6.9% of total body weight) after mating, and male weights decreased by a similar amount (27 ± 12 mg; 16.6 ± 7.8% of body weight). Weight changes of control insects during the same period were negligible (females lost 1 ± 1 mg, males lost 0.4 ± 0.3 mg, $N = 16$). There was a trend for increased weight change with increasing mating duration (virgin females, $R^2 = 0.4397$, $P = 0.013$, virgin males, $R^2 = 0.2481$, $P = 0.08$).

For experienced pairs, females gained an average of 24 ± 9 mg (14.8 ± 5.8% of body weight), whereas males lost 29 ± 12 mg (17.8 ± 7.3% of body weight) during copulation, and there appeared to be a correlation between weight transfer and mating duration (females, $R^2 = 0.2727$; males, $R^2 = 0.3214$). For the experiments with both virgin and experienced pairs, there was a small overall net weight loss, probably due to defecation by some of the test insects during the experiments.

Identification of Compounds from Aeration Extracts of Adult C. sayi. Typical gas chromatograms of female and male aeration extracts are shown in Figure 4. Compounds found in extracts from both sexes included (*E*)-2-hexenal, dodecane,

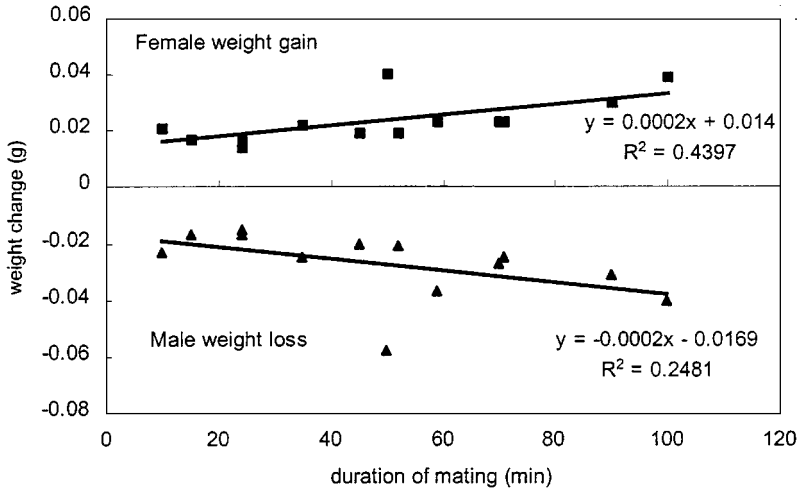


FIG. 2. Weight change of virgin *C. sayi* after mating. Females gained 0.024 ± 0.008 g (mean $\pm$ SD) in weight, and males lost 0.027 ± 0.012 g with every mating. $N = 13$. Results of the *t*-test on overall weight change versus control: $P = 3 \times 10^{-6}$ (female) and $P = 1 \times 10^{-7}$ (male). Linear regression was carried out on the weight change versus duration of mating for each sex. Female weight gain was correlated with duration of mating ($P = 0.0135$); male weight loss was not correlated with mating duration ($P = 0.083$).

(*E*)-2-octenal, tridecane, and (*E*)-2-octenyl acetate, all of which were tentatively identified by interpretation of their mass spectra and match-up with database mass spectra. These compounds also have been identified from the defensive secretions produced by the metathoracic glands in both sexes (Ho, 2000; Ho and Millar, 2001). Aeration extracts from green bean controls contained trace amounts of the common plant volatiles hexanol, (*E*)-2-hexenol, (*E*)-2-hexenal and linalool. Identifications were confirmed by match-up of mass spectra and GC retention times on two GC columns with those of authentic standards.

Aeration extracts from sexually mature males contained one major (peak B, Figure 4) and two minor male-specific compounds (peaks A and C, Figure 4). The relative proportions of these components were peak B, 100%; peak A, $0.45 \pm 0.09\%$ ($N = 3$); and peak C, $1.6 \pm 0.32\%$ ($N = 3$). The mass spectrum of the major component indicated a molecular weight of m/z 182, for a possible molecular formula of $C_{11}H_{18}O_2$. The compound was tentatively identified as methyl-3,7-dimethyl-2,6-octadienoate by comparison of the mass spectrum with the mass spectral database. The compound was confirmed to be the *E* isomer of this compound (methyl geranate) by comparison of its retention times on two GC columns and its mass spectrum with those of authentic standards of methyl

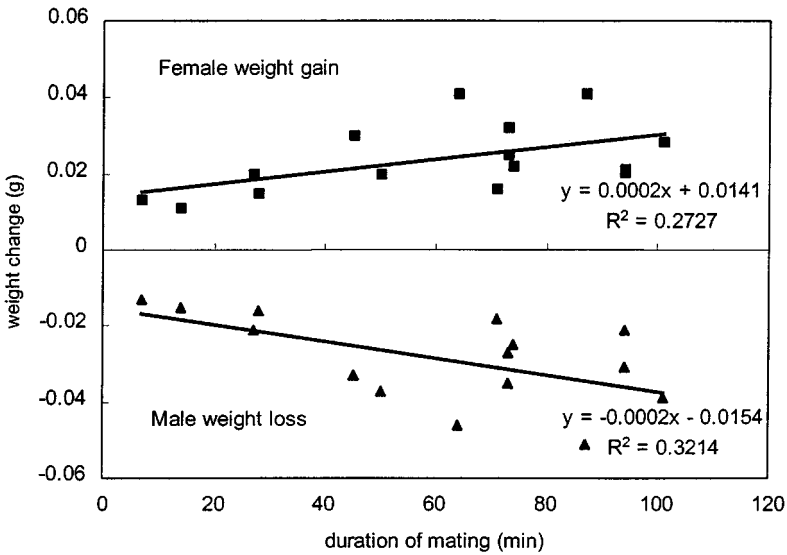


FIG. 3. Weight change of experienced *C. sayi* after mating. Females gained 0.024 ± 0.009 g (mean $\pm$ SD) in weight, and males lost 0.029 ± 0.012 g with every mating. $N = 15$. Results of the t test on overall weight change versus control: $P = 3 \times 10^{-6}$ (female) and $P = 4.9 \times 10^{-8}$ (male). Linear regression was carried out on the weight change versus duration of mating for each sex. Female weight gain was correlated with duration of mating ($P = 0.0458$), and male weight loss was correlated with mating duration ($P = 0.0275$).

(*E*)-2-3,7-dimethyl-2,6-octadienoate (methyl geranate) and methyl (*Z*)-2-3,7-dimethyl-2,6-octadienoate (methyl nerate).

The mass spectrum of the minor male-specific compound A exhibited a molecular ion at m/z 184, 2 mass units more than methyl geranate, and major fragment ions of m/z 69, 95, 110, and 152. The compound also had a shorter retention time than methyl geranate, suggesting that it might be the analog of methyl geranate lacking the double bond in conjugation with the carbonyl, that is, methyl 3,7-dimethyl-6-octenoate (methyl citronellate). This tentative identification was corroborated with a match with a reference spectrum of methyl citronellate in the mass spectral database. The identification was confirmed by comparison of the mass spectrum and retention times of the insect-produced compound with those of an authentic standard.

The mass spectrum of the third male-specific compound, peak C, exhibited a molecular ion at m/z 252, for a possible molecular formula of $C_{16}H_{28}O_2$, with major fragment ions of m/z 69 (possible C_5H_9 isoprene fragment), 109 (possible C_8H_{13} fragment), 123, 177, and 209. The possible presence of an isoprene unit, and the fact that the molecular weight was 68 mass units ($= C_5H_8$) higher than that

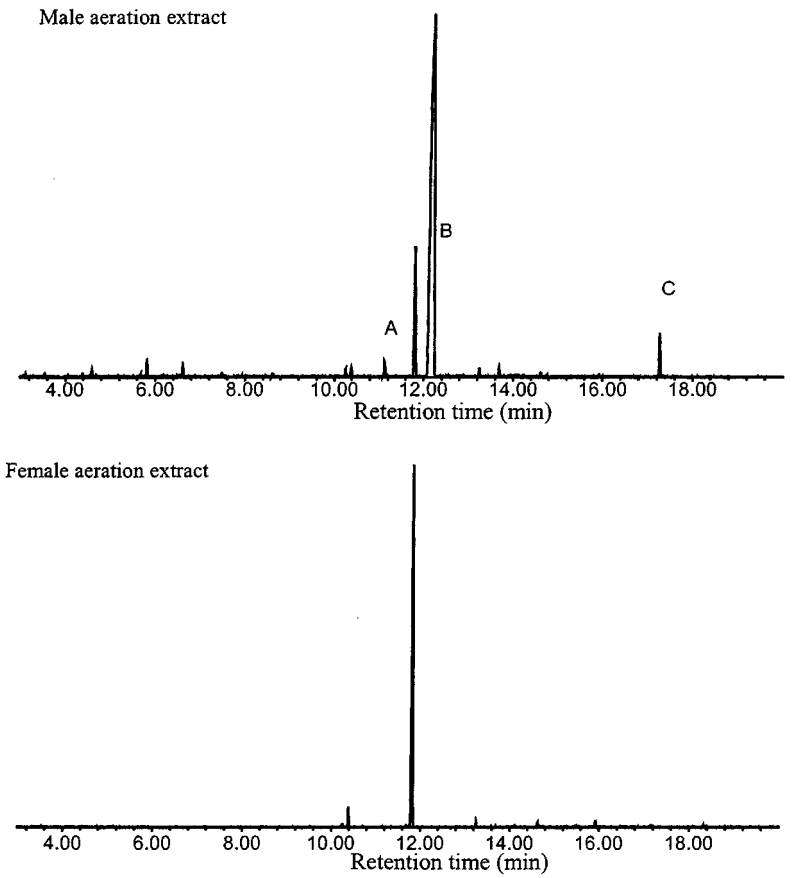


FIG. 4. Gas chromatograms of aeration extracts of female and male *C. sayi*. GC conditions: column DB5-MS (20 m × 0.2 mm ID), initial temperature 50°C (1 min), 10°C/min to 250°C, 250°C (10 min). Three compounds were found only in male aeration extract: A, methyl citronellate; B, methyl geranate; C, methyl (*E*)-6-2,3-dihydrofarnesoate.

of methyl citronellate suggested that this compound might be the sesquiterpenoid homolog, that is, methyl (*E*)-6-2,3-dihydrofarnesoate. The retention times of the insect-produced compound were significantly less than those of a methyl farnesoate standard (m/z 250) on two GC columns, further suggesting that the ester carbonyl was not conjugated. The identification was confirmed by comparisons of GC retention times and mass spectrum with those of an authentic standard.

An attempt to determine the enantiomeric composition of the insect-produced methyl citronellate and methyl (*E*)-6-2,3-dihydrofarnesoate by the formation of diastereomeric derivatives from the free acids (Huffer and Schreier, 1990) was not

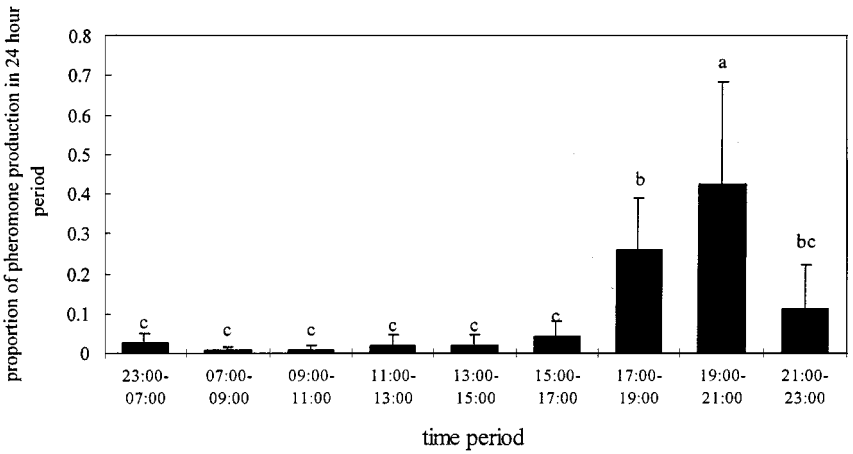


FIG. 5. Diurnal rhythm of production of male-specific compounds by male *C. sayi*. Five cohorts were aerated and the amount of the major male-specific compound, methyl geranate, produced per time interval is plotted as a proportion of the total amount produced in 24 hr. Two-way ANOVA for cohort effect, $F = 0.0419$, $DF = 4, 32$, $P = 0.9965$; for time interval effect $F = 6.9862$, $DF = 8, 32$, $P < 0.0001$. The maximum amount of the major male-specific compound, produced from 19:00–21:00 hr, was 51.6 ± 35.4 ng/bug hour. Bars surmounted by the same letter(s) are not significantly different (Student-Newman-Keuls test, $P < 0.05$).

successful due to the small amounts of the compounds in the extracts. Furthermore, we were unable to resolve racemic samples of these compounds on a capillary GC column with a chiral stationary phase (Cyclodex B, J&W Scientific).

Dynamics of Production of Male-specific Compounds. In aerations of 21 separate cohorts of male bugs, male-specific compounds were first detected between 9 and 12 days after the final molt. All cohorts produced sex-specific compounds, indicating that they had reached sexual maturity under the laboratory rearing conditions. The diurnal pattern of production of male volatiles is shown in Figure 5. Production was greatest during the late afternoon and evening hours, peaking during 19:00–21:00 hr.

Laboratory Bioassays. Female bugs were attracted to odors from live males in vertical Y-tube olfactometer bioassays (treatment = 20, control = 7; 4 nonresponders; chi-square test, $P = 0.012$). However, olfactometer bioassays with aeration extracts from males failed to elicit significant responses from females ($N = 24$) versus the solvent controls. Furthermore, the main male-specific component, methyl geranate, when tested as a single component at several doses, failed to elicit responses from females (20 μg, treatment 10 bugs, control 8 bugs, $P = 0.64$; 2 μg, treatment 5 bugs, control 5 bugs, $P = 1$; 0.2 μg, treatment

20 bugs, control 10 bugs, $P = 0.068$; χ^2 tests). Further tests with 0.2-μg doses of a three-component blend of (±)-methyl citronellate, methyl geranate, and (±)-methyl (*E*)-6-2,3-dihydrofarnesoate (0.5 : 100 : 2) resulted in attraction to the treatment versus a solvent control (treatment = 15, control = 3, $P = 0.0047$).

Field Bioassays. In the first field trial, 18 female *C. sayi* were observed on plants baited with the blend of male-specific *C. sayi* compounds versus a single bug on the control plants (ANOVA, $P = 0.057$). Although 10 male *C. sayi* were found on baited plants versus no males on control plants, the results were not significantly different [$P > 0.05$, multiresponse permutation procedure (MRPP)]. A second trial produced similar results, with more female bugs being collected on baited plants than on control plants (13 vs. 0, $P = 0.0044$, MRPP). No male bugs were found on either treatment or control plants in this bioassay.

A third bioassay testing the three-component blend versus the three possible two-component blends was indeterminate because the low numbers of bugs collected from baited plants precluded meaningful comparisons. However, a total of 18 females and 4 males were collected on plants baited with one of the four treatments, whereas no bugs of either sex were collected on control plants.

DISCUSSION

Reproductive Behavior. The close-range courtship of phytophagous stink bugs appears to follow characteristic steps, with all species examined to date exhibiting similar behaviors. These behaviors include a male approaching a female (over short distances), followed by antennation and head-butting of the female by the male, and end-to-end copulation (Fish and Alcock, 1973; Borges et al., 1987; Wang and Millar, 1997). The daily rhythms of reproductive activity also appear similar between species, with courtship and copulation being initiated primarily in the afternoon and evening [e.g., *Chlorochroa ligata* (Fish and Alcock, 1973); *Nezara viridula* (Harris and Todd, 1980); *Euschistus heros* (Borges et al., 1998a); *Thyanta pallidovirens* (Wang and Millar, 1997); *Acrosternum hilare* and *T. pallidovirens* (H. McBrien and J. Millar, unpublished data)]. Thus, late afternoon and evening were chosen as the optimum time to conduct both laboratory and field bioassays.

The pattern of *C. sayi* reproductive activity also coincided with the maximum production and release of pheromone by male bugs. However, the male-specific compounds were detected throughout the 24-hr monitoring periods, suggesting that the compounds were produced continuously, with only the production rate varied, or that the compounds were adsorbed on the cuticle, which then acted as a slow-release substrate. This pattern may have developed for several possible reasons. First, parasitoids of stink bugs use the male-produced compounds as kairomones (Aldrich, 1995), and timing peak production to the cooler parts of the

day with waning light levels may help male bugs avoid being parasitized (Harris and Todd, 1980). Second, because of the relatively long copulation times during which a copulating pair may be at greater risk of predation, peak production in the late afternoon and evening may be timed so that males and females find each other around dusk and then continue copulation under the cover of darkness. Third, peak pheromone production and mating activities may be timed for optimum temperature and humidity conditions. Mating activities in the heat of the day would increase the risk of desiccation, whereas cooler nighttime temperatures may hinder movement and activity.

Males transferred substantial weights of material to females during mating, with body weights of both sexes changing significantly after copulation. Weight changes occurred with both virgin and experienced insects, and mean duration of copulation was similar for virgin and experienced insects. Similar weight changes after mating occur with *T. pallidovirens* (Wang and Millar, 1997), and it has been documented that pentatomid females obtain nutrients from males, often in the form of large, nonfertilizing sperm (Schrader 1960; Mitchell and Mau, 1969; Mau and Mitchell, 1978; Kasule 1986). In *T. pallidovirens*, these materials resulted in enhanced egg production by females, increasing the reproductive output of both sexes (Wang and Millar, 1997). This increase in reproductive output should in turn encourage multiple copulations by both sexes, which is indeed a characteristic of pentatomid reproduction.

There are several possible explanations for male stink bugs being the pheromone-producing sex. For example, adult stink bugs are polyphagous and mobile, and it is known that they move into and out of habitats quickly. However, the details of the dynamics of these migrations are unknown, and it is possible that males are the pioneering sex in colonizing new habitats. Under such a scenario, males, upon finding a good habitat, might produce pheromone to attract females, with oviposition commencing within a day or so after copulation to the mutual benefit of both sexes.

Alternatively, stink bugs are attacked by a variety of parasitoids and predators, at least some of which use stink bug pheromones as kairomones to locate their prey (Aldrich, 1995). Because of the resultant risk, males may have evolved to be the pheromone producers, because a male that only survives long enough to mate once or a few times may still realize a large fraction of his potential reproductive output. Conversely, because females lay eggs at intervals throughout their adult lives, female reproductive output is maximized by longevity. Hence, the considerable risk of parasitization is primarily borne by the pheromone-producing males, for which longevity may be less important than attracting and copulating with females.

Male Specific Compounds. The male-specific compounds produced by *C. sayi* included three known terpenoid compounds, methyl geranate, methyl (*E*)-6-2,3-dihydrofarnesoate, and methyl citronellate. Methyl geranate and methyl citronellate have been identified frequently from the essential oils of plants, such as

species within *Lauraceae* (Ding et al., 1994). However, they are relatively uncommon in insects. Methyl geranate and analogs have been used as juvenile hormone mimics with the milkweed bug, *Oncopeltus fasciatus* (Brieger, 1971), to study structure–activity relationships. Methyl geranate was also collected from male Mediterranean fruit flies (*Ceratitis capitata* Wied.) (Flath et al., 1993), but because electroantennogram responses of female and male Medfly antennae to this compound were about the same, it was not considered to be a possible male pheromone (Jang et al., 1989). To our knowledge, methyl (*E*)-6-2,3-dihydrofarnesoate has not been found in nature and was originally synthesized as a possible treatment for liver disease (Yamatsu et al., 1981). Because of its structural similarity to insect juvenile hormones, this compound also has been used in the study of juvenile hormones (Campbell et al., 1998). However, a related compound, *trans*-2,3-dihydrofarnesol, found in labial glands from several species of bumblebees, may have a semiochemical role in scent marking of flight routes and possibly species recognition (Bergström et al., 1996).

Bioassays. Laboratory and field bioassays were complicated by the fact that females did not respond strongly to odors from conspecific males, to extracts from males, or to reconstructed blends of male specific compounds. Analogous problems have been reported during attempts to bioassay possible pheromone components for other phytophagous bug species. For example, two male-specific compounds, *trans*- and *cis*-(*Z*)-bisabolene epoxide, were first identified from the southern green stink bug, *Nezara viridula*, more than a decade ago (Aldrich et al., 1987; Baker et al., 1987), and blends of the two isomers are significantly more attractive to female bugs in laboratory bioassays than blank controls (Brézot et al., 1994). However, the attraction is comparatively weak. Furthermore, there has been only a single fragmentary report of field bioassays of the pheromone, despite the importance of this bug as a worldwide agricultural pest (Panizzi, 1997); only low numbers of bugs were attracted (Aldrich et al., 1993). Male-specific compounds also have been identified from a number of *Euschistus* spp. (Aldrich et al., 1991), but field bioassays resulted in collection of less than one bug per trap-day (Aldrich et al., 1991; Borges et al., 1998b). Laboratory bioassays with *Euschistus heros* (Borges et al., 1998a), *E. obscurus* (Borges and Aldrich, 1994), and *Piezodorus hybneri* (Leal et al., 1998) also demonstrated that attraction of female bugs to male volatiles or synthetic pheromone components was relatively weak, with less than 50% of the individuals tested being attracted to the test treatments.

We were able to demonstrate attraction of virgin female bugs to the odors from conspecific males, but not to aeration extracts from males, despite testing females from a number of cohorts on several occasions, using a variety of doses of extracts prepared from different cohorts of male bugs. There are several possible reasons for the failure of these bioassays. First, the crude extracts contained defensive chemicals in addition to the male-specific compounds, and these chemicals may have inhibited female responses. Second, the optimum dosage was not known,

and the production of male-specific compounds by the bugs was variable. Despite testing a number of different doses, there was no clear indication that any dose tested was attractive. The problem was compounded by the weak responses exhibited by females in the laboratory bioassays: even when the bioassay was working well, the best treatments were normally only two to three times as attractive as the controls.

Rather than spending further time on bioassays of crude extracts, the male-specific compounds were identified and synthesized to determine whether they had the expected biological activity. This approach allowed us to test doses varying by orders of magnitude and to standardize and reproduce blends over time. In the olfactometer bioassay, females exhibited attraction to the major male-specific compound and to the complete three-component blend of male-specific compounds. Further laboratory bioassays were not pursued because of declining populations of bugs in the colony.

Field Bioassays. Several reports indicate that many of the commonly used insect trap designs were not effective for phytophagous stink bugs and that bugs were frequently attracted to the vicinity of, but not into, traps (Aldrich et al., 1991; Sugie et al., 1996). In perhaps the most extreme example, James et al. (1996) caught no spined citrus bugs (*Biprorulus bibax*) in traps placed in citrus trees, but found that large numbers of bugs were attracted to trees containing traps. Consequently, instead of using traps of unknown efficacy, we decided to use a "trap plant" concept, in which the numbers of bugs on plants baited with pheromone lures or solvent controls would be counted. Before deploying bioassays, in a pilot experiment run to determine the optimum time to conduct bug counts, female bugs were not seen close to lures in the early morning when temperatures were still cool, nor were they seen in the upper half of the plants close to the lures during the heat of the day. The optimal period for attraction, in agreement with the laboratory studies and with previously reported data from other species, appeared to be late afternoon through to darkness. Consequently, field tests were evaluated from about 17:00 hr until dark.

Field test results followed the same general pattern as that seen in the laboratory, with weak attraction of adult female bugs to pheromone lures. Several other points also emerged. First, there were indications that lures also attract male bugs; the number of males on baited plants was always higher than the number near controls, in all tests run. However, there may be an alternate explanation for this apparent attraction; males searching for females may be attracted to or arrested on plants with females in response to cues associated with females. For example, males and females of several species of stink bugs are known to communicate over shorter distances by substrate-borne vibrational cues [*Nezara viridula* (Ota and Çokl, 1991), *N. antennata* (Konn et al., 1988), *N. viridula* and *Acrosternum hilare* (Çokl et al., 2001)]. Further studies will be required to determine whether males are indeed attracted to the synthetic male pheromone, or whether the

apparent attraction is actually arrestment or attraction in response to female-produced cues. There was no indication that the lures were attractive to immature bugs, as has been reported for at least one other stink bug species (Aldrich et al., 1987).

In total, the laboratory and field bioassay data provide strong evidence that one or more of the male-specific compounds are sex pheromone components. The relatively weak attraction of females to the pheromone suggests that other factors must also play a role in bringing the sexes together. For example, there is considerable evidence suggesting that sex attractant pheromones play a role in long-range attraction (Borges et al., 1987; Aldrich et al., 1987, 1991; James et al., 1994; Borges et al., 1998b). However, pheromones are probably not the only cues involved, because most phytophagous stink bugs are not strongly attracted into close proximity to pheromone sources or, at least, few phytophagous stink bugs have been caught in pheromone-baited traps (e.g., Aldrich et al., 1987, 1991; James et al., 1994). As described above, several reports suggest that at shorter ranges, stink bugs of both sexes may use vibrational signals transmitted through the substrate for intersexual communication (Ota and Çokl, 1991; Ryan and Walter, 1992). The possible effects of other sensory cues, such as visual signals, or cues associated with the host plants that may act in concert with insect-produced cues, remains to be investigated.

Because of the weak attraction to the male-produced compounds, and despite laboratory and field bioassay attempts extending over several years, it remains unclear whether the male-produced pheromone consists of the single component methyl geranate, or whether the true pheromone is actually a blend of methyl geranate and one or both of the minor male-specific components. Furthermore, all field bioassays to date have been carried out with racemic methyl dihydrofarnesoate and methyl citronellate, and it is entirely possible that the bugs may respond less well to the racemates than to the insect-produced enantiomers of these compounds. Efforts to address these questions are continuing, in parallel with efforts to develop an effective trap for this species.

Acknowledgments—The authors gratefully acknowledge financial support provided by the California Pistachio Commission, the Washington State Tree Fruit Agreement, and the University of California IPM Project.

REFERENCES

AHLQUIST, L., and STÄLLBERG-STENHAGEN, S. 1971. Studies on natural odoriferous compounds III. Synthesis of (+)- and (−)-3,7,11-trimethyl-6-*trans*,10-dodecadien-1-ol. *Acta Chem. Scand.* 25:1685–1694.

ALDRICH, J. R. 1995. Chemical communication in the true bugs and parasitoid exploitation. pp. 318–363, *in* R. Cardé and W. Bell (eds.). Chemical Ecology of Insects 2, Chapman & Hall, New York.

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and LOCKWOOD, J. A. 1987. Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteroptera: Pentatomidae). *J. Exp. Zool.* 244:171–175.

ALDRICH, J. R., HOFFMANN, M. P., KOCHANSKY, J. P., LUSBY, W. R., EGER, J. E., and PAYNE, J. A. 1991. Identification and attractiveness of a major pheromone component for Nearctic *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae). *Environ. Entomol.* 20:477–483.

ALDRICH, J. R., NUMATA, H., BORGES, M., BIN, F., WAITE, G. K., and LUSBY, W. R. 1993. Artifacts and pheromone blends from *Nezara* spp. and other stink bugs (Heteroptera: Pentatomidae). *Z. Naturforsch*. 48C:73–79.

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and BORGES, M. 1994. Identification of male-specific volatiles from Nearctic and Neotropical stink bugs (Heteroptera: Pentatomidae). *J. Chem. Ecol.* 20:1103–1111.

BAKER, R., BORGES, M., COOKE, N. G., and HERBERT, R. H. 1987. Identification and synthesis of (*Z*)-(1′*S*,3′*R*,4′*S*)-(−)-2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methyl-hepta-2,5-diene, the sex pheromone of the southern green stink bug, *Nezara viridula* (L.). *J. Chem. Soc., Chem. Commun.* 1987:414–416.

BERGSTRÖM, G., BERGMAN, P., APPELGREN, M., and SCHMIDT, J. O. 1996. Labial gland chemistry of three species of bumblebees (Hymenoptera: Apidae) from North America. *Bioorg. Med. Chem.* 4:515–519.

BIONDINI, M. E., BONHAM, C. D., and REDENTE, E. F. 1985. Secondary successional patterns in a sagebrush (*Artemisia tridentata*) community as they relate to soil disturbance and soil biological activity. *Vegetatio* 60:25–36.

BORGES, M., and ALDRICH, J. R. 1994. An attractant pheromone for the Nearctic stink bug, *Euschistus obscurus* (Heteroptera: Pentatomidae): Insight into a Neotropical relative. *J. Chem. Ecol.* 20:1095–1102.

BORGES, M., JEPSON, P. C., and HOWSE, P. E. 1987. Long-range mate location and close range courtship behaviour of the green stink bug, *Nezara viridula* and its mediation by sex pheromones. *Entomol. Exp. Appl.* 44:205–212.

BORGES, M., MORI, K., COSTA, M. L. M., and SUJII, E. R. 1998a. Behavioural evidence of methyl-2,6,10-trimethyltridecanoate as a sex pheromone of *Euschistus heros* (Heteroptera: Pentatomidae). *J. Appl. Entomol.* 122:335–338.

BORGES, M., SCHMIDT, F. G. V., SUJII, E. R., MEDEIROS, M. A., MORI, K., ZARBIN, P. H. G., and FERREIRA, J. T. B. 1998b. Field responses of stink bugs to the natural and synthetic pheromone of the Neotropical brown stink bug, *Euschistus heros* (Heteroptera: Pentatomidae). *Physiol. Entomol.* 23:202–207.

BRÉZOT, P., MALOSSE, C., MORI, K., and RENOU, M. 1994. Bisabolene epoxides in sex pheromone in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): role of *cis* isomer and relation to specificity of pheromone. *J. Chem. Ecol.* 20:3133–3147.

BRIEGER, G. 1971. Juvenile hormone mimics: Structure–activity relationships for *Oncopeltus fasciatus*. *J. Insect Physiol.* 17:2085–2093.

CAMPBELL, P. E., OAKESHOTT, J. G., and HEALY, M. J. 1998. Purification and kinetic characterisation of juvenile hormone esterase from *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 28:501–515.

CAFFREY, D. J., and BARBER, G. W. 1919. The Grain Bug. Bulletin No. 779, June 1919, United States Department of Agriculture, Bureau of Entomology, Washington, DC.

ÇOKL, A., MCBRIEN, H. L., and MILLAR, J. G. 2001. Comparison of substrate-borne vibrational signals of two stink bug species, *Acrosternum hilare* (Say) and *Nezara viridula* (L.) (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* in press.

DING, J., YU, X., DING, Z., CHENG, B., YI, Y., WU, Y., NANAO, H., and HISASHI, K. 1994. Essential oils of some *Lauraceae* species from the southwestern parts of China. *J. Essent. Oil Res.* 6:577–585.

ERMAN, W. F., WENKERT, E. and JEFFS, P. W. 1969. Base cleavage of β,γ-unsaturated bicyclic cyclobutanones. *J. Org. Chem.* 3:2196–2203.

EVANS, C. E., STADDON, B. W., and GAMES, D. E. 1990. Analysis of gland secretions of Pentatomoidea (Heteroptera) by gas chromatography–mass spectrometry techniques, pp. 321–328 *in* A. R. McCaffery, and I. D. Wilson (eds.). *Chromatography and Isolation of Insect Hormones and Pheromones*. Plenum Press, New York.

FISH, J., and ALCOCK, J. 1973. The behavior of *Chlorochroa ligata* (Say) and *Cosmopepla bimaculata* (Thomas) (Hemiptera: Pentatomidae). *Entomol. News* 84:260–268.

FLATH, R. A., JANG, E. B., LIGHT, D. M., MON, T. R., CARVALHO L., BINDER, R. G., and JOHN, J. O. 1993. Volatile pheromonal emission from the male Mediterranean fruit fly: Effects of fly age and time of day. *J. Agric. Food Chem.* 41:830–837.

FUJISAWA, T., SATO, T., KAWARA, T., NODA A., and OBINATA, T. 1980. A novel method for terpene synthesis by the ring-opening reaction of β-methyl-β-propiolactone. *Tetrahedron Lett.* 21:2553–2554.

HARRIS, V. E., and TODD, J. W. 1980. Male-mediated aggregation of male, female and 5th-instar southern green stink bugs and concomitant attraction of a tachinid parasite, *Trichopoda pennipes. Entomol. Exp. Appl.* 27:117–126.

HO, H.-Y. 2000. Studies on the sex pheromones of the pentatomid bugs *Chlorochroa ligata*, *Chlorochroa sayi*, and *Chlorochroa uhleri*, and the mirid bug *Lygus hesperus* (Hemiptera: Heteroptera). PhD thesis. University of California, Riverside, 236 pp.

HO, H.-Y., and MILLAR, J. G. 2000. Compounds in the metathoracic glands of adults and the dorsal abdominal glands of nymphs of the stink bugs *Chlorochroa uhleri, C. sayi* and *C. ligata* (Hemiptera: Pentatomidae). *Zool. Stud.* in press.

HOFFMANN, M. P., WILSON, L. T., and ZALOM, F. G. 1987. Control of stink bugs in tomatoes. *Calif. Agric.* May–June:4–6.

HUFFER, M., and SCHREIER, P. 1990. High resolution gas chromatographic resolution of chiral primary alcohols and acids as their diastereomeric 1-phenylethylamides. *J. Chromatogr.* 519:263–267.

JAMES, D. G., MORI, K., ALDRICH, J. R., and OLIVER, J. E. 1994. Flight-mediated attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to natural and synthetic aggregation pheromone. *J. Chem. Ecol.* 20:71–80.

JAMES, D. G., HEFFER, R., and AMAIKE, A. 1996. Field attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to synthetic aggregation pheromone and (*E*)-2-hexenal, a pentatomid defense chemical. *J. Chem. Ecol.* 22:1697–1708.

JANG, E. B., LIGHT, D. M., FLATH, R. A., NAGATA, J. T., and MON, T. R. 1989. Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata* to identified volatile constituents from calling males. *Entomol. Exp. Appl.* 50:7–19.

KASULE, F. K. 1986. Repetitive mating and female fitness in *Dysdercus cardinalis* (Hemiptera: Pyrrhocoridae). *Zool. J. Linn. Soc.* 88:191–199.

KOCIEÑSKI, P., and WADMAN, S. 1989. A highly stereoselective and iterative approach to isoprenoid chains: Synthesis of homogeraniol, homofarnesol, and homogeranylgeraniol. *J. Org. Chem.* 54:1215–1217.

KONN, M., OE, A., NUMATA, H., and HIDAKA, T. 1988. Comparison of the mating behavior between two sympatric species, *Nezara antennata* and *N. viridula* (Heteroptera: Pentatomidae), with special reference to sound emission. *J. Ethol.* 2:91–98.

LARUE, J. H., and JOHNSON, R. S. (eds.). 1989. Peaches, plums, and nectarines. Growing and handling for the fresh market. University of California, DANR publication 3331; UC Publications, Oakland, California.

LEAL, W. S., KUWAHARA, S., SHI, X., HIGUCHI, H., MARINO, C. E. B., ONO, M., and MEINWALD, J. 1998. Male released sex pheromone of the stink bug *Piezodorus hybneri. J. Chem. Ecol.* 24:1817–1829.

LEE, E. R., LAKOMY, I., BIGLER, P., and SCHEFFOLD, R. 1991. Reductive radical cyclizations of bromo acetals and (bromomethyl)silyl ethers of terpenoid alcohols. *Helv. Chim. Acta* 74:146–162.

MAU, R. F. L., and MITCHELL, W. C. 1978. Development and reproduction of the oriental stink bug *Plautia stali* (Hemiptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 71:756–757.

MCBRIEN, H. L., and MILLAR, J. G. 1999. Phytophagous bugs, pp. 277–304, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants, CAB International, Wallingford, UK.

MCCUNE, B., and MEFFORD, M. J. 1999. PC-ORD. Multivariate Analysis of Ecological Data, Version 4. MjM Software Design, Gleneden Beach, Oregon, http://www.ptinet.net/∼mjm

MCMURRY, J. E., and BOSCH, G. K. 1987. Synthesis of macrocyclic terpenoid hydrocarbons by intramolecular carbonyl coupling: bicyclogermacrene, lepidozene, and casbene. *J. Org. Chem.* 52:4885–4893.

MCPHERSON, J. E., and MCPHERSON, R. M. 2000. Stink Bugs of Economic Importance in America North of Mexico. CRC Press, Boca Raton, Florida.

METCALF, R. L., and METCALF, R. A. 1993. Destructive and Useful Insects. Their Habits and Control. McGraw-Hill, New York.

MILLAR, J. G. 1997. Methyl (2E, 4Z, 6Z)-deca-2,4,6-trienoate, a thermally unstable, sex-specific compound from the stink bug *Thyanta pallidovirens. Tetrahedron Lett.* 38:7971–7972.

MITCHELL, W. C., and MAU, R. F. L. 1969. Sexual activity and longevity of the southern green stink bug *Nezara viridula. Ann. Entomol. Soc. Am.* 62:1246–1247.

MORRILL, A. W. 1910. Plant-bugs injurious to cotton bolls. *U.S.D.A. Bur. Entomol. Bull.* 86:23–73.

NAGANO, H., NAGASAWA, T., and SAKUMA, M. 1997. Synthesis of 2-polyprenyl-substituted polyprenols and their conversion into phosphates. *Bull. Chem. Soc. Jpn.* 70:1969–1976.

OEHLSCHLAGER, A. C., SINGH, S. M., and SHARMA, S. 1991. Squalene synthetase inhibitors: Synthesis of sulfonium ion mimics of the carbocationic intermediates. *J. Org. Chem.* 56:3856–3861.

OHLENDORF, B. 1990. UC IPM Pest Management Guidelines. University of California Statewide Integrated Pest Management Project, University of California, Davis; Division of Agriculture and Natural Resources, publication 3339.

OTA, D., and ÇOKL, A. 1991. Mate location in the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae) mediated through substrate-borne signals in ivy. *J. Insect Behav.* 4:441–447.

PANIZZI, A. R. 1997. Wild hosts of pentatomids: Ecological significance and role in their pest status on crops. *Annu. Rev. Entomol.* 42:99–122.

RAO, C. G. 1980. A new rapid esterification procedure utilizing exceptionally mild reaction conditions. *Org. Prep. Proc. Int.* 12:225–228.

RUSSELL, E. E. 1952. Stink bugs on seed alfalfa in southern Arizona. Circular No. 903, July. United States Department of Agriculture, Washington, DC.

RYAN, M. A., and WALTER, G. H. 1992. Sound transmission in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Further evidence that signal transmission is substrate-borne. *Experientia* 48:1112–1115.

SATO, T., KAWARA, T., KAWASHIMA, M., and FUJISAWA, T. 1980. Copper-catalyzed reaction of Grignard reagents with β-propiolactones: A convenient method for the synthesis of β-substituted propionic acids. *Chem. Lett.* 1980:571–574.

SCHRADER, F. 1960. Evolutionary aspects of aberrant meiosis in some Pentatomidae (Heteroptera). *Evolution* 14:498–508.

SHIELDS, E. J. 1989. Artificial light: Experimental problems with insects. *Bull. Entomol. Soc. Am.* 00:40–44.

STRONG, F. E., SHELDAHL, J. A., HUGHES, P. R., and HUSSEIN, E. M. K. 1970. Reproductive biology of *Lygus hesperus* Knight. *Hilgardia* 40:105–147.

SUGIE, H., YOSHIDA, M., KAWASAKI, K., NOGUCHI, H., MORIYA, S., TAKAGI, K., FUKUDA, H., FUJIIE, A., YAMANAKA, M., OHIRA, Y., TSUTSUMI, T., TSUDA, K., FUKUMOTO, K., YAMASHITA, M., and SUZUKI, H. 1996. Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Appl. Entomol. Zool.* 31:427–431.

TAMAO, K., and ISHIDA, N. 1984. Silafunctional compounds in organic synthesis. 27. (Isopropoxydimethylsilyl)methyl Grignard reagent: A novel nucleophilic hydroxymethylating agent for aldehydes and ketones. *Tetrahedron Lett.* 25:4245–4248.

TOSCANO, N. C., and STERN, V. M. 1976. Dispersal of *Euschistus conspersus* from alfalfa grown for seed to adjacent crops. *J. Econ. Entomol.* 69:96–98.

WANG, Q., and MILLAR, J. G. 1997. Reproductive behavior of *Thyanta pallidovirens* (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 90:380–388.

YAMATSU, I., YUICHI, I., SHINYA, A., WATANABE, H., IGARASHI, T., SHIOJIRI, H., TANABE, Y., and KUNIKO, H. 1981. α,β-Dihydropolyprenyl derivatives, pharmacological preparations containing them and method for treating liver diseases. *Eur. Pat. Appl.* **EP** 41,235.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

COMPONENTS OF MALE AGGREGATION PHEROMONE OF STRAWBERRY BLOSSOM WEEVIL, *Anthonomus rubi* HERBST. (COLEOPTERA: CURCULIONIDAE)

P. J. INNOCENZI,[1,2,*] D. R. HALL,[2] and J. V. CROSS[1]

[1]*Horticulture Research International*
East Malling, West Malling, Kent ME19 6BJ, UK
[2]*Natural Resources Institute*
University of Greenwich
Chatham Maritime, Kent ME4 4TB, UK

(Received July 31, 2000; accepted February 9, 2001)

Abstract—The strawberry blossom weevil, *Anthonomus rubi*, is a major pest of strawberries in the United Kingdom and continental Europe. As part of a project to develop noninsecticidal control methods, the pheromone system of this species was investigated. Comparison of volatiles produced by field-collected, overwintering individuals of each sex led to identification of three male-specific compounds—(*Z*)- 2-(3,3-dimethylcyclohexylidene)ethanol, (*cis*)-1-methyl-2-(1-methylethenyl)cyclobutaneethanol, and 2-(1-methylethenyl)-5-methyl-4-hexen-1-ol (lavandulol)—in amounts of 6.1, 1.2, and 0.82 μg/day/male. The first two compounds are components of the aggregation pheromone of the boll weevil, *Anthonomus grandis*, grandlure II and grandlure I, respectively. Grandlure I was the (1*R*,2*S*)-(+) enantiomer and lavandulol was a single enantiomer, although the absolute configuration was not determined. Trace amounts of the other two grandlure components (*Z*)-(3,3-dimethylcyclohexylidene)acetaldehyde (grandlure III) and (*E*)-(3,3-dimethylcyclohexylidene)acetaldehyde (grandlure IV) were also detected. (*E*,*E*)-1-(1-Methylethyl)-4-methylene-8-methyl-2,7-cyclo-decadiene (germacrene-D), a known volatile from strawberry plants, *Fragaria ananassa*, was collected in increased amounts in the presence of pheromone-producing weevils. Male weevils only produced pheromone on *F. ananassa* and not on scented mayweed, *Matracaria recutita*, or cow parsley, *Anthriscus sylvestris*, although these are known food sources. In field trials using various combinations of synthetic grandlures I, II, III, and IV and lavandulol, significantly more weevils were caught in traps baited with blends containing grandlure I and II and lavandulol than in those baited with blends without lavandulol or unbaited controls. Addition of grandlure III and IV had no

*To whom correspondence should be addressed. e-mail: P.J.Innocenzi@gre.ac.uk

0098-0331/01/0600-1203$19.50/0

significant effect on attractiveness. Horizontal sticky traps were found to be more effective than vertical sticky traps or standard boll weevil traps. In mid-season females predominated in the catches, but later more males than females were trapped.

Key Words—Strawberry blossom weevil, *Anthonomus rubi*, Curculionidae, Coleoptera, aggregation pheromone, Grandlure, lavandulol, germacrene-D.

INTRODUCTION

The strawberry blossom weevil, *Anthonomus rubi* Herbst (Coleoptera: Curculionidae), is a major pest of strawberry crops in the United Kingdom and Europe (Cross and Easterbrook, 1998; Hoffman, 1954; Popov, 1996) and is sometimes a pest of raspberry (Alford, 1984). The weevil is univoltine, and adults emerge in late April from overwintering sites of leaf litter and soil adjacent to strawberry fields (Fenouhlet, 1907; Jary, 1932). It feeds initially on strawberry foliage before mating commences at the onset of flower bud formation. Diapausal adults emerge six to eight weeks after oviposition. It feeds in and around the strawberry crop for about two weeks until leaving the crop for an overwintering site in late July/early August (Jary, 1932; Morris, 1977). Strawberry crop loss is a result of the oviposition behavior of the female, which involves deposition of a single egg in an unopened strawberry bud, followed by severing of the stalk below the bud (Jary, 1932; Alford, 1984). A single female lays up to 50 eggs and potential crop losses are great even at low population levels. Owing to the small size of the weevil and the nature of the damage, effects on the crop are often unnoticed until it is too late to take remedial action. Current control involves the routine use of broad-spectrum insecticides, e.g., chlorpyrifos, which is unsatisfactory due to high cost, environmental affects, and negative public perception. Thus, a more cost-effective, benign, and acceptable control method is desirable.

Extensive work has been carried out on identification of pheromones and their use within the Curculionidae (Bartelt, 1999), exemplified by the decades of research conducted on the boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae) (Hardee and Mitchell, 1997). Following identification of four components of the male aggregation pheromone of *A. grandis*, given the trivial name grandlure, (Tumlinson et al., 1969), pheromones of several weevil species have been identified. These include two from within the same subfamily as *A. grandis*, the pepper weevil *A. eugenii* (Eller et al., 1994) and the pecan weevil *Curculio caryae* (Hedin et al., 1996, 1997), both of which produce male aggregation pheromones incorporating Grandlure components.

Although there have been no reports of pheromonal attraction for *A. rubi*, the successful identifications from within the same genus encouraged a preliminary trapping study with this species, the results of which led to the investigation of the composition of the pheromone.

METHODS AND MATERIALS

Insect Material. In 1998, adult *A. rubi* were collected from strawberry plants, *Fragaria ananassa*, at Horticulture Research International, East Malling, West Malling, UK, during early April, the time of weevil emergence. As there was no report of sexual dimorphism within the species, these were sexed by placing a group of four or five weevils together in a Petri dish containing filter paper, and separating the pairs that displayed mounting behavior. Males and females were maintained in separate plastic propagators (36 × 22 × 16 cm; B&Q, Eastleigh, Hants, UK) at 20°C and 16L:8D. Insects were provided with *F. ananassa* flowers that were changed every three days.

In 1999, during the second week of March, deciduous tree leaf-litter was collected in transparent polyethylene bags from beneath and around conifer trees adjacent to strawberry fields at Boughton Monchelsea, Kent. These strawberry fields had contained a large population of weevils the previous summer, just prior to migration to overwintering sites. The litter was maintained at 20°C and emerging weevils were transferred to individual holding chambers (13 × 8 × 5 cm) containing *F. ananassa* plants.

During the first week of April 1999, a second collection of *A. rubi* was made at Boughton Monchelsea. These were weevils recently emerged from overwintering and found on cow parsley, *Anthriscus sylvestris.* This date preceded the onset of strawberry bud development. Weevils were kept in individual containers on *A. sylvestris.* A third batch of blossom weevils was collected in the first week of August from a strawberry farm at Doddington, Kent. These individuals were found feeding on scented mayweed, *Matracaria recutita.* When placed together they showed no sign of sexual behavior and were thus presumed to be the diapausal first generation adults.

Collection of Volatiles. For collection of emitted volatiles, natural or synthetic sources were placed in a glass chamber (10 cm long × 3 cm diam.). A diaphragm pump (Capex Mk II, Charles Austen, Weybridge, Surrey, UK) was used to draw air at 1 liter/min into the chamber through an activated charcoal filter (20 cm long × 2 cm diam., 6–18 mesh) and out through a collection filter containing Porapak Q (200 mg, 50–80 mesh, PhaseSep) held between plugs of silanized glass wool in a Pasteur pipet. The Porapak was purified by Soxhlet extraction with chloroform for 8 hr, and filters were washed well with dichloromethane immediately before use. Adsorbed volatiles were removed from the filters with dichloromethane (pesticide grade; 1 × 0.5 ml, 1 × 1 ml). For pheromone identification, volatiles were collected from one to three weevils on *F. ananassa* plants for 24 hr at 20°C with a 16L:8D cycle. Volatiles were also collected from weevils on *A. sylvestris* and *M. recutita.* Collections were also made from plants alone for comparison.

Gas Chromatography (GC). GC Analyses were conducted using Carlo Erba Mega Series 5300 instruments equipped with fused capillary columns (25 m ×

0.32 mm ID) coated with polar CPWax 52CB (Carbowax 20 M equivalent; Chrompack) or nonpolar CPSil 5CB (methyl silicone; Chrompack). The carrier gas was helium at 50 kPa, and the oven temperature was held at 50°C for 2 min then programmed at 6°C/min to 240°C. Injection was splitless (200°C) and detection was by flame ionization detection (FID, 240°C). Data was captured and processed using EzChrom 6.1 software. Retention indices (RI) were calculated relative to the retention times of straight-chain hydrocarbons. Collections of the natural pheromone were quantified approximately by coinjection with undecane (10 ng) and comparison of peak areas without consideration of response factors.

For determination of enantiomeric composition of chiral materials, analyses were done using a Varian 3700 gas chromatograph with cyclodextrin capillary, column (CP-Chirasil-Dexcb; 25 m × 0.32 mm ID; Chrompack). The carrier gas was helium at 100 kPa and the oven temperature was held at 125°C. Injection was in split mode (50 : 1), and data handling was as above. Retention time comparisons and coinjection of natural and synthetic samples were used for confirmation of absolute configuration.

Gas Chromatography-Mass Spectrometry Analysis (GC-MS). GC-MS analyses were carried out with a fused silica capillary column (25 m × 0.25 mm ID) coated with polar CPWax 52CB or nonpolar CPSil 5CB linked directly to a Finnigan 700 ion trap detector (ITD) operated in electron impact or chemical ionization (*iso*butane) mode. GC conditions were as above for these columns. Spectra were compared with those in the NBS/NIH/EPA library, the ITD terpenoid library (Adams 1989), and a library generated from samples analysed previously at NRI.

Chemicals. (±)-*cis*-1-Methyl-2-(1-methylethenyl)cyclobutaneethanol (grandlure I, purity 98%), (*Z*)-2-(3,3,-dimethylcyclohexylidene)ethanol (grandlure II, purity 99.5%), and (*E*)-2-(3,3,-dimethylcyclohexylidene)ethanol (purity 99.5%) were obtained from Bedoukian, Danbury, Connecticut. (*Z*)-2-(3,3-Dimethylcycloheylidene)acetaldehyde (grandlure III) and (*E*)-2-(3,3-dimethylcyclohexylidene) acetaldehyde (grandlure IV) were obtained as a mixture of grandlures I–IV in relative proportions of 3:3:1:1 from Plato Industries, Houston, Texas. (±)-2-(1-Methylethenyl)-5-methyl-4-hexen-1-ol (lavandulol, 95% purity) was obtained from Fluka. (−)-(7*S*)-1-(1-Methylethyl)-4-methylene-8-methyl-2,7-cyclodecadiene (germacrene-D) was the major component (approximately 20%) in ylang ylang oil (Holland and Barrett, Nuneaton, Warwickshire, UK) and was purified to 80% purity by flash chromatography on silica gel eluted with hexane. Racemic germacrene-D from *Solidago altissima* was provided by Prof. Yoo Tanabe of Kwansei Gakuin University Japan (Nishii et al., 1997). (1*R*,2*S*)-(+)-*cis*-1-Methyl-2-1(1-methylethenyl)cyclobutaneethanol [(+)-grandlure I] was a gift from Prof. Kenji Mori of Tokyo University in Japan (Mori and Fukamatsu, 1992).

Pheromone Dispensers. Pheromone blends (200 mg) were dispensed from sachets prepared by heat sealing white polyethylene layflat tubing (2.5 cm ×

2.5 cm × 120 μm thick; International Pheromone Systems Ltd., Wirral, UK). For laboratory studies, dispensers were maintained in a laboratory wind tunnel (27°C, 8 kph windspeed) and release rates were measured by periodic weighing and by trapping of volatiles emitted over 1 hr at 27°C as above. Volatile collections were analyzed by GC on the nonpolar CPSil 5CB column using 1-undecanol (5 μg) as internal standard.

In the first field trial, grandlure was formulated as a 3:3:1:1 blend of grandlures I–IV in red laminate squares1 (2.5 cm × 2.5 cm × 2 mm thick; Plato Industries).

Trapping Trials. Trapping trials were carried out in fields belonging to commercial strawberry growers in Doddington, Kent, and West Peckham, Kent, or on-station at HRI, East Malling, West Malling, Kent. Unless otherwise stated, traps were white corrugated plastic sheets (20 × 20 cm) coated with polybutene adhesive on both surfaces (Oecos Ltd., Kimpton, Herts, UK) positioned horizontally on top of a wooden stake (60 cm). During initial trials carried out in 1998, trapped weevils were observed to be escaping, and additional adhesive was used during 1999. Live weevils or synthetic lures were placed in pink hair-rollers (Tesco, Cheshurst, Herts, UK), capped at both ends with white, sterilin tube lids, and secured to the center of the upper surface of the trap with a nail into the supporting stake. Live weevils used as bait were provided with a cut strawberry flower, and the flower and any dead weevils were replaced every second day. Traps were positioned 6 m apart in a 5 × 5 Latin square (live baiting experiment) or randomized complete block design with three replicates at each of three sites (synthetic pheromone experiments).

In a comparison of trap designs, the above horizontal sticky trap was compared with the standard boll weevil trap from Plato Industries (Mitchell and Hardee, 1974a) and a vertical white sticky trap (20 × 20 cm; Oecos Ltd., Kimpton, Herts, UK) positioned 60 cm above ground level in a 3 × 3 Latin square design.

Weevils trapped during the 1999 trials were placed in hexane to remove excess glue and stored in 70% ethanol before random samples were sexed using the coxal thorn method (Balachowsky and Mesnil, 1935; Innocenzi et al., 2001).

Statistical Analysis. Total trap catches were transformed to $\log_{10}(x + 1)$ to normalize the variance. Two-way analysis of variance (ANOVA) was carried out to test for any treatment × location interaction. After one-way ANOVA on the transformed data, differences between means were tested for significance at the 5% level by the least significant differences (LSD) test.

RESULTS

Attractiveness of Male and Female Weevils. A preliminary trapping trial was done at Hillberry Farm, East Malling, UK, from 5 to May 19, 1998, comparing catches of weevils in traps baited with a male *A. rubi* weevil, female weevil,

F. ananassa plant only, grandlure dispenser, and *F. ananassa* plant, or unbaited control. Fifteen weevils were caught in the five replicates, all on traps baited with live males. The small numbers were possibly a reflection of the low population due to the grower's pest management regime, as well as the inability of the sticky trap to retain the weevils. During each survey, the recorded weevils were observed walking freely on the sticky traps.

Comparison of Volatiles from Male and Female Weevils. Gas chromatograms of volatiles collected from *A. rubi* in 1998 did not show any consistent differences between the sexes. However, in 1999, GC analyses of volatiles from individuals collected as overwintering adults revealed three components

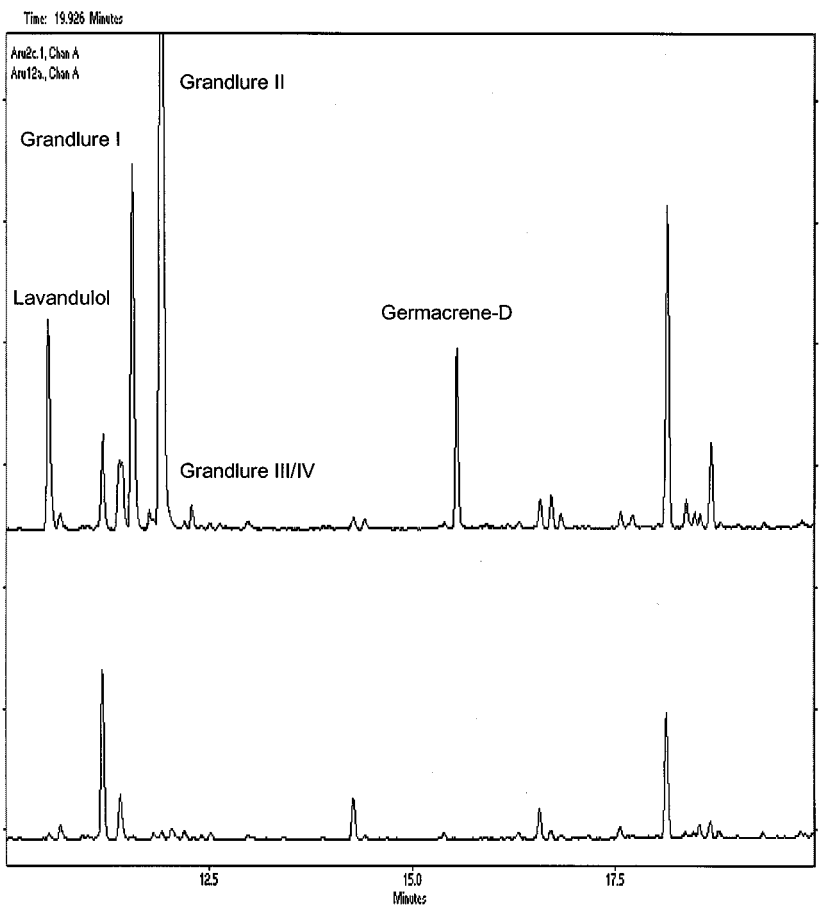


FIG. 1. Gas chromatograms (nonpolar CPSil 5CB column) of volatiles collected from male (upper) and female (lower) *A. rubi weevils.*

that were male-specific and another component found in relatively large amounts in male volatiles but only trace amounts in volatiles from females (Figure 1). Preliminary identifications were made by comparison of their EI mass spectra and library spectra and comparison of GC retention indices with published data. These preliminary identifications were confirmed by comparison of EI and CI mass spectra, retention indices on polar and nonpolar GC columns, and GC coinjection with the synthetic materials. The four components were found to be *cis*-1-methyl-2-(1-methylethenyl)cyclobutaneethanol (grandlure I), (*Z*)-2-(3,3-dimethyl-cyclohexylidene)ethanol (grandlure II), lavandulol, and germacrene-D (Figure 2), the latter being present in both male and female volatiles. These were produced in mean quantities per male weevil per day of 1.2, 6.1, 0.82 and 0.72 μg ($N = 8$), for grandlure I, grandlure II, lavandulol, and germacrene-D, respectively.

Further examination of GC-MS analyses of volatiles from male *A. rubi* showed the presence of trace amounts of the *E* isomer of grandlure II (approximately 1% of the *Z* isomer by GC-MS analysis), and also both (*Z*)-(3,3-dimethyl-cyclohexylidene)acetaldehyde (grandlure III) and (*E*)-(3,3-dimethylcyclohexylidene)acetaldehyde (grandlure IV) ($\leq$1% as compared with the (*Z*)-grandlure II by GC-MS) (Figure 2). The retention indices (RI) of the compounds identified on both polar and nonpolar GC columns are shown in Table 1. The *trans* isomer of grandlure I, fragranol (Bohlmann et al., 1973), was not available for comparison, but Yamazaki et al. (1994) reported this to elute clearly before the *cis* isomer on a Carbowax GC column.

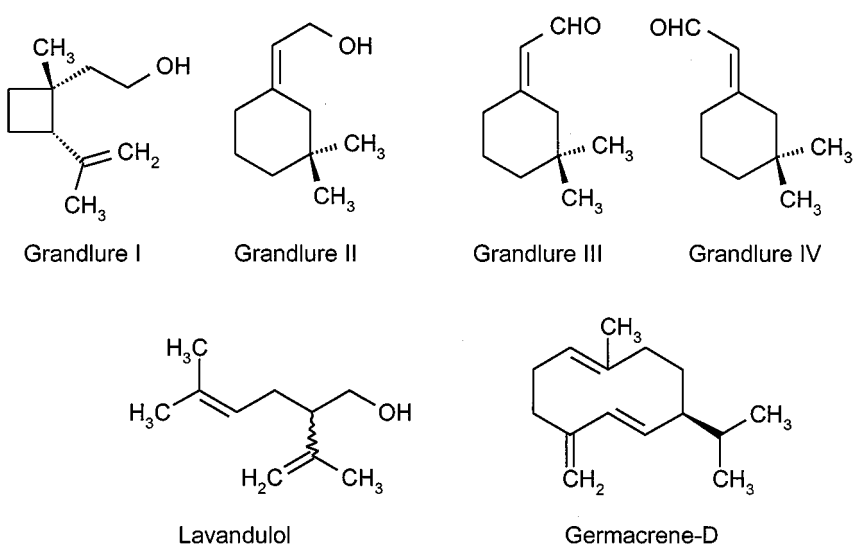


FIG. 2. Candidate pheromone components of male *A. rubi*.

TABLE 1. GC RETENTION INDICES (RI) OF PHEROMONE COMPONENTS

Chemical	RI	
	CPWax	CPSil
Grandlure I	1793	1190
(Z)-Grandlure II	1790	1203
(E)-Grandlure II	1801	1203
Grandlure III	1690	1219
Grandlure IV	1704	1228
Lavandulol	1677	1149

GC analyses of the volatiles from male *A. rubi* weevils on a chiral cyclodextrin column that separated the enantiomers of grandlure I (12.26 and 13.39 min) and lavandulol (7.17 and 7.44 min) showed that the naturally produced compounds were single enantiomers. Grandlure I was the (1*R*,2*S*)-(+) enantiomer by comparison of retention times and coinjection with the synthetic compound (12.26 min). The natural lavandulol cochromatographed with the second eluting enantiomer, but the absolute configuration is unknown as authentic samples of the enantiomers were not available. Racemic germacrene-D obtained from *Solidago altissima* extract gave two peaks on the cyclodextrin column with retention times of 19.76 and 20.58 min for the (+) and (−) enantiomers, respectively. The compound produced by male *A. rubi* weevils had the (−) configuration by retention time comparisons and coinjection with the *Solidago altissima* extract and (−)-germacrene-D obtained from ylang ylang oil (Nishii et al., 1997).

Volatile Collections from Weevils on Different Hosts. Volatiles from individual weevils were collected in the presence of *F. ananassa*. These weevils were then maintained on *F. ananassa* for two weeks, after which volatiles were collected from the same weevils in the presence of *A. sylvestris.* Volatiles were also collected from the weevils collected from *A. sylvestris* in the field in the presence of *A. sylvestris* or *F. ananassa*, the two plants being randomly assigned. The weevils collected from *M. recutita* underwent the same process in the presence of either *M. recutita* or *F. ananassa*. Volatiles were also collected from all three plants alone and from *F. ananassa* subjected to multiple cuts with microscissors.

Analyses of the collected volatiles by GC-MS showed that male weevils produced the above chemicals only in the presence of *F. ananassa*, and not in the presence of *A. sylvestris.* In GC-MS analysis of volatiles from weevils collected in August in the presence of either *F. ananassa* or *M. recucita*, the pheromone components were not detected. Volatiles from intact or cut *F. ananassa* contained small amounts of germacrene-D.

Pheromone Dispensers. Release rates from the sealed polyethylene sachet dispensers containing single components were constant (zero order). At 27°C and

8 kph windspeeed, rates were 1.0, 1.5, and 3.5 mg/day for grandlure I, grandlure II and lavandulol, respectively, as measured by weight loss of duplicate sachets.

The composition of the blends released from sachets containing the mixtures of components used in field trapping trials were measured by trapping and analysis of volatiles emitted. The mixtures were grandlure I + II (20:80), grandlure I + II + III + IV (25:58:8.5:8.5), Grandlure I + II and (±)-lavandulol (17:66:17), grandlure I + II + III + IV and (±)-lavandulol (ratio 19:47:7:7:20). The results over 36 days with a 200-mg initial loading are shown in Figure 3.

As expected from their individual release rates, the grandlure I + II blend released the two components in relative amounts similar to those in the starting

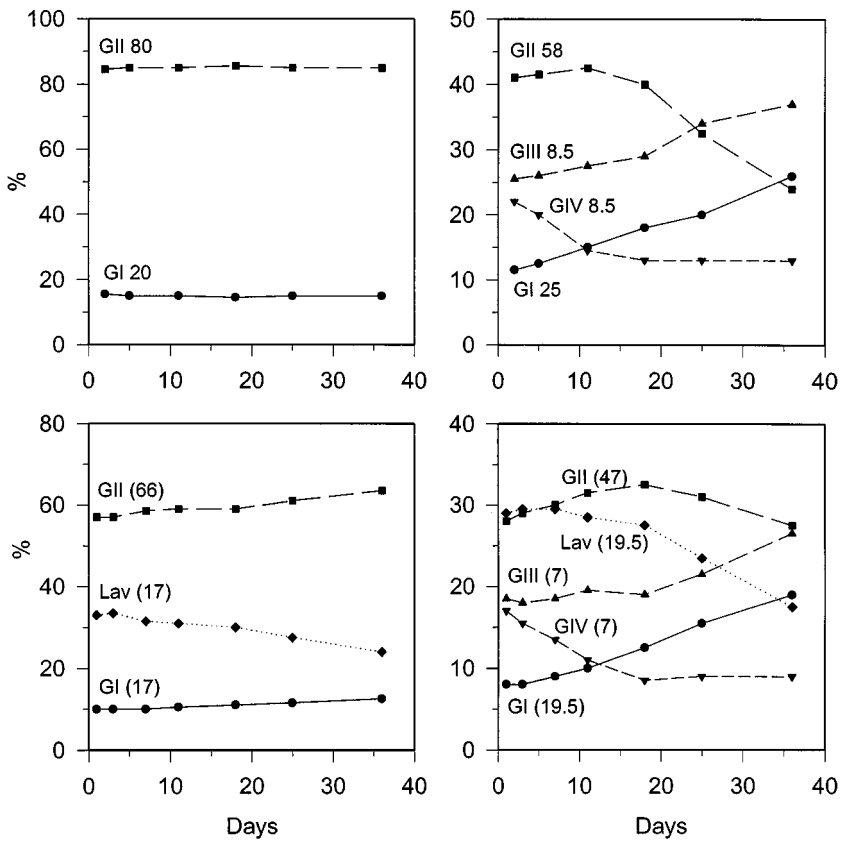


FIG. 3. Percentage composition of volatiles released from polyethylene sachet dispensers containing four different blends of synthetic pheromone components at 27°C and 8 kph windspeed. (GI = grandlure I; GII = grandlure II; GIII = grandlure III; GIV = grandlure IV; Lav = lavandulol; figures in parentheses are percentage in blend in sachet. Each sachet, replicated twice, contained 200 mg).

mixture, and this stayed constant during the period of measurement. With the three component grandlure I + II + lavandulol mixture, the lavandulol was released proportionately faster and the proportion of this component in the blend released declined.

The ratios of components released from the two mixtures containing the more volatile, and labile aldehyde components, grandlures III and IV, also deviated from the ratios in the starting mixture, especially in the presence of (±)-lavandulol. The blend released initially contained grandlures III and IV in higher relative proportions than in the blend in the sachet. However, whereas the proportion of grandlure III in the blend released increased during the study period, that of the *trans* isomer, grandlure IV, decreased over the first 20 days. This coincided with the appearance of additional GC peaks, suggesting some chemical transformation.

Trapping Trials. In 1999, attractiveness of the proposed pheromone components to *A. rubi* was evaluated in field trapping tests. A 3 × 5 randomized block design was set up at the three different sites using the four blends described above and a control, unbaited trap.

In the mid-season phase from mid-May to mid June 1999, the numbers of weevils caught in traps baited with blends of grandlure components alone (treatments B and D) were not significantly different from the numbers caught in unbaited traps (A) (Figure 4). Addition of (±)-lavandulol to either of these (treatments C

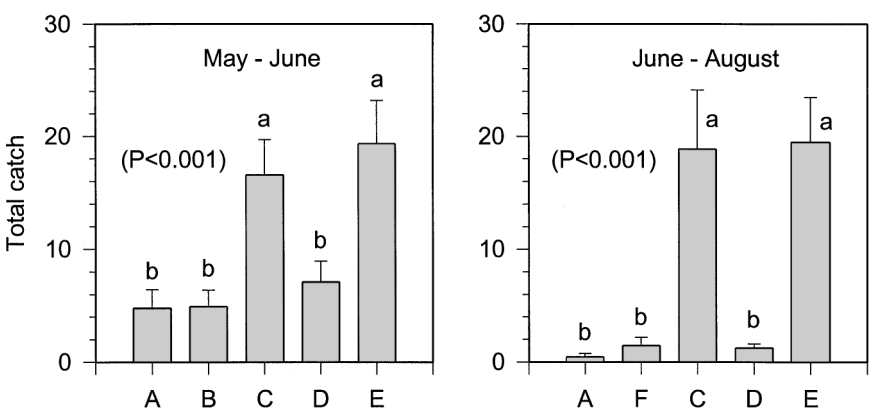


FIG. 4. Catches of *A. rubi* weevils in traps baited with different synthetic blends at three different sites, 1999 (three replicates at each site). Treatments in first experiment (40 days) were: A = control; B = grandlure I + II; C = Grandlure I, II, and (±)-lavandulol; D = Grandlure I, II, III + IV, E + Grandlure I, II, III, IV, and (±)-lavandulol; treatments in second experiment (34 days) were as previously except F = (±)-lavandulol. Standard error bars are shown for each treatment ($n = 9$); treatments with same letter above columns are not statistically different (LSD, $P = 0.05$); overall differences among treatments are shown in parantheses.

TABLE 2. CATCHES OF *A. rubi* WEEVILS IN THREE DIFFERENT TYPES OF TRAP[a]

Trap type	Total catch mean ± SE[b]
Horizontal sticky trap	15.5 ± 0.5 a
Vertical sticky trap	10.0 ± 2.0 b
Boll weevil trap	4.0 ± 0.0 c

[a] Lures contained grandlure I + grandlure II + grandlure III + grandlure IV + lavandulol; 3 replicates, May 23–July 25, 1999.
[b] Means followed by different letters are significantly different (data transformed to $\log_{10}(x + 1)$ followed by ANOVA and LSD test, $P = 0.05$).

and E) increased the catch ($P < 0.05$). Addition of grandlure III and IV had no significant effect on catches in the presence (E compared with C) or absence (D compared with B) of (±)-lavandulol.

Prior to the late-season phase from mid-June to early August, the lures were renewed, treatments rerandomized, and grandlure I + II (B) replaced by (±)-lavandulol (F) alone. Similar results were obtained with the two blends containing grandlure components and (±)-lavandulol (C, E) catching more weevils than the other treatments (Figure 4). Traps baited with grandlure I–IV (D) or (±)-lavandulol (F) did not catch more than unbaited traps (A).

The horizontal sticky trap was found to be the most effective trap design for trapping weevils when compared with a vertical sticky trap or the boll weevil trap (Table 2).

Sex of Weevils. Both male and female *A. rubi* were caught in sticky traps containing the attractive blends. However, the male to female ratio varied during the trapping period with females predominating in mid-season, but more males than females caught late season (Table 3).

TABLE 3. *A. Rubi* WEEVILS CAUGHT WITH SYNTHETIC LURES DURING TWO TRAPPING PERIODS[a]

	Catch [% (*N*)] [b]			
	May–June 1999		June–August 1999	
Sex	Lure A	Lure B	Lure A	Lure B
Male	36 (20)	40 (26)	75 (9)	80 (24)
Female	64 (36)	60 (39)	25 (3)	20 (6)

[a] Actual Numbers Sampled are in parentheses.
[b] Lure A: grandlure I + II + lavandulol (1:4:1); lure B: grandlure I + II + III + IV + lavandulol (19:47:7:7:20).

DISCUSSION

As with the three previously reported pheromones of weevil species in the subfamily Curculioninae (Tumlinson et al., 1969; Eller et al., 1994; Hedin et al., 1997), males of the strawberry blossom weevil, *A. rubi*, produce components of grandlure, the pheromone of the boll weevil, *A. grandis.* In addition to all four grandlure components, they also produce the acyclic monoterpene, lavandulol. Both grandlure I and lavandulol produced by males were found to be enantiomerically pure, although the absolute configuration of the latter has yet to be resolved. The grandlure I was shown to be the (1*R*,2*S*)-(+)- enantiomer as in *A. grandis*, where it has been shown that the opposite enantiomer is behaviorally inactive (Dickens and Mori, 1989). Trapping results with racemic materials showed that a minimum combination of grandlure I, grandlure II, and lavandulol was necessary to attract significant numbers of male and female *A. rubi.* Addition of small amounts of the aldehydes, grandlures III and IV, had no consistent significant effect on catches.

Germacrene-D was present in all headspace analyses incorporating strawberry plants, *F. ananassa.* This is a known component of *F. ananassa* volatiles (Hamilton-Kemp et al., 1990), but the amounts collected increased more than 10-fold in the presence of a pheromone-producing male. This increase may be due to production by the weevils or a plant response to feeding-induced damage. Although microscissor-damaged flowers and/or leaves did not produce elevated amounts of germacrene-D, this type of artificial approach to mimic insect-plant feeding has been shown to be unrepresentative (Loughrin et al., 1996; Dicke, 1999). Whether insect or plant produced, further investigation of germacrene-D is warranted because pheromone–host-plant synergism is widely reported (Landolt and Phillips, 1997) and the attractiveness of the pheromone is enhanced by host volatiles in many species of Curculionids (Dickens, 1989; Ridgway et al., 1990; Giblin-Davis et al., 1994, 1996; Cerda et al., 1999).

Volatile collections on the different host plants—strawberry and cow parsley, *A. sylvestris*—showed that males only produced the pheromone in the presence of *F. ananassa.* Observations in the laboratory and field confirmed that *A. sylvestris* is a food source. This suggests that male *A. rubi* may produce the pheromone from chemicals derived from the oviposition host-plant or that the male only signals when a suitable oviposition host has been located, the pheromone components being produced *de novo.* Pheromone biosynthesis in *A. grandis* is possible both from plant-derived precursors (Thompson and Mitlin, 1979) and by *de novo* synthesis (Mitlin and Hedin, 1974).

With *A. rubi*, the male/female mid-season response pattern to the pheromone mirrors that found for *A. grandis* (Hardee et al., 1970) and *A. eugenii* (Eller et al., 1994), in that catches during this mating and ovipositing period were dominated by females. However, late season catches were unlike the 1:1 sex ratio

reported in the American curculionids in that over 75% of the *A. rubi* catch was male. *A. rubi* produces one generation a year that is diapausal, and the late season catches were likely to include weevils in this physiological state. This is supported by concurrent collection of males and females that showed no mating behavior, and from which no pheromone components were collected. Villavaso and Earle (1974) and Mitchell and Hardee (1974b) reported that diapausal boll weevils were attracted to live, reproductive males and to the synthetic pheromone. Caution is necessary when relating the sex ratio of synthetic lure catches to the actual field sex ratio. Mitchell and Hardee (1974b), researching *A. grandis*, found synthetic lure/live-baited catches gave a different sex ratio to field-sampled collections.

Comparison of the three trap designs, white horizontal sticky, white vertical sticky, and boll weevil trap, showed that white horizontal sticky traps were more effective at trapping *A. rubi* weevils. This may in part be due to the weevil's attraction to white (unpublished results) and/or the ability of *A. rubi* to escape from the boll weevil traps, as reported by Eller et al. (1994) for *A. eugenii.* Over 95% of the weevils were trapped on the underside of the horizontal sticky surface, which suggests weevils fly to the vicinity of the pheromone source, then walk to it, and rarely fly directly onto it. This supports the hypothesis that flying is arrested on encountering high concentrations of aggregation pheromone (Dethier et al., 1960; Kennedy, 1977; Plarre and Vanderwel, 1999). Short-range pheromones (Hedin et al., 1979) as well as visual and acoustical cues may then take over. However, under laboratory conditions, *A. rubi* males mount all individuals encountered, regardless of sex, and the existence of a short-range, female-produced pheromone is considered to be unlikely.

Henson et al. (1976) studied the chemical stability of each grandlure component in isolation and found grandlures I and II to be stable, while grandlures III and IV formed several products, including the corresponding acids. In this work, the alcohols were stable in the absence of the aldehydes, but breakdown products were formed when they were mixed with the aldehydes. Mass spectral data suggested that the two predominant compounds were dehydration products of grandlures I and II, possibly catalysed by the acids formed from oxidation of grandlures III and IV.

The effect on trap catches of the individual lavandulol enantiomers and germacrene-D will be addressed in future studies, as will also optimizing of the component ratios and release rates of existing blends.

Acknowledgments—Thanks are due to Dr. Alan Cork for helpful comments regarding earlier drafts of the paper, Martin Ridout for advice on statistical methods, and to the growers Graham Cuthbert, Don Pascoe, and Harry Wooldridge who provided sites for the field trials. Professor Kenji Mori kindly provided a sample of (+)-grandlure I and Professor Yoo Tanabe generously supplied racemic germacrene-D. This project is funded by the Natural Resources Institute, University of Greenwich, and MAFF.

REFERENCES

ADAMS, R. P. 1989. Identification of Essential Oils by Ion Trap Mass Spectroscopy. Academic Press Limited, London.

ALFORD, D. V. 1984. A Colour Atlas of Fruit Pests: Their Recognition, Biology and Control. Wolfe Publishing London.

BALACHOWSKY, A., and MESNIL, L. 1935. Les Insectes Nuisibles aux Plantes Cultivées. Ministry of Agriculture, Paris, France.

BARTELT, R. J. 1999. Weevils, pp. 91–112, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Wallingford, UK.

BOHLMANN, F., ZDERO C., and FAASS, U. 1973. Natürlich vorkommende Terpenderivate, XXVI. Uber die inhaltstoffe von *Artemesia fragrans* Willd. *Chem. Ber.* 106:2904–2909.

CERDA, H., FERNANDAZ, G., LOPEZ, A., and VARGA, J. 1999. Olfactory attraction of the sugar cane weevil (Coleoptera: Curculionidae) to host plant odors, and its aggregation pheromone. *Fla. Entomol.* 82:103–112.

CROSS, J. V., and EASTERBROOK, M. A. 1998. Integrated management of flower pests of strawberry. *IOBC/WPRS Bull.* 21:81–87.

DETHIER, V. G., BARTON BROWN, L., and SMITH, C. N. 1960. The designation of chemicals in terms of the responses they elicit from insects. *J. Econ. Entomol.* 53:134–136.

DICKE, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomol. Exp. Appl.* 91:131–142.

DICKENS, J. C. 1989. Green leaf volatiles enhance aggregation pheromone of boll weevil, *Anthonomus grandis. Entomol. Exp. Appl.* 52:191–203.

DICKENS, J. C., and MORI, K. 1989. Receptor chirality and behavioral specificity of the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), for its pheromone, (+)-grandisol. *J. Chem. Ecol.* 15:517–527.

ELLER, F. J., BARTELT, R. J., SHASHA, B. S., SCHUSTER, D. J., RILEY, D. G., STANSLEY, P. A., MULLER, T. F., SHULER, K. D., JOHNSON, B., DAVIS, J. H., and SUTHERLAND, C. A. 1994. Aggregation pheromone for the pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae): Identification and field activity. *J. Chem. Ecol.* 20:1537–1555.

FENOULHET, G. 1907. The black *Anthonomus* (*Anthonomus rubi*) attacking the strawberry. *J. South-East. Agricul. Coll. Wye* 16:105–108.

GIBLIN-DAVIS, R. M., WEISSLING, T. J., OEHLSCHLAGER, A. C., and GONZALEZ, L. M. 1994. Field response of *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) to its aggregation pheromone and fermenting plant volatiles. *Fla Entomol.* 77:164–177.

GIBLIN-DAVIS, R. M., OEHLSCHLAGER, A. C., PEREZ, A., GRIES, G., GREIS, R., WEISSLING, T. J., CHINCHILLA, C. M., PENA, J. E., HALLETT, R. H., PIERCE, H.D., JR., and GONZALEZ, L.M. 1996. Chemical and behavioural ecology of palm weevils (Curculionidae: Rhynchophorinae). *Fla. Entomol.* 79:153–167.

HAMILTON-KEMP, T. R., LOUGHRIN, J. H., and ANDERSEN, R. A. 1990. Identification of some volatile compounds from strawberry flowers. *Phytochemistry* 29:2847–2848.

HARDEE, D. D., and MITCHELL, E. B. 1997. Boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae): A summary of research on behaviour as affected by chemical communication. *Southwest. Entomol.* 22:466–491.

HARDEE, D. D., CROSS, W. H., HUDDLESTON, P. M., and DAVICH, T. B. 1970. Survey and control of the boll weevil in West Texas with traps baited with males. *J. Econ. Entomol.* 63:1041–1048.

HEDIN, P. A., MCKIBBEN, G. H., MITCHELL, E. B., and JOHNSON, W. L. 1979. Identification and field evaluation of the compounds comprising the sex pheromone of the female boll weevil. *J. Chem. Ecol.* 5:617–627.

HEDIN, P. A., PAYNE, J. A., CARPENTER, T. L., and NEEL, W. 1996. Sex pheromones of the male and female pecan weevil, *Curuculio caryae:* Behavioural and chemical studies. *Environ. Entomol.* 8:521–523.

HEDIN, P. A., DOLLAR, D. A., COLLINS, J. K., DUBOIS, J. G., MULDER, P. G., HEDGER, G. H., SMITH, M. W., and EIKENBARY, R. D. 1997. Identification of male pecan weevil pheromone. *J. Chem. Ecol.* 23:965–977.

HENSON, R. D., BULL, D. L., RIDGWAY, R. L., and IVIE, G. W. 1976. Identification of the oxidative decomposition products of the boll weevil pheromone, grandlure, and the determination of the fate of grandlure in soil and water. *J. Agric. Food Chem.* 24:228–231.

HOFFMANN, A. 1954. Faune de France. Editions Paul Lechevalier, Paris.

INNOCENZI, P. J., HALL, D. R., CROSS, J. V., and GREEN, S. V. 2001. Sexing adults of the strawberry blossom weevil, *Anthonomus rubi* Herbst. (Coleoptera: Curculionidae). *J. Appl. Entomol.* In press.

JARY, S. G. 1932. The strawberry blossom weevil *Anthonomus rubi* (Herbst). *J. South-East. Agric. Coll. Wye* 30:171–182.

KENNEDY, J. S. 1977. Olfactory responses to distant plants and other odor sources, pp. 67–91, *in* H. H. Shorey and J. J. McKelveyn (eds.). Chemical Control of Insect Behaviour: Theory and Application. John Wiley, New York.

LANDOLT, P. J., and PHILLIPS, T. W. 1997. Host plant influences on sex pheromone behaviour of phytophagous insects. *Annu. Rev. Entomol.* 42:271–391.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON-KEMP, T. R., and BYERS, M. E. 1996. Role of feeding-induced plant volatiles in aggregative behaviour of the Japanese beetle (Coleoptera: Scarabaeidae). *Environ. Entomol.* 25:1188–1191.

MITCHELL, E. B., and HARDEE, D. D. 1974a. In-field traps: A new concept in survey and suppression of low populations of boll weevils. *J. Econ. Entomol.* 67:506–508.

MITCHELL, E. B., and HARDEE, D. D. 1974b. Seasonal determination of sex ratios and condition of diapause of boll weevils in traps and in the field. *Environ. Entomol.* 3:386–388.

MITLIN, N., and HEDIN, P. A. 1974. Biosynthesis of Grandlure, the pheromone of the boll weevil, *Anthonomus grandis*, from acetate, mevalonate, and glucose. *J. Insect Physiol.* 20:1825–1831.

MORI, K., and FUKAMATSU, K. 1992. A new synthesis of (+)-grandisol. *Liebigs Ann. Chem.*1992: 489–493.

MORRIS, M. G. 1977. The British species of *Anthonomus* Germar (Col., Curculionidae). *Entomol. Mon. Mag.* 112:19–40.

NISHII, Y., YOSHIDA, T., and TANABE, Y. 1997. Enantiomeric resolution of a germacrene-D derivative by chiral high-performance liquid chromatography. *Biosci. Biotech. Biochem.* 61:547–548.

PLARRE, R., and VANDERWEL, D. C. 1999. Stored-product beetles, pp. 149–198, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Wallingford, UK.

POPOV, S. Y. 1996. Possibility of monitoring the population density of the strawberry blossom weevil, *Anthonomus rubi* Herbst (Coleoptera, Curculionidae), on strawberry by two methods: counting clipped buds and using pheromones. *Entomol. Rev.* 75:104–109.

RIDGWAY, R. L., INSCOE, M. N., and DICKERSON, W. A. 1990. Role of the boll weevil pheromone in pest management. pp. 437–471, *in* R. L. Ridgway, R. M. Silverstein and M. N. Inscoe (eds.). Behavior-Modifying Chemicals for Insect Management: Applications of Pheromones and other Attractants. Marcel Dekker, New York.

THOMPSON, A. C., and MITLIN, N. 1979. Biosynthesis of the sex pheromone of the male boll weevil from monoterpene precursors. *Insect Biochem.* 9:293–294.

TUMLINSON, J. H., HARDEE, D. D., GUELDNER, R. C., THOMPSON, A. C., HEDIN, P. A., and MINYARD, J. P. 1969. Sex pheromones produced by male boll weevils: Isolation, identification and synthesis. *Science* 166:1010–1012.

VILLAVASO, E. J., and EARLE, N. W. 1974. Attraction of female boll weevils to diapausing and reproducing males. *J. Econ. Entomol.* 67:171–172.

YAMAZAKI, S., FUJITSUKA, H., TAKARA, K., and INOUE, T. 1994. Utilization of selenium-directed [2 + 2] cycloadditions: Concise synthesis of fragranol. *J. Chem. Soc. Perkin Trans. 1* 6:695–700.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

NOVEL CHEMISTRY OF ABDOMINAL DEFENSIVE GLANDS OF NYMPHALID BUTTERFLY *Agraulis vanillae*

GARY N. ROSS,[1] HENRY M. FALES,[2,*] HELEN A. LLOYD,[2,5] TAPPEY JONES,[3] EDWARD A. SOKOLOSKI,[2] KIMBERLY MARSHALL-BATTY,[2] and MURRAY S. BLUM[4]

[1]*6095 Stratford Avenue, Baton Rouge, Louisiana 70808*

[2]*Laboratory of Biophysical Chemistry, NHLBI, NIH*
Bethesda, Maryland 20892

[3]*Department of Chemistry*
Virginia Military Institute
Lexington, Virginia 24450

[4]*Department of Entomology*
University of Georgia
Athens, Georgia 30602

[5]*Present address: Sequim, Washington 98382*

(Received October 5, 2000; accepted February 12, 2001)

Abstract—Abdominal defensive glands of both sexes of the Gulf fritillary butterfly, *Agraulis vanillae (Linnaeus) (Nymphalidae:Heliconiinae)* emit a pronounced odor when disturbed. We have identified 6-methyl-5-hepten-2-one; oleic, palmitic, and stearic esters of the corresponding alcohol 6-methyl-5-hepten-2-ol; hexadecyl acetate; 1,16-hexadecanediol diacetate; and 1,15-hexadecanediol diacetate in the glandular exudate. Since we have determined that free-flying birds or birds in a butterfly conservatory discriminate against *A. vanillae* as prey, we suggest that the constituents in the glands may play a defensive role against potential avian predators.

Key Words—*Agraulis vanillae*, defensive glands, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol esters, 1,15-hexadecanediol diacetate 1,16-hexadecanediol diacetate.

INTRODUCTION

The Gulf fritillary butterfly, *Agraulis vanillae* (Nymphalidae), was first described by Linnaeus (1758), who characterized it as a species whose brilliant coloration

*To whom correspondence should be addressed.

0098-0331/01/0600-1219$19.50/0

made it very conspicuous. *Agraulis* is the most primitive genus in the large neotropical subfamily Heliconiinae, a taxon that is famous for the development of mimicry theory utilizing both brightly colored unpalatable and palatable species of butterflies (Müller, 1886; Ross, 1995). This generally tropical butterfly is also unusual in its marked northern extension that reaches to Georgia, the Carolinas, and southern Missouri and Nebraska (Glassberg, 1999) in the United States (Emsley, 1965). The wing pattern is known for its striking contrast coloration with silver below the wings and triangles and polygons of alternating silver, yellow, red, and brown above. Tyrosine is responsible for the black and brown colors via melanin formation, while tryptophan forms the red, light brown, and tan portions via 3-hydroxykynurenine and ommochromes as shown by incorporation of these radiolabeled precursors (Nijhout and Koch, 1991).

Eltringham (1925) has discussed the observation of Müller (1886) on the emission of a "strong and nauseous smell" emanating from glands in the anal valvulae of the males and from a yellow gland extruded on the abdominal dorsum of the females of *A. vanillae.* Müller (1886) called this species the Maracuja butterfly because the larvae generally fed on the maracuja (*Passiflora edulis*, Passifloriaceae), also known as the passion flower, on which it does considerable damage. The odor of the exudate from the females was said to resemble that of phenylcarbylamine (phenylisocyanide) (Eltringham, 1925), but then again "not unpleasant" and "more complex than phenylcarbamylamine." We report below the chemical nature of some of the compounds in the exudates of male and female *A. vanillae.* The above workers all described the repellent functions of these odorous secretions, and we have made additional observations with predatory birds in view of the possible unpalatability of both females and males of *A. vanillae.*

METHODS AND MATERIALS

Extraction and Subsequent Survival. Eight males and five females were collected from a personal butterfly garden belonging to Ross (1999) in Baton Rouge, Louisiana on July 22, 1999. The butterflies were initially placed individually in glassine envelopes for temporary storage. After being brought inside, each butterfly was handheld with pressure exerted on the thorax and upper abdomen. The pressure forced extrusion of the glands, which were then punctured with a No.1 stainless steel insect pin. Squares of filter paper (7–8 mm) held with a watchmaker's pair of forceps were touched to the secretions for collection. The papers were then stored in small glass vials, three for the females and two for the males, each containing 1.0 ml of methyl alcohol. Separate pins were used for the males and females. Forceps were cleaned first with soap and water and then with methyl alcohol between sampling from males and females. Following extraction (milking), the butterflies were returned to their glassine envelopes, placed within lock-proof sandwich bags (males and females separate), and then stored in a refrigerator until

the following day. Milkings were done on four consecutive days; however, males produced virtually no secretions on days 3 and 4, whereas females continued to produce, albeit to a lesser degree, through day 4. By day 5, four of the males and one of the females had died and milkings were discontinued.

The remaining eight live individuals were returned to the garden. Surprisingly, after 15 min of resting in the summer sun, the butterflies began to fly, feebly at first, but in time, more naturally. Since the garden is full of flowers, including lantana, a favorite of *Agraulis*, most of the released butterflies began to feed. When checked 3 hr later, three males and three females were still present and flying about with no apparent difficulties. The following day only one male and two females were seen. The females were flying about a *Passiflora* vine attempting to oviposit, but the milking procedures had apparently damaged some critical component of their reproductive tracts since no deposition of eggs could be observed. Two females continued to remain within the garden for a total of four days.

Chemical Analysis. Mass spectra were run using an LKB 2091 mass spectrometer operating in the EI mode with a 15-m $\times$ 0.25-mm DB-1 column with 0.25-μm film thickness operating from 78 to 300°C at 8°C/min, with a helium flow rate of 10 ml/min. Samples (1 μl) of the extracts dissolved in methanol were injected at 250°C using a splitless injector.

Samples of 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol were obtained from Aldrich (Milwaukee, Wisconsin). Hexadecyl acetate and 1,16-hexadecanediol diacetate were prepared by reacting equimolar amounts of the corresponding alcohols (Aldrich) with an excess of acetic anhydride in pyridine overnight. Evaporation of the excess reagents and solvent left the crude acetates that were used without further purification. The 1,2- and 2,3-hexadecanediol acetates were similarly prepared from samples of the diols prepared from the corresponding hydrocarbons in conjunction with elucidation of the structures of diols found in chicken uropygial glands (Haahti and Fales, 1967). 1,3-Hexadecanediol diacetate was similarly prepared by acetylation of the corresponding diol prepared by treating commercial tetradecanal from Fluka (Milwaukee, Wisconsin) with commercial vinylmagnesium bromide (Aldrich). The resulting crude 1-hexadecen-3-ol was treated with an excess of borane dimethylsulfide complex (Aldrich). The usual oxidative work-up with sodium hydroxide and hydrogen peroxide provided a sample of crude 1,3-hexadecanediol that was ca. 80% pure by gas chromatography.

1,15-Hexadecanediol diacetate was prepared by allowing an excess of 2.6 M methyllithium in ether (Aldrich) to react with 1 meq of ω-pentadecalactone (Aldrich) dissolved in 20 ml of hexane. After standing 10 min, dilute hydrochloric acid was added and the solution extracted with more ether. After drying with molecular sieves (3Å, Fluka), the extracts were evaporated and a solid residue obtained. This was easily recrystallized from 50% toluene in hexane, but NMR revealed it to be a mixture. A portion of the sample was then separated on 100-μm-thick silica gel thin layer chromatography plates (Aldrich) with 2% ethanol

in chloroform. Three zones with retention factors (R_f) 0.63, 0.28, and 0.20 were visualized with methyl red and their contents recovered by scraping and extraction with 10% ethanol in chloroform. The zone at $R_f = 0.63$ was found by MS to be recovered ω-pentadecalactone. The zone with $R_f = 0.28$ melted at 45–62°C and was found by GC-MS to be the desired 1-hexadecanol-15-one [*m/z* (%): 41(39), 43(100), 55(49), 67(14), 69(20), 71(29), 96(12), 180(3), 223(2), 226(1), 256(3, M^+)] contaminated with about 30% ethyl tetradecanoate, presumably from an impurity of that acid in the lactone. Reduction of the keto alcohol with sodium borohydride in ethanol overnight formed 1,15-hexadecanediol [*m/z* (%): 41(75), 43(60), 45(95), 55(100), 57(44), 67(40), 69(67), 71(28), 95(54), 96(41), 109(24), 111(25), 123(14), 124(15), 125(10), 137(8), 194(4), 196(6), 243(5), 258(0, M^+)], and acetylation with pyridine and acetic anhydride produced the diacetate, whose mass spectrum and retention times were identical with the unknown diol diacetate shown as peak 3 in Figure 2 below. The lower band of $R_f = 0.20$ was found to be the 2-methyl-1,15-hexadecanediol, mp 63–67°C, formed by secondary reaction of the methyl ketone with excess methyllithium [*m/z* (%): 69(100), 71(55), 81(34), 83(78), 95(40), 97(45), 109(23), 111(18), 123(10), 125(8), 168(4), 180(3), 196(10), 221(4), 224(4), 239(9), 257(13, M-CH_3), 272 (0, M^+)].

Predatory Birds vs. Gulf Fritillary. Potential avian predators in the presence of large populations of *A. vanillae* were observed in two situations. In the first, 50–70 butterflies were released weekly during 1996 into the "Butterflies in Flight" exhibit at the Audubon Zoological Garden, New Orleans, Louisiana (the releases were normal protocol for populating the conservatory). The exhibit included 60–70 free-flying birds, including aggressive insectivorous species such as red-rumped caciques [*Cacicus haemorrhous* (Icteridae)] and superb starlings [*Lamprotornis superbus* (Sturnidae)]. Avian predation on *A. vanillae* and other butterflies was monitored almost daily for nine months by G. N. Ross as well as by zoo employees and volunteers for approximately 60 hr, which included examination of the ground for detached wings.

Avian predation was also monitored in an outdoor butterfly garden ($\sim$350 m^2) in Baton Rouge, Louisiana, between 1997 and 1999 (Ross, 1999). *A. vanillae*, which utilizes *Passiflora incarnata* as a host plant (Ross 1996, 2000 b), was the most common butterfly species by a factor of at least 10-fold. A variety of known avian predators were present at all times in addition to a diversity of invertebrate species with well-known predatory propensities. Efforts were made to directly observe *A. vanillae* being utilized as a prey species or indirectly in terms of detached wings being present on the ground.

RESULTS

Abdominal Glands and Their Secretions. The female possesses a pair of posterior abdominal scent glands on the dorsum between abdominal segments 7

and 8. These glands are bright yellow in color, spherical in shape (1.9–2.4 mm in diameter), and appear spongy in texture. The paired structures are normally inverted. However, when a butterfly is captured or disturbed, the glands are quickly everted. Additionally, the dorsal membrane between abdominal segments 6 and 7 is partially extruded and appears bright yellow in color. If held within 15 cm, a strong, very sweet odor is easily detected. If a gland is punctured with a fine pin, a sizable quantity of bright yellow, clear fluid is emitted. This fluid has an acrid taste, dries rapidly, and retains its color. The odor is not detectable after the fluid dries.

The male possesses a pair of bilobed, spherical structures (glands) 1.4–1.8 mm in diameter within the valvae (claspers) of the abdominal tip. The structures appear as bright yellow swollen pads when the claspers are extended, which occurs with only the slightest provocation. The fragrance is more acrid and less sweet (more alcoholic) than that produced by the females. If a gland is punctured, a small amount (but less so than that of females) of clear yellowish fluid is released. The fluid has the same fragrance as that emitted by an unaltered male. The taste is acrid and similar to that of females, dries rapidly, and retains its color. The odor dissipates on drying

Chemical Analysis. In spite of the subtle differences in odor described above, samples of the fluid obtained from either male or female abdominal glands provided essentially the same gas chromatograms and mass spectral analyses. Figure 1 shows a chromatogram of the male exudate where the early-eluting peak (peak 1) was identified as 6-methyl-5-hepten-2-one via a search of the NIST-NIH-EPA database

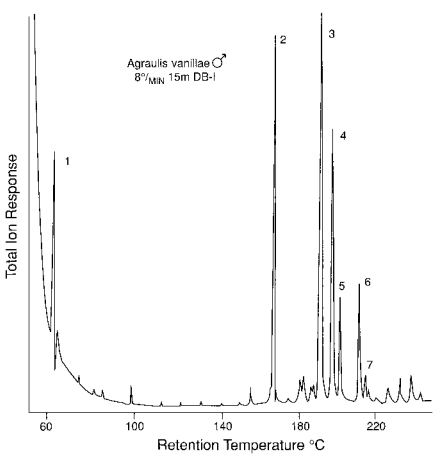


FIG. 1. Gas chromatogram of a methanol extract of male abdominal glands of *A. vanillae*: peak 1, 6-methyl-5-hepten-2-one; 2, hexadecyl acetate; 3, 1,15-pentadecanediol; 4, 1,16-hexadecanediol diacetate; 5, palmitoyl ester of 6-methyl-5-hepten-2-ol; 6, oleoyl ester of 6-methyl-5-hepten-2-ol; 7, stearoyl ester of 6-methyl-5-hepten-2-ol.

(NIST/NIH/EPA Mass Spectral Library, Standard Reference Data Program of the National Institute of Standards and Technology, Gaithersburg, Maryland) and by comparison of its mass spectrum and retention time with an authentic sample (Aldrich). Hexadecyl acetate (peak 2) was readily identified from its "acid plus one" peak at *m/z* 61 and its M-60 ion at *m/z* 224, and its presence also confirmed by comparison of its spectrum and retention time with an authentic sample. Peak 3 is discussed in detail below. Peak 4 was identified as 1,16-hexadecanediol diacetate through the database and by comparison with an authentic sample.

Mass spectra of peaks 5, 6, and 7 all closely resembled each other with abundant ions appearing at *m/z* 55, 69, 95, and 110 (Figure 2, peak 5). When searched in the database, these ions produced the spectrum of 5-[(1,5-dimethyl-4-hexenyl)oxy]-3-methyl-2-pentenoic acid ethyl ester where they were clearly due to the (1,5-dimethyl-4-hexenyl)oxy- moiety. This fragment corresponds to the alkoxy residue of 6-methyl-5-hepten-2-ol that is, in turn, the reduction product of the ketone found in peak 1. Peaks 5, 6, and 7 then represent homologous long-chain esters of that compound. Examination of minor peaks at higher mass identified these as palmitic acid (*m/z* 256), oleic acid (*m/z* 282), and stearic acids (*m/z* 284), respectively. To confirm the first of these assignments (peak 5), the palmitoyl ester was prepared from 6-methyl-5-hepten-2-ol (Aldrich) and palmitoyl chloride in pyridine. The product was indeed identical in retention time and mass spectrum with that obtained from the insect in peak 5. These compounds do not appear to have been encountered previously.

The mass spectrum of peak 3 resembled that of peak 4 (1,16-hexadecanediol diacetate) in many respects, including the loss of two molecules of acetic acid to form ions at *m/z* 222. However, peak 3 shows an ion at *m/z* 87 not seen in the 1,16-diol, and this suggests the linkage $CH_3CH = OAc^+$. In fact, one of us (H.M.F) earlier noted a series of 2,3-alkanediols in the uropygial glands of birds (Haahti and Fales, 1967), so 2,3-hexadecanediol diacetate was prepared from the diol synthesized in connection with that study. The 2,3-diol diacetate (Figure 2, bottom spectrum) did indeed show cleavage between the acetoxy groups producing ions at *m/z* 87 and *m/z* M-87 at 255, but its spectrum was not identical with that of peak 3. An isomeric ion $CH_2(OAc)CH_2^+$ of *m/z* 87 is theoretically available also from 1,3-hexadecanediol diacetate, so this compound was synthesized by the action of vinylmagnesium bromide on tetradecanal followed by hydroboration, oxidation, and acetylation. This compound (spectrum not shown) showed an abundant α-cleavage peak containing both acetate units at *m/z* 159 while the intensity at *m/z* 87 was only 8%, so it was also ruled out. By now it was clear that the two acetate units are more remotely located in the unknown. Based on the ion at *m/z* 87, as well as the presence in the extract of 1,16-hexadecanediol diacetate, we considered a likely candidate to be the 1,15-diol isomer. This diol was prepared by reacting commercially available ω-pentadecalactone (Aldrich) with methyllithium to form 1-hexadecanol-15-one

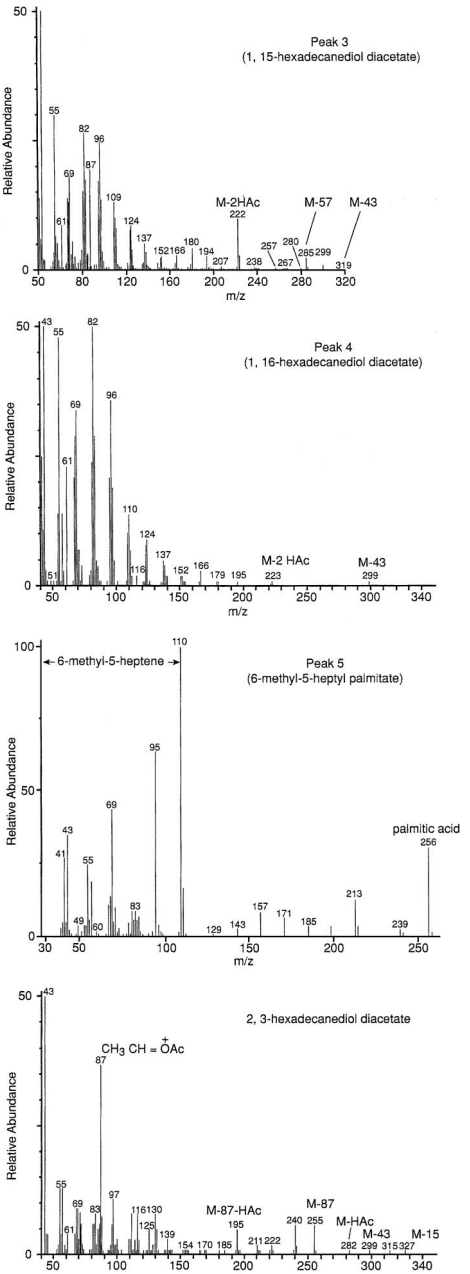


FIG. 2. Mass spectra of peaks 3, 4, and 5 in the gas chromatogram of the abdominal glands of *A. vanillae.* 2,3-Hexadecanediol acetate is presented for comparison to other diacetates (peaks 3 and 4).

(with much of the tertiary alcohol 2-methyl-2,15-pentadecanediol as by-product). Reduction of the ketone with sodium borohydride and acetylation with acetic anhydride gave the desired 1,15-pentadecanediol diacetate, which was indeed identical in retention time and mass spectrum with the compound represented by peak 3.

Predation on Agraulis vanillae. Predation on adults of *A. vanillae*, flying in a butterfly house, was never observed during the nine-month observation period. More than 400 butterflies were released into the exhibit building that also contained a population of free-flying insectivorous birds, including two species of aggressive predators; the superb starling and the red-rumped cacique. In addition, evidence of detached wings were never found on the ground. However, all species, other than the those belonging to the subfamily Heliconiinae, were eagerly attacked and eaten. (In time, many of the predatory birds had to be removed from the exhibit because they were decimating all nonheliconian butterflies.) Thus, in an enclosed environment, containing a significant population of predatory birds, adults of *A. vanillae* are discriminated against as prey items.

Similar results were obtained in an outdoor butterfly garden in which *A. vanillae* was the dominant butterfly species by a factor of at least 10. Different species of butterflies were observed to be fed upon by invertebrate predators that included spiders (Araneidae), assassin bugs (Reduviidae), dragonflies (Libellulidae), wasps (Vespidae), ambush bugs (Physmatidae), and praying mantids (Mantidae). Vertebrate predators included anole lizards and a variety of free-flying birds. Predation on butterfly species, especially *Phyciodes tharos* (Nymphalidae:Melitaeinae), *Hermeuptychia hermes* (Satyridae), and *Papilio polyxenes* (Papilionidae) was pronounced but was never observed with *A. vanillae.* In spite of its abundance, *A. vanillae* is obviously not a commonly utilized prey species and is avoided by both invertebrate and vertebrate predators.

DISCUSSION

The only truly volatile component found in both sexes in this study was 6-methyl-5-hepten-2-one, a well-known ant alarm pheromone and defensive allomone (Tomalski et al., 1987), and this compound may be objectionable to birds as well. Use of the volatile ketone as a solitary defensive compound by both cockroaches and beetles (unpublished data) clearly points to a similar role in adult *A. vanillae.* To us, the odor of this ketone, which can be detected easily when the butterfly is disturbed, does not recall the "phenylcarbamylamine" cited by Eltringham (1925), but, of course, this is a subjective evaluation.

The simultaneous occurrence of this compound in its alcohol form in the extract where it is conjugated to palmitic, oleic, and stearic acids may be significant. It is possible that this constitutes a method for its storage until required, whereupon it may be available via the sequential action of a lipase and oxidase from another

source or gland. The higher molecular weight diol diacetates may also be involved here by dissolution of the ketone, reducing its loss through evaporation. An alternative possibility for the diol diacetates, for which we have no evidence, is that they might have a function in the butterfly similar to that of the diols in the preen glands of fowl, i.e., by rendering their wing coverings water repellent (Haahti and Fales, 1967). However, extensive investigations on the Heliconiinae, implemented more than 100 years ago (Müller, 1886), emphasized that the secretions of the abdominal glands of these nymphalids clearly possessed a defensive function that afforded the butterflies considerable protection from predators. Although we cannot state unambiguously that the glandular components of *A. vanillae* are responsible for the apparent immunity of these butterflies from avian predation, we suspect that the compounds in this abdominal exudate may play an important role in defensive contexts. For example, we have observed that several ant species are effectively repelled by the defensive secretion of the cockroach *Neostylopyga rhombifolia* (unpublished data). This exudate is essentially pure 6-methyl-5-hepten-2-one. In addition to deterring these predatory invertebrates, we have also observed that the ketone-rich secretion of *N. rhombifolia* (Blattidae) effectively repels the American chameleon, *Anolis carolinensis*, a very aggressive vertebrate predator. These studies indicate that 6-methyl-5-hepten-2-one possesses deterrent properties against at least some invertebrate and vertebrate predators.

Our studies on the level of avian predation on free-flying *A. vanillae* clearly demonstrate that this eminently aposematic lepidopteran is virtually immune to the predatory activities of insectivorous birds. This was particularly striking in a butterfly conservatory where these lepidopterans shared their flying space with birds that fed readily on butterfly species other than *A. vanillae* (the same was true in the butterfly garden). Observations of *A. vanillae* versus birds in the butterfly exhibit indicated that birds took evasive action when they closed on the butterflies. It appears that these insects possess a powerful organoleptic shield that appears to insulate them from at least some avian and nonavian predators as well. Since the odor of 6-methyl-5-hepten-2-one can be detected in the presence of feeding *A. vanillae*, it is possible that the secretion of the abdominal glands may normally leak, thus enabling this aposematic species to advertise its presence freely through olfaction. Producing this ketone in sufficient quantity in order to be olfactorily aposematic may be possible if these butterflies are indeed generating its alcoholic precursor from the rich store of fatty acid esters of 6-methyl-5-hepten-2-ol. Additionally, it appears as if *A. vanillae* has morphological strategies that allow individuals to recover easily from an attack, another indicator that the species is protected.

The extraordinary tenacity of the individual butterflies that had been subjected to the milking procedures is probably yet another indication that *A. vanillae* is a protected species. Potential predators must learn which species to avoid, and learning requires sampling. Consequently it is in the best interest of a protected

prey to be able to survive an occasional attack and so most protected species have developed morphological strategies that bestow some degree of toughness (Ross, 2000a.).

REFERENCES

ELTRINGHAM, H. 1925. VI. On the abdominal glands in *Heliconius* (Lepidoptera). *Trans. Entomol. Soc. London. Parts I and II.* 269–275.

EMSLEY, M. G. 1965. Speciation in *Heliconius* (Lep., Nymphalidae): Morphology and geographic distribution. *Zoologica* 50:191–254.

GLASSBERG, J. 1999. Butterflies Through Binoculars: The East. Oxford University Press, New York, 242 pp.

HAAHTI, E. O. A., and FALES, H. M. 1967. The uropygials: Identification of the unsaponifiable constituent of the diester wax from chicken preen glands. *J. Lipid Res.* 8:131–137.

LINNAEUS, C. 1758. Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Editio decima, reformata. Laurentii Salvii, Holmiae. 1. p. 482.

MÜLLER, W. 1886. Südamerikanische Nymphalidenraupen: Versuch eines natürlichen Systems der Nymphaliden. *Zool. Jb.* 1:417–678.

NIJHOUT, H. F., and KOCH, P. B. 1991. The distribution of radiolabeled pigment precursors in the wing patterns of nymphalid butterflies. *J. Res. Lepid.* 30:1–13.

ROSS, G. N. 1995. *Everything you wanted to know about butterflies. 100+ questions and answers by Gary Noel Ross, PhD.* Gary N. Ross, Baton Rouge, Louisiana, 52 pp.

ROSS, G. N. 1996. Gulf fritillary. *La. Conserv.* 48:18–20.

ROSS, G. N. 1999. Butterfly gardens as scientific laboratories. *Butt. Gard. News* 4:1, 4–7.

ROSS, G. N. 2000a. The tough guys! *Butt. Gard. News* 5:3–5.

ROSS, G. N 2000b. Ten secrets to good butterfly gardening. *La. Gard.* Sept. 8–9.

TOMALSKI, M. D., BLUM, M. S., JONES, T. H., FALES, H. M., HOWARD, D. F., and PASSERA, L. 1987. Chemistry and functions of exocrine glands of the ants *Tapinoma melanocephalum* and *T. erraticum. J. Chem. Ecol.* 13:253–263.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

LACK OF INTRASPECIFIC AGGRESSION IN THE ANT *Tetramorium bicarinatum*: A CHEMICAL HYPOTHESIS

CYRIL ASTRUC,[1,2] CHRISTIAN MALOSSE,[3] and CHRISTINE ERRARD[1,*]

[1]*Institut de Recherche sur la Biologie de l'Insecte, C.N.R.S.*
Faculté des Sciences et Techniques
Parc de Grandmont, F-37200 Tours, France

[2]*Laboratoire d'Éthologie Expérimentale et Comparée, C.N.R.S.*
Université Paris Nord
F-93420 Villetaneuse, France

[3]*Unité de Phytopharmacie et Médiateurs chimiques,*
I.N.R.A. de Versailles
F-78026 Versailles, France

(Received October 25, 1999; accepted February 13, 2001)

Abstract—*Tetramorium bicarinatum* (Myrmicinae) is an ant species frequently found in tropical and subtropical areas, particularly in Africa, Southeast Asia (Japan), and South America (Brazil). The species is polygynous, reproduces by budding, and has sterile workers. Since the nests are widely distributed in a given area, the problem arises of territorial defense against conspecifics. Because not all ants defend territories, we assessed the defensive behavior of *T. bicarinatum* workers through intraspecific and interspecific aggressiveness tests. A detailed behavioral study of the interactions between workers from several different colonies of *T. bicarinatum* (originating from Japan and Brazil) showed that workers do not discriminate against conspecific nonnestmate individuals, but they are highly aggressive towards allospecifics (*Myrmica rubra*, Myrmicinae). The results suggest that each colony from this ant species possesses a similar colonial odor. Chemical analyses of the cuticular hydrocarbons of these species were made with gas chromatography coupled to mass spectrometry. Results showed that the different colonies of *T. bicarinatum* possess a common chemical profile mainly composed of straight-chain alkanes and alkenes, while *M. rubra* possess more methyl-branched alkanes. We suggest that methyl alkane cues play a determining role in colonial recognition and that these results could explain the underlaying basis of the lack of intraspecific aggressiveness in *T. bicarinatum.*

Key Words—Formicinae, intraspecific aggressiveness, cuticular hydrocarbon profile, *Tetramorium bicarinatum.*

*To whom correspondence should be addressed.

0098-0331/01/0600-1229$19.50/0

INTRODUCTION

Nestmate recognition is a fundamental feature of social insect behavior and allows alien individuals to be rejected from the colony. The capacity to discriminate nestmates from nonnestmates prevents robbery, predation, and parasitism; enables the monopolization of resources in defended territories; and helps in eliminating neighboring competitors (Wilson, 1971).

Nestmate recognition is due to nonvolatile olfactory cues borne on each individual's cuticle, cues that are shared by all nestmates and vary among nests (Howse, 1975; Hölldobler and Michener, 1980; Howard, 1993; Lenoir et al., 1999). Among the cuticular lipids of ants that serve to prevent desiccation and regulate cuticular permeability (Hadley, 1980), hydrocarbons are dominant (Lahav et al., 1999; for reviews see Breed, 1998; Vander Meer and Morel, 1998; Lenoir et al., 1999). Several authors have hypothesized that some methyl-branched hydrocarbons could be involved in intracolonial recognition in ants (Bonavita-Cougourdan et al., 1987, 1990; Provost et al., 1992), and in social wasps (Dani et al., 1996; Gamboa et al., 1996).

Each colony member possesses its own cues (or labels) that are correlated with genotype (endogenous source) (for reviews see Hölldobler and Michener, 1980; Blum, 1987; Breed and Bennett, 1987; Vander Meer and Morel, 1998). These cues are influenced by exogenous sources from the physical and social environment, such as air, soil, food, nest materials, and colony structure (Carlin and Hölldobler, 1986; Gamboa et al., 1991). Examples in the literature of nestmate recognition stress the importance of exogenous sources in *Acromyrmex octospinosus* (Jutsum et al., 1979), endogenous sources in *Pseudomyrmex ferruginea* (Mintzer and Vinson, 1985), and a combination of both in *Solenopsis invicta* (Obin and Vander Meer, 1988). Thus, numerous combinations of endogenous and exogenous sources are possible as odor determinants constituting the specific chemical signature by which individuals recognize one another as members of the same colony (Vander Meer and Morel, 1998).

Each colony member also possesses a reference model of the chemical signature (or sensory template located in the brain or in the antennal receptor cells) encoding the labels that characterize nestmates. The acceptance or rejection of an individual can be explained by a decision rule, depending on the degree to which the chemical signature of the encountered individual and the reference model of its partner overlap. This rule can be used to determine appropriate subsequent behavior (Breed and Bennett, 1987; Errard and Hefetz, 1997). Moreover, since the odor is dynamic because of a constantly changing chemical environment as well as within-colony individual genetic variations, template plasticity must follow (Vander Meer et al., 1989). For example, in a monogynous species (where all the individuals of a colony are strongly related), the template must reflect the cue changes in order to permit, at any given time, the resident colony individuals

to exhibit aggressive behavior towards intruders such as conspecific, but nonnestmate individuals. In contrast, polygynous species often do not defend a territory and do not recognize conspecific nonnestmates as intruders (Vander Meer and Morel, 1998). Furthermore, Provost and Cerdan (1990) showed there is a correlation between the number of queens and the degree of closure of the colony. These authors showed that monogynous colonies of the ant *Messor barbarus* are more closed than colonies that have been made experimentally polygynous. Keller and Passera (1989) suggested in *Linepithema humile* that the presence of several queens in the same colony (polygynous species) may result in a less distinct colony odor (more variable genetic cues) and decreased intercolonial aggressiveness. This suggestion was confirmed by Morel et al. (1990), who have shown that in the fire ant *Solenopsis invicta* polygynous residents do not recognize monogynous or polygynous intruders as different; however, monogynous residents are aggressive towards both types of intruders. Thus, in this case, the cues for polygynous and monogynous intruders fit within the polygynous template. Vander Meer et al. (1989) reported a generalized cue/template model where environmental cues were expected to vary quantitatively and qualitatively, whereas heritable cues would vary only in the relative intensity of the compounds involved. Later, it was suggested (Vander Meer and Morel, 1998) that the difference between monogynous and polygynous nestmate recognition lies in the template, which in the latter case is broader and less distinct than that of their monogynous counterparts.

On the other hand, Provost et al. (1994) have shown that *Messor barbarus* workers belonging to monogynous colonies were able to discriminate between intruders from other monogynous colonies and those from polygynous ones. They observed that one set of hydrocarbons (*n*-alkanes, mono-, di-, and trimethyl alkanes) was characteristically associated with workers from monogynous colonies. It also emerged that another set of hydrocarbons could be said to characterize the digynous and trigynous colonies.

In the present study, we tested the intra- and interspecific defensive behaviors of the ant *Tetramorium bicarinatum* (Formicidae, Myrmicinae) to understand the mechanisms underlying its widespread success. *T. bicarinatum* is a species originating in Southeast Asia and is characterized as an accomplished tramp (Bolton, 1980). Its current biotope extends through all the tropical and subtropical areas, particularly in Africa, South America, and Japan, having been distributed throughout the world by humans (Bolton, 1980). This species has the following characteristics: it is polygynous (about 50 queens for 1200 workers), the sterile workers are small (3–3.5 mm), it reproduces by budding, and the nests are widely distributed over a given area (Bolton, 1980 and personal observations). *Myrmica rubra* (Formicidae, Myrmicinae), which is an allospecific intruder, is a polygynous and populous ant species (about 73 workers per queen and 1080 workers per colony) that is found in Europe, especially Northern Europe (Bernard, 1968).

In order to understand the basis of the discrimination process, we analyzed intraspecific and interspecific aggressiveness among different *T. bicarinatum* colonies and *M. rubra* colonies, and the cuticular hydrocarbon profiles of workers from each colony.

METHODS AND MATERIALS

Biological Material

The species studied, *T. bicarinatum* and *M. rubra*, were collected from different biotopes. Three colonies of *T. bicarinatum* were collected in Brazil: the Itabuna site (B1) is located 20 km from the Ilheus site (B2); both are 1000 km from the Crasto site (B3). Two colonies of *T. bicarinatum* were collected in Japan: the Kume site (J1) is an island 500 km from the Meijy site (J2). Two colonies of *M. rubra* were collected in France: the Gif-sur-Yvette site (F1) is 200 km from the Sainte Marie Dumont site (F2). The different *T. bicarinatum* colonies had 2–3 queens and 25–70 workers and brood, and the *M. rubra* colonies had 3–4 queens and 300–400 workers and brood (Table 1).

After collection, these mother colonies were reared in the laboratory in artificial plaster nests and maintained under standard conditions (23°C ± 2°C, 70% relative humidity, and a photoperiod of 10D : 14L). They were regularly fed with a honey–apple mixture and mealworms *ad libitum.*

Tolerance Tests

In order to analyze the degree of tolerance among individuals from the different colonies, we removed workers from the mother colonies. These ants were

TABLE 1. ORIGIN OF COLONIES

Ant species and colony symbol	Ants origin	Geographic area	Date collected
T. bicarinatum			
J1	Japan	Kume (Okinawa)	Sep 1991
J2	Japan	Meijy (Tokyo)	Oct 1996
B1	Brazil	Itabuna (Bahia)	Sep 1994
B2	Brazil	Ilheus (Bahia)	Sep 1994
B3	Brazil	Crasto (Sergipe)	Sep 1994
Myrmica rubra			
F1	France	Gif-sur-Yvette (Essonne)	Jun 1997
F2	France	Sainte Marie Dumont (Calvados)	Jun 1998

marked with a color dot on the abdomen (one color for each colony), and nestmates were placed in the same experimental vial (Falcon, 15 ml, 12 × 1.5 cm). The different vials were placed in a neutral arena (35 × 25 cm) equidistant from a central source of food and opened. Twenty-four hours later we recorded the numbers and the identities of dead workers, alive and uninjured workers, and injured and fighting workers. We also recorded their respective localization in the different vials and in the arena as a function of their origin.

Intraspecific Intercolonial Tolerance Tests. For *T. bicarinatum*, three vials were placed in the arena, the first containing 50 workers from J1, the second 50 workers from B1, and the third 50 workers from B2.

For *M. rubra*, two vials were placed in the arena, the first containing 50 workers from F1 and the second 50 workers from F2.

Interspecific Tolerance Test. One vial contained 50 *M. rubra* workers from F1 and the second 50 *T. bicarinatum* workers from B1.

Aggressiveness Tests

For each confrontation, six workers were removed from the mother colonies. An individual marked with a color dot on the abdomen served as the test ant. It was confronted with five other individual nestmates or nonnestmates in a neutral arena (Petri dish 5 cm in diameter).

The different types of confrontations are shown (Table 2): intracolonial intraspecific confrontations or control encounters (confrontations with conspecific ants belonging to the same nest); intercolonial intraspecific confrontations (confrontations between conspecifics taken from Brazilian and Japanese colonies of *T. bicarinatum* and between French colonies of *M. rubra*; and interspecific

TABLE 2. DIFFERENT TYPES OF ENCOUNTERS[a]

Intracolonial intraspecific	Intercolonial intraspecific	Interspecific
B1(B1)	B1(B2)-B2(B1)	B1(F1)-F1(B1)
B2(B2)	B1(B3)-B3(B1)	J1(F1)-F1(J1)
B3(B3)	B2(B3)-B3(B2)	
J1(J1)	B1(J1)-J1(B1)	
J2(J2)	B2(J1)-J1(B2)	
F1(F1)	B3(J1)-J1(B3)	
F2(F2)	B1(J2)-J2(B1)	
	J1(J2)-J2(J1)	
	F1(F2)-F2(F1)	

[a] (See Table 1 for the origin of colonies). The colony origin of the test ant (marked) is in parenthesis.

confrontations (confrontation between *T. bicarinatum* from Brazil or Japan with *M. rubra* allospecific individuals).

For 3 min (until the first physical contact with the marked ant), we recorded the reaction of the ants towards the marked ant according to the following index of aggression: 1 = inspection and antennal contact, 2 = threat as indicated by mandibular opening, 3 = biting, 4 = biting with stridulations, 5 = curling the abdomen in stinging attempts, 6 = stinging. The frequency and duration of each behavioral component were recorded by using a microcomputer (Psion event recorder, Aware, Paris, France) and the overall aggressiveness (AI) exhibited in each encounter was calculated as follows (Errard and Hefetz, 1997):

$$AI = \frac{\sum_{i=1}^{n} AI_i * t_i}{T}$$

where AI_i represents the index of aggression, t_i the duration of each act, and T the total interaction time.

We conducted 15 replications for each type of encounter. Individuals were tested only once in a given encounter to avoid the possible effects of familiarization. The results were analyzed by using ANOVA (Statistica for Windows 95).

Chemical Analysis

For extractions, 10 workers from each colony of both species (*T. bicarinatum* and *M. rubra*) were killed by freezing. Each ant's cuticular compounds were extracted by immersion in 2 ml of pentane for 10 min, and the total pentane extract was drawn off and evaporated. Eicosane (n-C_{20}) was used as an internal standard. Extracts were redissolved in 50 μl of pentane, of which 2 μl were injected into a HP 5890 series II Plus gas chromatograph with a flame ionization detector equipped with a capillary column (Chrompack CPSIL 5 WCOT, 25 m $\times$ 0.25 mm ID). The injector was of the split-splitless type, and the injection was performed for 15 sec in splitless mode. The carrying gas was helium at 1 bar, and the temperature was programmed from 100°C to 280°C at 5°C/min. The chromatograph was coupled to a HP computer equipped with a HP 5890 Chemstation that provided the data.

Cuticular washes from each group of 50 *T. bicarinatum* workers were pooled and analyzed by GC-MS on a Varian 3300 linked to a nermag R10-10C quadrupole mass spectrometer. Spectra were recorded in electron ionization mode at 70 eV with a mass range of 40–540 atomic mass unit (amu). The samples were run on an AT-5 (Alltech) capillary column (30 m $\times$ 0.32 mm ID, 0.3-μm film thickness, Ross injector heated at 280°C) whose temperature was programmed from 120°C to 300°C at 8°C/min. The carrier gas was helium at 1 bar.

For *M. rubra* species, we used previous identifications of cuticular hydrocarbons (Bagnères and Morgan, 1991; Vienne, 1993).

The identified peaks were recorded, numbered, and used for chemical analysis. For each cuticular profile, the relative value of each with respect to the total was calculated and expressed as a percentage.

We used a hierarchical cluster analysis (Ward's method, Euclidean distance, Statistica for Windows 95) to estimate the similarity (or the divergence) of the chemical profiles of the different colonies. The statistical analysis was performed on 39 extracts (27 *T. bicarinatum* extracts: 5 J1, 5 J2, 6 B1, 6 B2, 5 B3, and 12 *M. rubra* extracts: 5 F1, 7 F2) and 68 peaks.

RESULTS

Tolerance Tests

In the intraspecific tests with *T. bicarinatum* workers taken from the three colonies, J1, B1, and B2, we observed that, after 24 hr in the same area, all workers were alive and none was injured (Table 3). A new distribution of the ants was observed, some of them having moved from their vials and changed places with nonnestmates. Workers from different mother colonies seemed to mix without any aggressive behavior.

The results of the intraspecific tests with *M. rubra* workers taken from the two colonies, F1 and F2, are also depicted in Table 3. After 24 hr, we observed numerous

TABLE 3. TOLERANCE TESTS[a]

Tolerance test	Vial number	Number and origin of live workers in each vial ($t = 0$)	Number, origin and localization of workers after 24 hr			
			Dead	Wounded outside	Fighting	Alive
T. bicarinatum	Vial 1	50 J1	0 J1	0 J1	0 J1	17 ± 2 J1 18 ± 2 B1 15 ± 3 B2
	Vial 2	50 B1	0 B1	0 B1	0 B1	20 ± 8 J1 23 ± 5 B1 11 ± 5 B2
	Vial 3	50 B2	0 B2	0 B2	0 B2	13 ± 4 J1 9 ± 5 B1 24 ± 2 B2
M. rubra	Vial 1	50 F1	21 ± 9 F1	17 ± 5 F1	3 ± 3 F1	0 F1 10 ± 5 F2
	Vial 2	50 F2	19 ± 7 F2	17 ± 8 F2	3 ± 2 F2	0 F2 8 ± 5 F1

[a] Tolerance test between *T. bicarinatum* workers taken from three different colonies: B1 and B2 (Brazil) and J1 (Japan) (mean $\pm$ SEM, $N = 3$) and between *M. rubra* workers taken from two different colonies: F1 and F2 (France) (mean $\pm$ SEM, $N = 3$).

dead individuals, while the majority of live workers were outside the vials. In these experiments, nonnestmates never cohabited peacefully, as was observed in the case of *T. bicarinatum* species.

In the interspecific tests, all *M. rubra* workers were killed by *T. bicarinatum* workers while all *T. bicarinatum* workers were uninjured and alive.

Aggressiveness Tests (Table 2)

In all control encounters (intracolonial confrontations), the interactions among nestmate individuals were not aggressive. Each individual recognized and tolerated its marked nestmates. Aggressiveness indices revealed that the control encounters in the seven different experiments were not significantly different ($F = 4.48$; dl 5,89; *NS*). It also seems that the color dot we marked the ants with did not affect the behavior of the other ants (visual cues) (Figure 1).

In all intercolonial, intraspecific confrontations (Figure 1), *T. bicarinatum* workers did not react aggressively towards nonnestmates ($AI = 0.57 \pm 0.03$). The encounters among the ants originating from Brazil ($AI = 0.46 \pm 0.04$) were not different from the control encounters ($F = 24.07$; dl 4,80; *NS*). In encounters involving ants originating from Japan, J1 workers from Kume island were not aggressive towards J2 workers ($AI = 0.87 \pm 0.09$), but the duration of antennal interactions increased relative to control encounters ($F = 46.47$; dl 2,192; $P < 0.001$) and the encounters between Brazilian populations ($F = 34.61$; dl 1,108; $P < 0.001$).

In contrast, *M. rubra* presented intraspecific intercolonial aggressiveness with some biting behaviors ($AI = 2.89 \pm 0.17$), more than that noted in all intraspecific *T. bicarinatum* encounters ($F = 432.93$; dl 1,253; $P < 0.001$) as depicted in Figure 2.

Interspecific confrontations between Brazilian or Japanese *T. bicarinatum* individuals towards *M. rubra* workers were more aggressive (much biting, $AI = 3.12 \pm 0.10$) than *M. rubra* workers towards *T. bicarinatum* workers ($AI = 1.74 \pm 0.11$, $F = 75.46$; dl 1,57; $P < 0.001$) (Figure 2).

Myrmica rubra workers seemed more aggressive in intraspecific encounters (threatening behaviors) than in interspecific encounters towards *T. bicarinatum* from Brazil or Japan, towards which they only exhibited longer antennations ($F = 30.70$; dl 1,58; $P < 0.001$). In contrast, *T. bicarinatum* workers were far more aggressive towards *M. rubra* workers than towards alien *T. bicarinatum* workers, which were ignored ($F = 636.68$; dl 1,252; $P < 0.001$).

Chromatogram Analysis

Each colony possesses a specific chemical profile: 32 compounds for *T. bicarinatum* from Kume (J1) and Meijy (J2); 31 compounds for *T. bicarinatum*

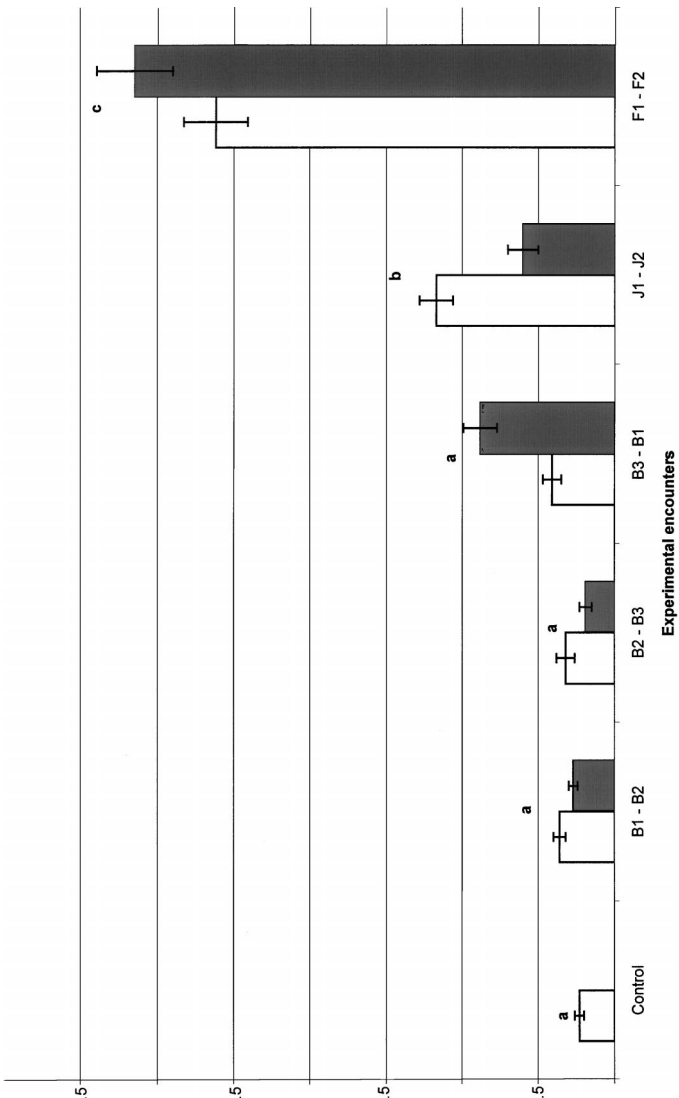


FIG. 1. Aggression of *T. bicarinatum* and *M. rubra* towards allocolonial intraspecific individuals according to the geographic area. *Tetramorium bicarinatum* encounters: B1–B2 (B1 towards B2 in white and B2 towards B1 in gray), and the *idem* for the other confrontations B2–B3, B1–B3, J1–J2. *Myrmica rubra* encounters: F1–F2 (F1 towards F2 in white and F2 toward F1 in gray). Different letters represent the groups, which differed significantly (ANOVA, $P < 0.001$, $N = 15$).

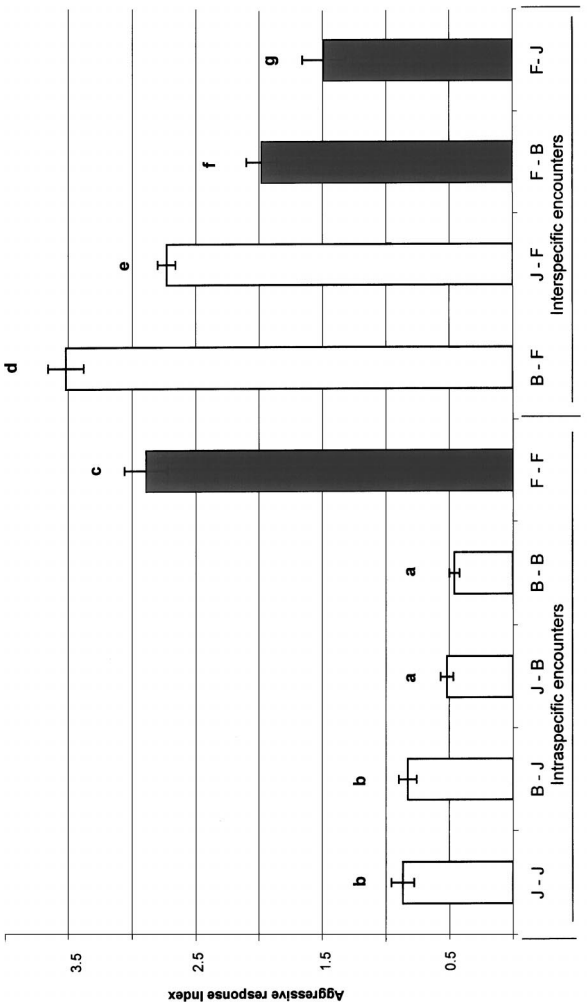


FIG. 2. Aggression of *T. bicarinatum* and *M. rubra* towards allocolonial conspecific individuals and towards allospecific individuals. Intraspecific encounters: J-J: intercolonial encounters between Japanese *T. bicarinatum* workers; B–J and J–.B: intercolonial encounters between Brazilian *T. bicarinatum* workers and Japanese *T. bicarinatum* workers and Brazilian *T. bicarinatum* workers; B–B. intercolonial encounters between Brazilian *T. bicarinatum* workers. F–F: intercolonial encounters between French *M. rubra*. Interspecific encounters: B–F and J–F (encounters between Brazilian and Japanese *T. bicarinatum* workers and *M. rubra* workers; F–B and F–J (encounters between *M. rubra* and Brazilian and Japanese *T. bicarinatum* workers. Different letters represent the groups, which differed significantly (ANOVA, $P < 0.001$, $N > 15$).

from Itabuna (B1), Ilheus (B2) and Crasto (B3); and 64 compounds for *M. rubra* from F1 and F2.

For *T. bicarinatum*, the most easily quantifiable hydrocarbons were (Table 4, Figure 3): 16 *n*-alkanes, (*n*-C_{13}–*n*-C_{15}, *n*-C_{17}, *n*-C_{19}–*n*-C_{30}), 18 mono- or di-alkenes

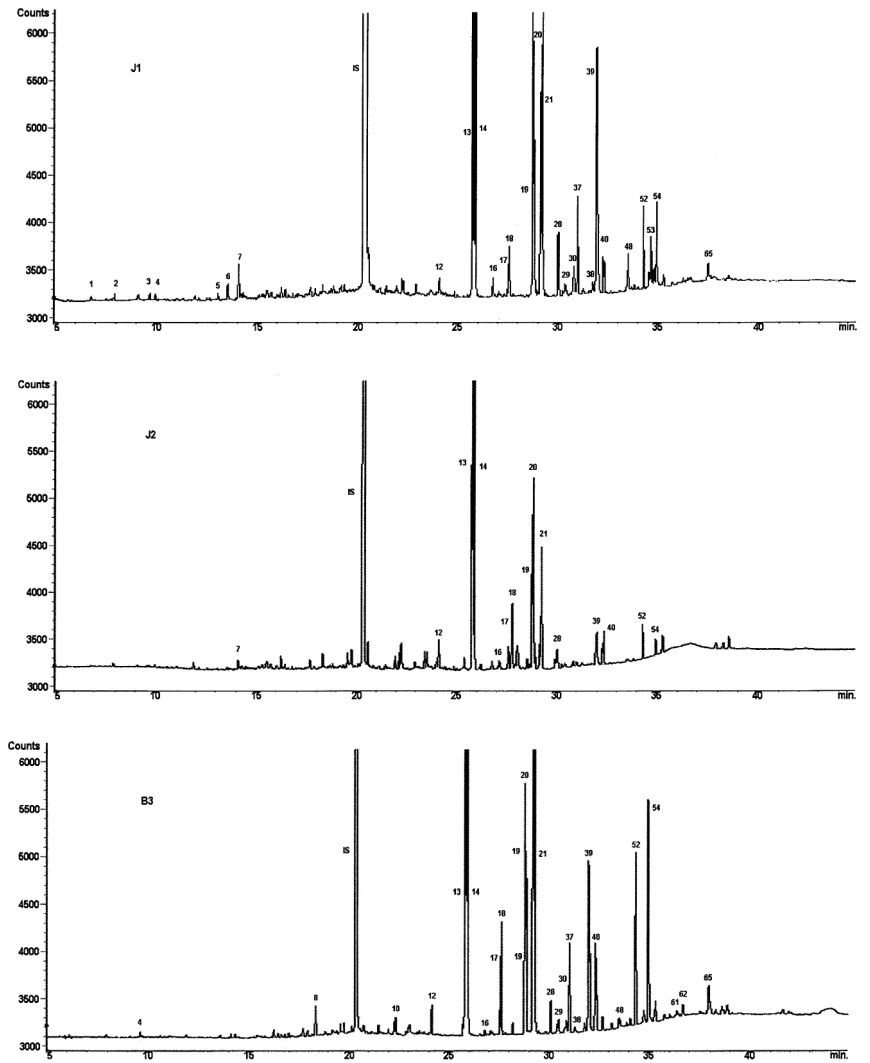


FIG. 3. Gas chromatograms of the cuticular hydrocarbon profiles of *T. bicarinatum* workers from colonies J1 (Kume, Japan), J2 (Meijy, Japan) and B3 (Crasto, Brazil).

TABLE 4. CHEMICAL IDENTITY OF MAJOR CUTICULAR HYDROCARBONS OF *Myrmica rubra* AND *Tetramorium bicarinatum* WORKERS[a]

		M. rubra		*T. bicarinatum*				
Peak	Compound	F1 (Essonne)	F2 (Calvados)	J1 (Kume)	J2 (Meijy)	B1 (Itabuna)	B2 (Ilheus)	B3 (Crasto)
1	*n*-C_{13}			0.220 ± 0.136	T			
2	*n*-C_{14}			0.264 ± 0.193	T			
3	*n*-$C_{15:1}$			0.268 ± 0.157	T	0.510 ± 0.117	T	T
4	*n*-C_{15}			4.610 ± 1.276	T	2.236 ± 1.571	0.265 ± 0.224	0.236 ± 0.1216
5	*n*-$C_{17:2}$			0.223 ± 0.123	T			
6	*n*-$C_{17:1}$			0.234 ± 0.187	T	0.582 ± 0.131	T	T
7	*n*-C_{17}			0.258 ± 0.230	0.168 ± 0.068	0.389 ± 0.120	T	T
8	*n*-C_{19}					T	T	0.184 ± 0.080
9	*n*-C_{20}					T	T	0.211 ± 0.080
10	*n*-C_{21}					T	T	0.188 ± 0.078
11	7 + 9 + 11Me-C_{21}	0.918 ± 0.501	0.601 ± 0.419					
12	*n*-C_{22}			3.833 ± 1.872	2.818 ± 0.249	0.707 ± 0.140	0.757 ± 0.062	0.832 ± 0.175
13	*n*-$C_{23:1}$			11.737 ± 0.244	25.248 ± 1.633	19.830 ± 2.229	16.446 ± 0.610	17.624 ± 1.948
14	*n*-C_{23}	1.102 ± 0.144	0.975 ± 0.112	21.410 ± 1.087	25.656 ± 1.950	22.256 ± 5.383	31.731 ± 1.383	31.384 ± 6.726
15	7 + 9 + 11Me-C_{23}	0.460 ± 0.177	0.509 ± 0.277					
16	*n*-$C_{24:2}$			0.368 ± 0.098	T	0.186 ± 0.067	T	0.116 ± 6.726
17	*n*-$C_{24:1}$			0.934 ± 0.187	0.928 ± 0.037	0.692 ± 0.098	0.949 ± 0.166	0.881 ± 0.105
18	*n*-C_{24}	0.973 ± 0.161	0.754 ± 0.179	2.108 ± 0,181	2.794 ± 1.891	1.893 ± 0.410	1.066 ± 0.131	1.212 ± 0.271
19	*n*-$C_{25:2}$			9.091 ± 1.257	8.480 ± 2.163	11.582 ± 1.541	10.053 ± 1.173	5.707 ± 1.272
20	*n*-$C_{25:1}$	3.227 ± 0.293	2.108 ± 0,240	8.589 ± 1.035	12.743 ± 1.855	12.502 ± 1.069	12.952 ± 0.718	11.429 ± 1.192
21	*n*-C_{25}	10.699 ± 0.695	8.246 ± 0.421	9.960 ± 0.481	8.028 ± 0.319	12.085 ± 1.315	10.658 ± 0.499	10.612 ± 0.859
22	9 + 11 + 13Me-C_{25}	2.909 ± 0.178	3.224 ± 0.096					
23	7Me-C_{25}	1.296 ± 0.160	1.742 ± 0.059					
24	5Me-C_{25}	1.756 ± 0.285	1.418 ± 0.068					
25	9,13DiMe-C_{25}	1.081 ± 0.262	0.937 ± 0.241					
26	3Me-C_{25}	0.169 ± 0.139	0.359 ± 0.170					
27	5,11DiMe-C_{25}	0.583 ± 0.152	0.884 ± 0.177					
28	*n*-$C_{26:2}$			1.294 ± 0,193	3.830 ± 1.261	T	T	0.858 ± 0.147
29	*n*-$C_{26:1}$			0.368 ± 0.095	T	T	T	0.197 ± 0.087
30	*n*-C_{26}	1.177 ± 0.102	1.122 ± 0.236	0.855 ± 0.224	T	0.398 ± 0.094	0.630 ± 0.087	0.702 ± 0.093

31	5,9,13TriMe-C_{25}	0.527 ± 0.263	0.619 ± 0.182					
32	10+12+14MeC_{26}	1.265 ± 0.292	1.833 ± 0.069					
33	6Me-C_{26}	1.032 ± 0.466	0.597 ± 0.115					
34	5Me-C_{26}	1.031 ± 0.734	0.546 ± 0.097					
35	10,12DiMe-C_{26}	0.100 ± 0.064	0.373 ± 0.127					
36	6,10DiMe-C_{26}	0.058 ± 0.040	0.232 ± 0.131					
37	unknown			2.770 ± 0.315	T	1.615 ± 0.104	2.507 ± 0.364	2.490 ± 0.469
38	*n*-$C_{27:2}$			1.093 ± 1.062	T	1.897 ± 0.186	1.831 ± 0.136	0.216 ± 0.150
39	*n*-$C_{27:1}$	3.716 ± 0.281	2.502 ± 0.158	7.129 ± 0.030	1.428 ± 0.116	2.273 ± 0.170	1.935 ± 0.120	3.346 ± 0.934
40	*n*-C_{27}	3.856 ± 0.388	2.834 ± 0.112	2.540 ± 0.051	2.158 ± 0.292	1.213 ± 0.115	2.525 ± 0.228	1.765 ± 0.278
41	9+11+13Me-C_{27}	19.414 ± 2.350	21.271 ± 1.264					
42	7Me-C_{27}	1.830 ± 0.239	1.887 ± 0.323					
43	5Me-C_{27}	4.563 ± 0.294	3.532 ± 0.165			T	T	0.588 ± 0.107
44	11,15DiMe-C_{27}	3.231 ± 0.415	3.251 ± 0.332					
45	3Me-C_{27}	2.965 ± 0.371	2.885 ± 0.194					
46	7,16DiMe-C_{27}	T	T					
47	5,11DiMe-C_{27}	3.455 ± 0.410	3.548 ± 0.280					
48	*n*-$C_{28:2}$			2.087 ± 1.091	T	4.397 ± 2.297	1.044 ± 0.238	1.561 ± 0.595
49	*n*-C_{28}	2.909 ± 0.357	1.313 ± 0.126	0.765 ± 0.179	T	T	T	0.244 ± 0.156
50	5,9,13TriMe-C_{27}	0.073 ± 0.073	0.205 ± 0.140					
51	10+12+14Me-C_{28}	2.502 ± 0.271	2.696 ± 0.196					
52	*n*-$C_{29:2}$			2.351 ± 0.523	2.872 ± 0.135	1.791 ± 0.183	3.519 ± 0.255	2.330 ± 0.234
53	*n*-$C_{29:1}$	0.171 ± 0.126	0.384 ± 0.105	1.077 ± 0.075	T	T	T	0.508 ± 0.165
54	*n*-C_{29}	1.279 ± 0.135	1.303 ± 0.164	1.548 ± 0.204	1.705 ± 0.162	0.963 ± 0.350	1.133 ± 0.098	2.357 ± 0.291
55	9+11+13+15Me-C_{29}	8.675 ± 0.330	9.439 ± 0.352					
56	7Me-C_{29}	0.586 ± 0.152	0.931 ± 0.176					
57	5Me-C_{29}	1.545 ± 0.203	1.296 ± 0.098					
58	13,17DiMe-C_{29}	1.759 ± 0.143	2.352 ± 0.171					
59	3Me-C_{29}	1.687 ± 0.508	1.532 ± 0.118					
60	5,11DiMe-C_{29}	1.627 ± 0.040	1.796 ± 0.095					
61	*n*-$C_{30:2}$			T	T	T	T	0.526 ± 0.156
62	*n*-C_{30}	0.213 ± 0.058	0.860 ± 0.266	T	T	T	T	0.275 ± 0.142
63	11+13+15Me-C_{30}	0.358 ± 0.156	0.896 ± 0.146					
64	5Me-C_{30}	0.543 ± 0.292	0.748 ± 0.188					
65	n-$C_{31:2}$ + n-$C_{31:1}$			1.454 ± 0.668	T	T	T	1.120 ± 0.174
66	11+13+15Me-C_{31}	1.379 ± 0.352	2.790 ± 0.496					
67	5Me-C_{31}	1.174 ± 0.116	1.937 ± 0.350					
68	13,17DiMe-C_{31}	0.128 ± 0.128	0.730 ± 0.460					

[a](Me, DiMe, and TriMe correspond to methyl, dimethyl and trimethyl, respectively). Values indicate, for each compound, the mean relative percentage (± SEM) for a 10-min extraction with pentane (T indicates trace). Values are calculated for F1 workers ($N = 5$), F2 workers ($N = 7$), J1 workers ($N = 5$), J2 workers ($N = 5$), B1 workers ($N = 6$), B2 workers ($N = 6$), and B3 workers ($N = 5$).

(*n*-$C_{15:1}$, *n*-$C_{17:2}$, *n*-$C_{17:1}$, *n*-$C_{23:1}$, *n*-$C_{24:2}$, *n*-$C_{24:1}$, *n*-$C_{25:2}$, *n*-$C_{25:1}$, *n*-$C_{26:2}$, *n*-$C_{26:1}$, *n*-$C_{27:2}$, *n*-$C_{27:1}$, *n*-$C_{28:2}$, *n*-$C_{29:2}$, *n*-$C_{29:1}$, *n*-$C_{30:2}$, *n*-$C_{31:2}$, *n*-$C_{31:1}$), and one methyl-alkane (5Me-C_{27}). Twenty-eight compounds are common to the different colonies of *T. bicarinatum* but *n*-C_{13}, *n*-C_{14}, *n*-$C_{15:1}$, and *n*-$C_{17:2}$ were specific to the Japanese colonies (J1 and J2), and *n*-C_{19}, *n*-C_{20}, *n*-C_{21}, 5Me-C_{27} were specific to the Brazilian colonies (B1, B2, B3).

For *M. rubra* species, the most quantifiable hydrocarbons were: 8 *n*-alkanes (*n*-C_{23}–*n*-C_{30}), 3 *n*-alkenes (*n*-$C_{25:1}$, *n*-$C_{27:1}$, *n*-$C_{29:1}$), 40 mono-methyl alkanes, 10 di-methyl alkanes, and 2 trimethyl alkanes (Table 4).

The cuticular hydrocarbon composition of the ants studied was species specific. The profiles of the five different colonies of *T. bicarinatum* appear similar, and they differ from the *M. rubra* profiles (F1 and F2) that possess 39 monomethyl, 10 dimethyl, 2 trimethyl alkanes *M. rubra*-specific compounds.

Hierarchical Cluster Analysis (HCA)

The schematic representation of the clustering obtained with the HCA algorithm on the mean chemical data of each colony (Figure 4A) shows the nodes separating into three groups. The first node (68.02) separates *T. bicarinatum* colonies (J1, J2, B1, B2, B3) from *M. rubra* colonies (F1, F2), and the second (15.97), which divides Japanese *T. bicarinatum* colonies (J1, J2) from Brazilian *T. bicarinatum* colonies (B1, B2, B3), is situated at a less significant level. This cluster analysis reflects the proximity between Brazilian and Japanese colonies.

From the detailed clustering obtained with the HCA algorithm on individual *T. bicarinatum* data (Figure 4B), two groups emerged (first node = 74.23), a group including Brazilian individuals (B1, B2 and B3) and a second including Japanese individuals. The second node (49.78) separates Japanese individuals from Kume (J1) from those from Meijy (J2), while the third node (35.83) divides Brazilian individuals from Crasto (B3) from those from Itabuna (B1) and Ilheus (B2). This cluster analysis reflects the geographical proximity of B1 and B2, both collected in Bahia state.

DISCUSSION

The behavioral and chemical results of this study corroborate the hypothesis that recognition processes are based on the discrimination of colonial odor. Our results show that *T. bicarinatum* societies are open, in the sense intended by Le Masne (1952), because workers exhibit no aggressive behavior towards alien conspecifics. In fact, intraspecific confrontations never exceed antennation behaviors, and nonnestmate conspecifics mix peacefully with different partners that have

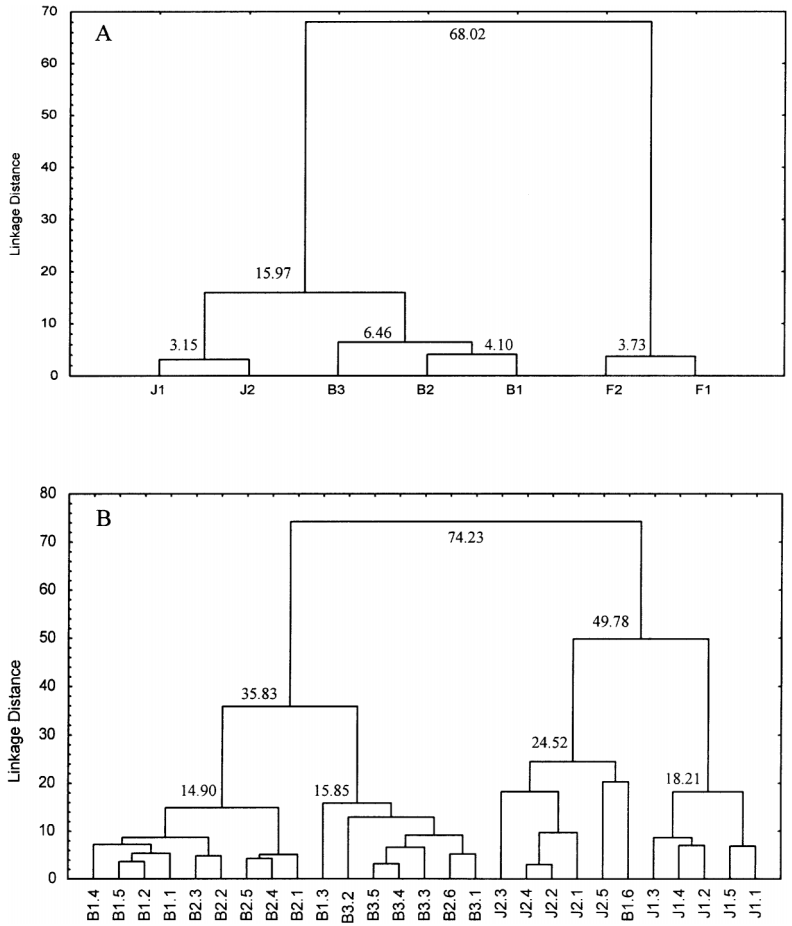


FIG. 4. Hierarchical cluster analysis (Ward's method, Euclidean distances) conducted on (A) the mean of the relative proportions of the 68 peaks of the workers cuticular profiles from the two colonies of *Myrmica rubra* (F1 and F2) and from the five colonies of *Tetramorium bicarinatum* from Japan (J1 and J2) and from Brazil (B1, B2 and B3); (B) the relative proportion of the 35 peaks in *T. bicarinatum* workers from Japan (J1: 1, 2, 3, 4, 5; J2: 1, 2, 3, 4, 5) and from Brazil (B1: 1, 2, 3, 4, 5, 6; B2: 1, 2, 3, 4, 5, 6; B3: 1, 2, 3, 4, 5).

numerous allogrooming and trophallaxis behaviors. In contrast, *M. rubra* societies appeared more discriminating in intraspecific confrontations that frequently ended in the death of individuals. *Myrmica rubra* interacted aggressively with conspecifics (coming from nests 200 km apart) through threatening and biting behaviors. These results confirm those by Cammaerts and Cammaerts (1984), who

showed that *M. rubra* alien queens are less accepted by workers, the more distant their nest.

Behavioral results demonstrating that *T. bicarinatum* workers do not act aggressively towards alien conspecifics support the observations by Provost and Cerdan (1990) on *Messor barbarus*, who showed that there was a positive correlation between the number of queens in a colony and its alien conspecific tolerance. Therefore, with high population levels (about 12,500 workers) and numerous queens (250–400 workers per queen) per colony, *T. bicarinatum* has the profile of a polygynous species, where there is no territorial defense and where alien conspecifics are not recognized as intruders (Vander Meer and Morel, 1998). In fact, the cuticular hydrocarbon profiles of *T. bicarinatum* from the different colonies reveal only a slight divergence, workers being tolerated and left alone by alien conspecific individuals. The longer antennal contacts observed in encounters involving Japanese workers from Kume Island are presumably due to the insularity of this colony. This has led to divergent cuticular hydrocarbon profiles. Similar results were observed by Dahbi and Lenoir (1998) in the ant *Cataglyphis iberica*. These authors showed that when workers were separated for three months (in groups with or without a queen), after regrouping, nestmate recognition remained unchanged but they observed longer mutual antennal contacts between previously separated workers without aggressive reactions. They concluded that this extension in antennal duration was probably due to the slight divergence observed in the cuticular chemical profiles of the workers during separation.

However, in spite of a lack of intraspecific aggressiveness, *T. bicarinatum* workers exhibit pronounced interspecific aggressiveness towards *M. rubra* workers. Since we have shown that the chemical cues of *T. bicarinatum* do not match the chemical cues of *M. rubra*, this suggests that *T. bicarinatum* workers are able to discriminate these chemical profile differences.

Since the cuticular profile of *M. rubra* is characterized by many branched alkanes (mono-, di-, and trimethyl alkanes), we suggest that colony recognition is strongly correlated to the composition of cuticular hydrocarbons, thus characterizing the chemical signature of *M. rubra*. This suggestion supports some previous studies. For example, several authors have shown that some methyl-branched hydrocarbons seem to be particularly involved in intracolonial recognition. In the social wasp *Polistes dominulus*, Dani et al. (1996) suggested that dimethyl-branched hydrocarbons seem to be important in distinguishing females according to their colony of origin. In *Polistes fuscatus*, behavioral and chemical analyses concluded that methyl-branched alkanes were relatively colony specific and that straight-chain alkanes were not (Gamboa et al., 1996). In the ant *Camponotus vagus*, Bonavita-Cougourdan et al. (1987) demonstrated that colony recognition was strongly correlated with the variation of relative proportions of dimethyl alkanes in different colonies. Provost et al. (1992) showed that in *Messor barbarus* the straight chain alkanes and certain dimethyl alkanes seem to be characteristic of the colony

signature. This, however, may not be the case for other ant genera. For example, the dimethyl alkanes present on the cuticular surface of several *Cataglyphis* ant species did not change drastically, showing that they do not have a communicative role, and, therefore, were not subjected to the presumed interspecific competition pressure (Dahbi et al., 1996).

Because these results suggest that branched hydrocarbons, particularly dimethyl alkanes, are important in distinguishing nestmates according to their colony, we hypothesize that the presence of these branched alkanes in the chemical signature of *M. rubra* could be implicated in discrimination by *T. bicarinatum.*

This hypothesis is reinforced by the results of encounters between *M. rubra* and *T. bicarinatum* that show that *M. rubra* workers discriminate conspecific alien individuals, while *T. bicarinatum* individuals induce only longer antennal contacts without any aggressive behaviors. As *T. bicarinatum* do not possess branched hydrocarbons, we suggest that *M. rubra* workers do not strongly attack *T. bicarinatum* workers because they do not recognize them as intruders. On the other hand, *M. rubra* discriminate alien conspecific workers whose chemical profile differed only quantitatively from their own.

Recently, it was experimentally demonstrated for the first time (Holway et al., 1998) that the lack of territoriality in the unicolonial Argentine ant (*Linepithema humile*) might account for their high population density. These authors demonstrated that a reduced intraspecific aggressiveness allows spatially separate colonies to fuse and achieve higher worker densities compared to colonies that exhibit intraspecific aggressiveness. They concluded that there is a relationship between reduced intraspecific aggressiveness and the concomitant abandonment of territorial behavior, unique to introduced populations of the Argentine ant, and that this contributes to the elevated population densities directly responsible for its widespread success as an invader. These authors proposed that the loss of intraspecific aggressiveness in this species might result either from the lack of stimuli due to the high degree of genetic relatedness among introduced colonies or from an innate loss of aggressiveness due to a break down in nestmate recognition.

We can surmise that *T. bicarinatum* species previously displayed intraspecific competition (possessing cuticular branched hydrocarbons like other Myrmicinae) and that this competitive ability has been lost during evolution, along with the branched alkanes. Two possible scenarios can be proposed. (1) The loss of intraspecific competition might have preceded the branched alkane loss. In this case, the chemicals have disappeared because they were not subjected to the presumed selective pressure. (2) The loss of branched alkanes might have preceded, leading to a lack of intraspecific competition, but with the alkanes being kept to prevent desiccation and regulate cuticular permeability. Whatever the case, *T. bicarinatum* has maintained the ability to discriminate cuticular branched hydrocarbons, as demonstrated by its aggressive behaviors towards *M. rubra* species.

Our study shows that *T. bicarinatum* workers exhibit a lack of territoriality against conspecifics over small and large spatial ranges (Brazilian and Japanese colonies). Moreover, in *T. bicarinatum*, the insignificant intraspecific aggressiveness seems to go along with strong interspecific competition, contributing to patterns of distribution and abundance in ant communities. In the same way, Human and Gordon (1999) showed that the aggressive behaviors of the Argentine ant may contribute to its competitive success against several native ants species, which tended to retreat more frequently. These behavioral characteristics of *T. bicarinatum* led us to confirm that this species is a highly successful tramp species (Bolton, 1980).

In his review, Passera (1994) concluded that tramp species are similar to polydomous ants with which they share the attributes of unicoloniality, colony reproduction by budding, or reduced nuptial flight, but that these attributes are more strongly expressed in tramp species. To these characteristics of polydomous species, tramp species add small size, monomorphism, and short queen longevity, so that finally only tramp species are truly domestic (dispersed throughout the world by human commerce and live in close association with humans), ensuring their ecological success.

Finally, if the lack of intraspecific aggressiveness is indeed due to the absence of branched alkanes, our results provide the chemical signature of an invasive species. This hypothetical additional character of tramp species could allow them to be better tolerated by other species, while remaining aggressive against all allospecific intruders, and could represent an important advantage for the invasive strategies these species are seen to deploy. If a lack of dimethyl alkanes contributes to the characteristics of a tramp species, surely other examples might also be available.

Acknowledgments—We are very grateful to Pierre Jaisson and Thibaut Monin for providing the ants. We would like to thank Alain Lenoir and Abraham Hefetz for proofreading the manuscript, and anonymous referees for constructive comments on this manuscript. We also thank Jerôme Orivel (Laboratoire d'Ethologie Expérimentale et Comparée, Université Paris Nord, F-93430 Villetaneuse) for the *M. rubra* GC analysis and Andrea Dejean for revising the English.

REFERENCES

BAGNÈRES, A. G., and MORGAN, E. D. 1991. The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 47:106–111.

BERNARD, F. 1968. Les Fourmis (Hymenoptera Formicidae) d'Europe occidentale et septentrionale. Faune de l'Europe et du bassin méditerranéen. Masson, Paris.

BLUM, M. S. 1987. Specificity of pheromonal signals: A search for its recognitive bases in terms of a unified chemiosociality, pp. 401–405, *in* J. Eder and H. Rembold (eds.), Chemistry and Biology of Social Insects. Peperny, Munich.

BOLTON, B. 1980. The ant tribe Tetramoriini. The genus *Tetramorium* Mayr in the Oriental and Indo-Australian regions, and in Australia. *Bull. Br. Mus.* 40(3):192–384.

BONAVITA-COUGOURDAN, A., CLÉMENT, J. L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus* Scop. *J. Entomol. Soc.* 22:1–10.

BONAVITA-COUGOURDAN, A., CLÉMENT, J. L., and POVEDA, A. 1990. Les hydrocarbures cuticulaires et les processus de reconnaissance chez les fourmis: Le code d'information complexe de *Camponotus vagus*. *Actes Colloq. Insectes Soc.* 6:273–280.

BREED, M. D. 1998. Chemical cues in kin recognition: criteria for identification, experimental approaches, and the honey bee as an example, pp. 57–77, *in* R. K. Vander Meer, M. D. Breed, K. E. Espelie, and M. L. Winston (eds.). Pheromone Communication in Social Insects. Westview Press, Boulder, Colorado.

BREED, M. D., and BENNETT, B. 1987. Kin recognition in highly eusocial insects, pp. 243–285, *in* D. J. C. Fletcher and C. D. Michener (eds.). Kin Recognition in Animals. John Wiley & Sons, New York.

CAMMAERTS, M. C., and CAMMAERTS, R. 1984. Adoption ou élimination de reines étrangères par les ouvrières de *Myrmica rubra* L. (Formicidae). Devenir de sociétés orphelines. *Ann. Sc. Nat. Zool. Paris* 6:207–220.

CARLIN, N. F., and HÖLLDOBLER, B. 1986. The kin recognition system of carpenter ants (*Camponotus* spp.) I. Hierarchical cues in small colonies. *Behav. Ecol. Sociobiol.* 19:123–134.

DAHBI, A., and LENOIR, A. 1998. Queen and colony odour in the multiple nest ant species, *Cataglyphis iberica* (Hymenoptera, Formicidae). *Insectes Soc.* 45:301–313.

DAHBI, A., LENOIR, A., TINAUT, A., TAGHIZADEH, T., FRANCKE, W., and HEFETZ, A. 1996. Chemistry of the postpharyngeal gland secretion and its implication for the phylogeny of Iberian *Cataglyphis* species (Hymenoptera: Formicidae). *Chemoecology* 7:163–171.

DANI, F. R., FRATINI, S., and TURILLAZZI, S. 1996. Behavioural evidence for the involvement Dufour's gland secretion in nestmate recognition in the social wasp *Polistes dominulus* (Hymenoptera: Vespidae). *Behav. Ecol. Sociobiol.* 38:311–319.

ERRARD, C., and HEFETZ, A. 1997. Label familiarity and discriminatory ability of ants reared in mixed groups. *Insectes Soc.* 44:189–198.

GAMBOA, G. J., REEVE, H. K., and HOLMES, W. G. 1991. Conceptual issues and methodology in kin-recognition research: A critical discussion. *Ethology* 88:109–127.

GAMBOA, G. J., GRUDZIEN, T. A., ESPELIE, K. A., and BURA, E. A. 1996. Kin recognition pheromones in social wasps: Combining chemical and behavioural evidence. *Anim. Behav.* 34:685–695.

HADLEY, N. F. 1980. Surface waxes and integumentary permeability. *Am. Sci.* 68:546–553.

HÖLLDOBLER, B., and MICHENER, C. D. 1980. Mechanisms of identification and discrimination in social Hymenoptera, pp. 35–38, *in* H. Markl (ed.). Evolution of Social Behaviour: Hypotheses and Empirical Tests. Verlag Chemie, GmbH. Weinheim.

HOLWAY, D. A., SUAREZ, A. V., and CASE, T. J. 1998. Loss of intraspecific in the success of a widespread invasive social insect. *Science* 282:949–952.

HOWARD, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp. 179–226, *in* D. W. Stanley-Samuelson and D. L. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

HOWSE, P. E. 1975. Chemical defenses of ants, termites and other insects: some outstanding questions, pp. 23–40, *in* C. Noirot, P. E. Howse, and G. Le Masne (eds.). Pheromones and Defensive Secretions in Social Insects. Congress IUSSI, Dijon, France.

HUMAN, K. G., and GORDON, D. M. 1999. Behavioral interactions of the invasive Argentine ant with native ant species, *Insectes Soc.* 46:159–163.

JUTSUM, A. R., SAUNDERS, T. S., and CHERRETT, J. M. 1979. Intraspecific aggression in the leaf-cutting ant *Acromyrmex octospinosus*. *Anim. Behav.* 26:1064–1071.

KELLER, L., and PASSERA, L. 1989. Influence of the number of queens on nestmate recognition and attractiveness of queens to workers in the Argentine ant *Iridomyrmex humilis* (Mayr). *Anim. Behav.* 37:733–740.

LAHAV, S., SOROKER, V., VANDER MEER, R. K., and HEFETZ, A. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften* 86:246–249.

LE MASNE, G. 1952. Classification et caractéristiques des principaux types de groupements sociaux réalisés chez les invertébrés, pp. 19–70, *in* CNRS (ed.). 34ème Colloq. Int. CNRS: Structure et Physiologie des Sociétés Animales. Paris.

LENOIR, A., FRESNEAU, D., ERRARD, C., and HEFETZ, A. 1999. Individuality and colonial identity in ants: the emergence of the social representation concept, pp. 219–237, *in* C. Detrain, J. L. Deneubourg, and J. M. Pasteels (eds.). Information Processing in Social Insects. Birkhauser, Basel.

MINTZER, A., and VINSON, S. B. 1985. Kinship and incompatibility between colonies of the Acacia ant *Pseudomyrmex ferruginea. Behav. Ecol. Sociobiol.* 17:75–78.

MOREL, L., VANDER MEER, R. K., and LOFGRENN, C. S. 1990. Comparison of nestmate recognition between monogyne and polygyne populations of *Solenopsis invicta* (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am.* 83:642–647.

OBIN, M. S., and VANDER MEER, R. K. 1988 Source of nestmate recognition cues in the imported fire ant *Solenopsis invicta* Buren. *Anim. Behav.* 36:1361–1371.

PASSERA, L. 1994. Characteristics of tramp species, pp. 23–41, *in* D. F. Williams (ed.). Exotic Ants. Biology, Impact, and Control of Introduced Species. Westview Press, Boulder, Colorado.

PROVOST, E., and CERDAN, P. 1990. Experimental polygyny and colony closure in the *Messor barbarus* L. *Behaviour* 115:114–126.

PROVOST, E., CERDAN, P., BAGNERES, A. G., MORGAN, E. D., and RIVIERE, G. 1992. Role of the queen in *Messor barbarus* colony signature, pp. 195–202, *in* J. Billen (ed.). Biology and Evolution of Social insects. Leuven University Press, Leuven, Belgium.

PROVOST, E., RIVIÈRE, G., ROUX, M., BAGNÈRES, A. G., and CLÉMENT, J. L. 1994. Cuticular hydrocarbons whereby *Messor barbarus* ant workers putatively discriminate between monogynous and polygynous colonies. Are workers labeled by queens? *J. Chem. Ecol.* 20:2985–3003.

VANDER MEER, R. K., and MOREL L. 1998. Nestmate recognition in ants, pp. 79–103, *in* R. K. Vander Meer, M. Breed, M. Winston, and K. E. Espelie (eds.). Pheromone Communication in Social Insects. Westview Press, Boulder, Colorado.

VANDER MEER, R. K., SALIWANCHIK, D., and LAVINE, B. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta.* Implications for Nestmate Recognition. *J. Chem. Ecol.* 15:2115–2125.

VIENNE, C. 1993. Organisation sociale et reconnaissance interindividuelle dans les colonies mixtes artificielles de fourmis. Doctoral Thesis, University Paris Nord, Villetaneuse, France.

WILSON, E. O. 1971. The Insect Societies. Harvard University Press, Cambridge, Massachusetts.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

AVOIDANCE OF CONSPECIFIC INJURY-RELEASED CHEMICAL CUES BY FREE-RANGING *Gammarus lacustris* (CRUSTACEA: AMPHIPODA)

BRIAN D. WISENDEN,* SCOTT G. POHLMAN, and ERIN E. WATKIN

Department of Biology
Minnesota State University Moorhead
Moorhead, Minnesota 56563

(Received August 28, 2000; accepted February 13, 2001)

Abstract—Behavioral responses to chemical cues have been demonstrated for a range of aquatic animals. Injury-released chemical alarm cues from conspecifics are released when a prey's predator is actively foraging. Detection of these cues elicits antipredator behaviors that reduce the probability of predation. Amphipod crustaceans in the genus *Gammarus* are widespread denizens of ponds and streams. Antipredator responses by *Gammarus* to conspecific alarm cues, and subsequent reduction of predation risk, are known from experiments in the laboratory. Here, we verify laboratory findings by demonstrating an avoidance response to alarm cues using a field population of *G. lacustris.* We used small traps baited with sponge blocks containing either water (control) or injury-released cues from *Gammarus.* We repeated the experiment twice. In both experiments, significantly fewer *Gammarus* were captured in traps with alarm cue sponges than in traps with water sponges. Predatory leeches *Dina parva* were attracted to *Gammarus* traps in the first experiment but not the second experiment. In the second experiment, we measured the individual weight of captured amphipods. Two size classes were present; small (1–5 mg) and large (35–108 mg). Both sizes contributed to the avoidance response. Within the large size class, small individuals were significantly less responsive to the alarm cue than large individuals, implying that small adult *Gammarus* may have different cost/benefit decision criteria for risk assessment than large *Gammarus.*

Key Words—*Gammarus*, amphipod, crustacea, alarm cue, alarm signal, predator–prey, avoidance behavior, leech, *Dina parva.*

*To whom correspondence should be addressed. e-mail: wisenden@mnstate.edu

0098-0331/01/0600-1249$19.50/0

INTRODUCTION

Predator–prey interactions in aquatic environments are mediated, in part, by chemical cues released from various sources including disturbed and/or injured prey, and predators (Smith, 1992; Chivers and Smith, 1998; Kats and Dill, 1998; Tollrain and Havell, 1999; Wisenden 2000). Injury-released chemical alarm cues are released after a successful predatory attack and indicate imminent and proximate danger to conspecifics of the prey (see Chivers and Smith, 1998, for review). Aquatic taxa ranging from protozoa to amphibia use alarm cues to detect and avoid predators. Most of these studies have been conducted in the laboratory setting. While laboratory studies provide a carefully controlled environment in which to test specific hypotheses, the results lack the realism of the natural habitat. Field verification of laboratory findings is an important part of the scientific study of chemical ecology.

Amphipods in the genus *Gammarus* occur commonly in aquatic environments. They are small (<2 cm) detritivores that associate closely with the substratum and fall prey to a wide variety of predators. The substratum is often highly structured and water clarity often limited, reducing the reliability of visual information in assessing predation risk. *Gammarus* are well suited for the study of chemically mediated predator–prey interactions because they can be studied in the laboratory and in the field.

Laboratory populations of *Gammarus* show antipredator behavior in response to injury-released alarm cues of conspecifics (Williams and Moore, 1985; Mathis and Hoback, 1997; Wudkevich et al., 1997). In a laboratory study, latency to first capture by predatory green sunfish *Lepomis cyanellus* was significantly longer for *Gammarus* exposed to conspecific alarm cues than *Gammarus* exposed to water or heterospecific alarm cues (Wisenden et al., 1999).

It has not yet been established that free-living *Gammarus* respond with antipredator behavior to conspecific alarm cues in their natural habitat. We tested a field population of *Gammarus* for an avoidance response to alarm cues modeled after similar experiments on small fishes (e.g., Mathis and Smith, 1992; Chivers and Smith, 1994; Wisenden et al., 1995). Small sponge blocks soaked in water (control) or injury-released alarm cues of *Gammarus* were placed in small invertebrate traps. If field *Gammarus* avoid alarm cues, then we predicted that traps with alarm cues would catch fewer *Gammarus* than traps with water.

Recognition of conspecific alarm cues as indicators of risk generally occurs at a very early stage of development and requires no previous experience (Pfeiffer, 1963; Waldman, 1982; Magurran, 1989). However, there is some evidence from fishes that large individuals avoid alarm cues more effectively than small individuals (Mathis and Smith, 1992; Chivers and Smith, 1994; Chivers et al., 1995). Large, old individuals are more experienced than small, young individuals and may improve or acquire recognition of danger cues with age, particularly for responses to heterospecific alarm cues (Chivers et al., 1995). Alternatively, larger individuals

may be more vulnerable to predation and, thus, exhibit a lower response threshold than small individuals (Mathis and Hoback, 1997).

To investigate the effect of size on recognition of conspecific alarm cues we weighed captured *Gammarus.* We predicted that if aversion of alarm cue was acquired with age, or if cost/benefit decision criteria change with size, then large *Gammarus* would be caught in alarm cue traps in lower proportions than small *Gammarus.*

METHODS AND MATERIALS

Experiment 1. Alarm cue was prepared from adult *Gammarus lacustris* sampled from Erhard Pond in fall of 1997, located approximately 70 km SE of Moorhead, Minnesota, USA (46°30′N, 96°05′W). Erhard Pond does not contain any fish species. A stock solution of alarm cues was prepared be reducing 15 adult *Gammarus* (mean weight ± SE = 52.1 ± 1.6 mg, $N = 15$) to a fine pulp with mortar and pestle and diluting to a final volume of 37.5 ml with dechlorinated tap water. Small blocks of cellulose sponge (2 × 2 × 1.5 cm) each received 1.5 ml of the stock solution of alarm cues. This method of stimulus preparation is similar to the one successfully used by Wudkevich et al. (1997) and Wisenden et al. (1999). We thus prepared 15 sponges of *Gammarus* alarm cue and froze them at −20°C. Fifteen additional sponge blocks were soaked in 2.5 ml of dechlorinated tap water to control for the effect of sponge and water and then frozen at −20°C. Sponges were kept on ice during transport to the study site and remained frozen until used in the experiment to guard against any degradation of the cue.

We placed 30 invertebrate traps in Erhard Pond approximately 5 m apart along the shore, at a depth of about 0.5 m. The traps consisted of a 1-q (946-ml) wide-mouth Mason jar fitted snugly with a plastic funnel inserted into the jar opening with the apex of the funnel directed inward. The funnel was held in place by rubber bands attached to hooks on the outside of the funnel and the jar. Traps were laid on their side on the pond bottom. Inside each jar we placed a sponge soaked in either alarm cue or water. Traps were set in pairs (one control trap and one experimental trap simultaneously) at 5-min intervals, then pulled exactly 1 hr later at 5-min intervals in such a way that time in the water was held constant and equal among trap pairs and sponge types. Trap contents were stored and returned to the laboratory for counting.

Experiment 2. We repeated the experiment in spring 2000 at the same study site using similar methods. Adult *Gammarus* to be used for alarm stimulus were collected a few days prior to the field test and brought to the laboratory. Some of the adults were coupled in precopulatory amplexus. For each sponge (3.5 × 3 × 2 cm), we individually crushed a male–female pair (mean ± SE combined weight of each male–female pair = 176.9 ± 4.2 mg; $N = 17$) to a fine pulp with a mortar and pestle and diluted to a final volume of 10 ml with dechlorinated

water. Thus, stimulus strength in the second experiment was about four times stronger than in the first experiment. We prepared each of 17 sponges with 10 ml of alarm cue and another 17 sponges each with 10 ml of dechlorinated water (control). All sponge blocks were frozen at -20°C, and transported to the study site on ice to keep them frozen until needed.

Thirty-four traps were placed in the littoral zone within 1 m from the water's edge along the shoreline of Erhard Pond at approximately 5-m intervals. The traps used in the second experiment were commercial traps (MT3 minnow traps, Aquatic Ecosystems Inc.) and differed slightly from those used in the first experiment. The commercial traps consisted of 1-q (946-ml) wide-mouth Mason jars with inverted plastic funnels adapted to thread directly onto the jar mouth. The traps were laid on their side on the pond bottom as in the first experiment. Each trap contained one sponge block (control or alarm cue) and was set and retrieved in control–alarm pairs at 5-min intervals as for the first experiment. Time in the water was exactly 1 hr. Trap contents were returned to the laboratory to be counted and weighed to the nearest milligram. Large *Gammarus* were weighed individually. *Gammarus* <5 mg were too small to weigh individually. Small *Gammarus* for each trap were combined and weighed en masse to determine average weight.

Statistical Analysis. Statistical tests for avoidance behavior are one-tailed because we predicted a priori that *Gammarus* would avoid conspecific alarm cue. All other statistical tests are two-tailed.

RESULTS

Experiment 1. The median number (and 25th percentiles) of *Gammarus* per trap was 90 (50–117.5) and 40 (30–66.5) for control and alarm cue traps, respectively (Figure 1). Significantly more *Gammarus* were captured in control traps than alarm cue traps (Wilcoxon Mann Whitney test: $z = 1.95$, $P < 0.026$). These data are consistent with an avoidance response to conspecific alarm cues.

Leeches *Dina parva* entered traps with *Gammarus* alarm cue significantly more frequently than control traps (Figure 2). There was a median (and 25th percentiles) of 0 (0–1) and 2 (0.5–3) leeches in control and alarm cue traps, respectively (Wilcoxon Mann Whitney test: $z = 2.57$, $P = 0.025$). Five of 15 control traps caught at least one leech, whereas 11 of 15 alarm cue traps caught at least one leech (Figure 3). There was no correlation between number of *Gammarus* and number of leeches per trap for all traps combined ($R^2 = 0.0003$, $F = 0.007$, $P = 0.931$), within alarm cue traps ($R^2 = 0.0022$, $F = 0.029$, $P = 0.866$) or within control traps ($R^2 = 0.0007$, $F = 0.008$, $P = 0.927$).

Experiment 2. The median number (and 25th percentiles) of *Gammarus* per trap was 6 (5–9) and 3 (2–5) for control and alarm traps, respectively (Figure 4). Significantly more *Gammarus* were captured in control traps than in alarm cue traps (Wilcoxon Mann Whitney test: $z = 2.84$, $P = 0.002$). These data, collected

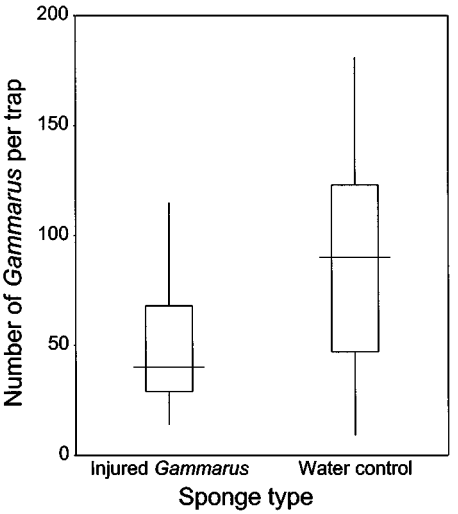


FIG. 1. Box and whisker plot of median, 25th percentiles, and range of number of *Gammarus* caught in the first experiment. Traps were baited with sponges containing either *Gammarus* injury-released alarm cues or water (control).

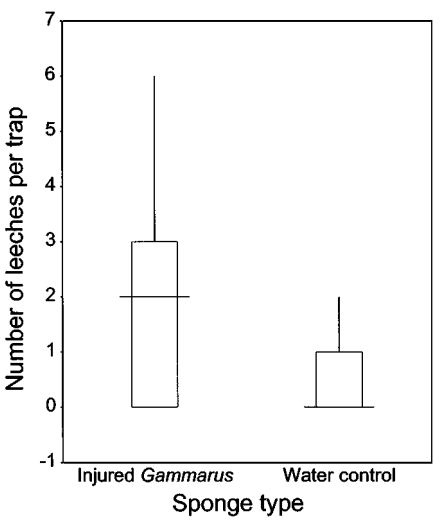


FIG. 2. Box and whisker plot of median, 25th percentiles, and range of the number of leeches caught in the first experiment. Traps were baited with sponges containing either *Gammarus* injury-released alarm cues or water (control).

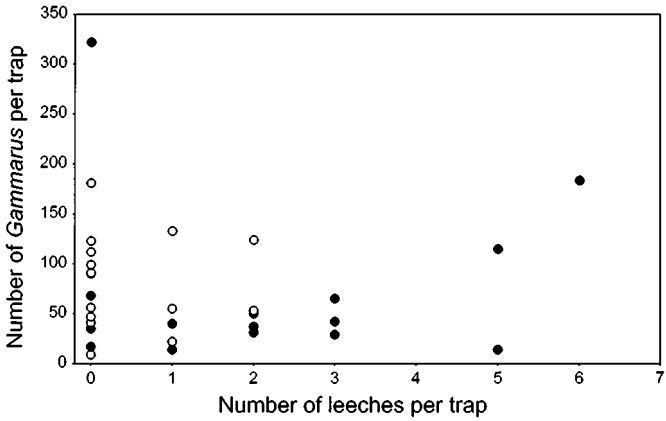


FIG. 3. Number of *Gammarus* per trap plotted against the number of leeches per trap in the first experiment. Open symbols, traps with water sponges; closed symbols, traps with alarm cue sponges.

at a different time of year and with slightly different gear and stimulus strength, corroborate data from the first experiment. Only four of 17 alarm cue traps and three of 17 control traps caught leeches. Because the majority of traps caught no leeches, the median (±25th percentiles) number of leeches entering both trap types was 0 (0–0) (Wilcoxon Mann Whitney test: $z = 1.29$, $P = 0.197$).

There were two distinct size classes in the catch (Figure 4). Small (1–5 mg) and large (35–108 mg) *Gammarus* both contributed to the overall avoidance response.

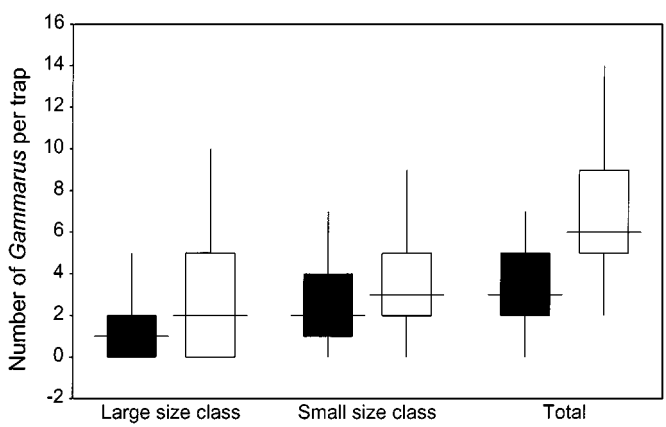


FIG. 4. Box and whisker plot of median, 25 percentiles and range of the number of *Gammarus* per trap in the second experiment. The large size class ($N = 72$) ranged from 32 to 108 mg, the small size class ($N = 104$) ranged from an average of 1 to 5 mg. Solid bars, alarm cue traps; open bars, control traps.

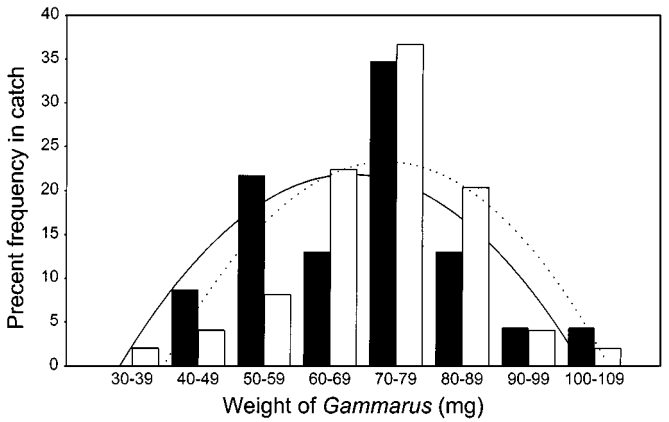


FIG. 5. Percent of the catch in each trap type in the second experiment represented by the large size class of *Gammarus* ranging from 32 to 108 mg. Fitted lines are second-order polynomial functions. Alarm cue traps, solid bars and solid line; control traps, open bars and dashed line.

However, the effect of alarm cue was not significant within the small size class (Wilcoxon Mann Whitney test: $z = 1.09$, $P = 0.276$) or (barely) the large size class (Wilcoxon Mann Whitney test: $z = 1.95$, $P = 0.051$) partly due to the low overall numbers of animals captured in the second experiment.

Within the large age class, small individuals did not avoid traps containing alarm cue as much as large individuals (Figure 5). We divided the size range of *Gammarus* into equal thirds creating small (32–57.3 mg) and large (82.7–108 mg) size categories with the adult size class. Small *Gammarus* represented 30.4% of the individuals caught in alarm cue traps, whereas small *Gammarus* represented only 10.2% of the catch in control traps ($\chi^2 = 4.44$, $P < 0.05$). Large *Gammarus* represented 17.4% and 20.4% of catches in alarm cue and control traps, respectively ($\chi^2 = 0.09$, $P > 0.9$). Similar analysis of length distribution of the small size class (<5 mg) was precluded by the low accuracy of weight measurements at the limit of the range of our electronic balance. We do not have individual weights for small *Gammarus.*

DISCUSSION

These data provide an important verification of laboratory findings. Field populations of *Gammarus lacustris* avoided injury-released conspecific alarm cues in two separate experiments at different times of year and by using slightly different methods. Chemical alarm cues serve as an important indicator of predation risk

for *Gammarus* in the field, and presumably antipredator behavioral responses such as avoidance behavior serve to decrease the probability of predation (Hews, 1988; Mathis and Smith, 1993; Wisenden et al., 1999).

Responses by very small *Gammarus* contributed to the overall avoidance response, suggesting that recognition of conspecific injury-released cues as indicators of predation risk is either innate or acquired at a very early stage of development. Innate responses to conspecific alarm cues are thought to be the case for various fish species (Pfeiffer, 1963; Waldman, 1982; Magurran, 1989), but to our knowledge this is the first such evidence for an aquatic arthropod.

Within the adult size class of *Gammarus*, small individuals were disproportionately represented in alarm cue traps. We do not interpret this to indicate acquired recognition of conspecific alarm cues over time and experience (cf. Chivers et al., 1995) because very small juvenile *Gammarus* responded to the alarm cue.

Large *Gammarus* may respond most strongly to conspecific alarm cues because they have a greater cost–benefit trade-off than small *Gammarus*. Large adult *Gammarus* are more responsive to risk of fish predation than small adult *Gammarus* (Mathis and Hoback, 1997). Although fish predators are absent from our study site, a variety of waterfowl use the site and presumably prey on *Gammarus*. Largex *Gammarus* would be easier to detect by tactile probing of the vegetation and provide more energy per unit of foraging time.

Alternatively, cost–benefit trade-offs in risk assessment could depend on foraging opportunities for individuals at varying levels of social hierarchy, competitive ability, or somatic resources. Trade-offs between risk and foraging are well known (e.g., Milinski and Heller, 1978; Milinski, 1985; Godin and Sproul, 1988; Lima and Dill, 1990). Hungry (Smith, 1981; Brown and Smith, 1996), and low condition (Wisenden, Rush, and Sargent unpublished) fish do not respond to conspecific alarm cues with an overt behavioral response. Small *Gammarus* may tolerate more predation risk while foraging because of subordinate status or weak competitive abilities. Social interactions, dominance hierarchies, and territorial behavior among *Gammarus* are not well understood with respect to risk avoidance.

Leeches are significant predators of *Gammarus* and detect prey by chemical and tactile means (Dahl and Greenberg, 1997; Dahl, 1998). Leeches were attracted to traps with injury-released *Gammarus* cues, leaving open the possibility that *Gammarus* avoided alarm cue traps because of the presence of leeches rather than recognition of alarm cues per se. However, there was no correlation between the number of leeches and the number of *Gammarus* caught per trap. Moreover, leeches were attracted to the *Gammarus* cue in the first experiment only, and not in the second experiment, where cue concentration was four times higher. Thus, *Gammarus* aversion to the alarm cue in the first experiment was likely attributable to the alarm cue and not leeches.

Ostariophysan fishes possess specialized epidermal cells that contain an alarm pheromone. This pheromone is released when the skin is damaged; it signals alarm

to conspecifics and also serves as an attractant to predators (Mathis et al., 1995). Attracting predators can benefit the individual sending the signal because secondary predators attempt piracy or cannibalism that afford the prey an opportunity for escape (Chivers et al., 1996). The predator attraction hypothesis is the only mechanism for the evolution of ostariophysan alarm substance cells to receive experimental support. Leech attraction to injury-released cues of *Gammarus* raises the possibility that a similar alarm signal/predator attractant cue may be released from injured *Gammarus.* Leeches do not engulf their prey. Presumably, there is the potential for large amounts of internal fluids from *Gammarus* to be released in the process of being consumed by a leech, inadvertently informing other *Gammarus* of predation risk, but also attracting other leeches. Although special glands or cells that may contain a specialized pheromone are not known for *Gammarus*, chemical deterrents are known to occur in a number of aquatic arthropods in the insect orders Coleoptera and Hemiptera (Scrimshaw and Kerfoot, 1987). Further study is required to determine if *Gammarus* possess specialized structures and the nature of competitive foraging among two or more leeches.

In addition to verifying laboratory results and revealing potential size-dependent behavioral decision criteria, these data establish a convenient field technique for further testing the role of chemical cues in mediating predator prey interactions in aquatic invertebrates. It is our hope that future work will be stimulated in this direction.

Acknowledgments—We thank the Kevin Brennan of the U.S. Fish and Wildlife Service for permission to use Erhard Pond as a study site for this project. Mac Butler and Kyle Zimmer kindly loaned us the traps used in the first experiment. Funding for this research was provided by Minnesota State University Moorhead.

REFERENCES

BROWN, G. E., and SMITH, R. J. F. 1996. Foraging trade-offs in fathead minnows (*Pimephales promelas*): Acquired predator recognition in the absence of an alarm reaction. *Ethology* 102:776–785.

CHIVERS, D. P., and SMITH, R. J. F. 1994. Intra- and interspecific avoidance of areas marked with skin extract from brook sticklebacks (*Culaea inconstans*) in a natural habitat. *J. Chem. Ecol.* 20:1517–1524.

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator/prey systems: A review and prospectus. *Ecoscience* 5:338–352.

CHIVERS, D. P., BROWN, G. E., and SMITH, R. J. F. 1996. The evolution of chemical alarm signals: attracting predators benefits signal senders. *Am. Nat.* 148:649–659.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, R. J. F. 1995. The role of experience in the response of fathead minnows (*Pimephales promelas*) to skin extract of Iowa darters (*Ethoestoma exile*). *Behaviour* 132:665–674.

DAHL, J. 1998. The impact of vertebrate and invertebrate predators on a stream benthic community. *Oecologia* 117:217–226.

DAHL, J., and GREENBERG, L. 1997. Foraging rates of a vertebrate and an invertebrate predator in stream enclosures. *Oikos* 78:459–466.

GODIN, J.-G. J., and SPROUL, C. D. 1988. Risk taking in parasitized sticklebacks under threat of predation: Effects of energetic need and food availability. *Can. J. Zool.* 66:2360–2367.

HEWS, D. K. 1988. Alarm response in larval western toads, *Bufo boreas*: Release of larval chemical by a natural predator and its effect on predator capture efficiency. *Anim. Behav.* 36:125–133.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Ecoscience* 5:361–394.

LIMA, S. L., and DILL, L. M. 1990. Behavioural decisions made under the risk of predation: A review and prospectus. *Can. J. Zool.* 68:610–640.

MAGURRAN, A. E. 1989. Acquired recognition of predator odour in the European minnow (*Phoxinus phoxinus*). *Ethology* 82:216–223.

MATHIS, A., and HOBACK, W. W. 1997. The influence of chemical stimuli from predators on precopulatory pairing by the amphipod, *G. pulex pseudolimnaeus. Ethology* 103:33–40.

MATHIS, A., and SMITH, R. J. F. 1992. Avoidance of areas marked with a chemical alarm substance by fathead minnows (*Pimephales promelas*) in a natural habitat. *Can. J. Zool.* 70:1473–1476.

MATHIS, A., and SMITH, R. J. F. 1993. Chemical alarm signals increase the survival time of fathead minnows (*Pimephales promelas*) during encounters with northern pike (*Esox lucius*). *Behav. Ecol.* 4:260–265.

MATHIS, A., CHIVERS, D. P., and SMITH, R. J. F. 1995. Chemical alarm signals: Predator-deterrents or predator-attractants? *Am. Nat.* 145:994–1005.

MILINSKI, M. 1985. Risk of predation of parasitized sticklebacks (*Gasterosteus aculeatus*) under competition for food. *Behaviour* 93:203–216.

MILINSKI, M., and HELLER, R. 1978. Influence of a predator on the optimal foraging behaviour of sticklebacks (*Gasterosteus aculeatus*). *Nature* 275:642–644.

PFEIFFER, W. 1963. Alarm substances. *Experientia* 19:113–168.

SCRIMSHAW, S., and KERFOOT, W. C. 1987. Chemical defenses of freshwater organisms: Beetles and bugs, pp. 240–262, *in* W. C. Kerfoot, and A. Sih, (eds.). Predation: Direct and Indirect Impacts on Aquatic Communities. University Press of New England, Hanover, New Hampshire.

SMITH, R. J. F. 1981. Effect of food deprivation on the reaction of Iowa darters (*Etheostoma exile*) to skin extract. *Can. J. Zool.* 59:558–560.

SMITH, R. J. F. 1992. Alarm signals in fishes. *Rev. Fish Biol. Fish.* 2:33–63.

TOLLRIAN, R., and HAVELL, C. D. 1999. The evolution of inducible defenses: current ideas, pp. 306–321, *in* R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible Defenses. Princeton University Press, Princeton, New Jersey.

WALDMAN, B. 1982. Quantitative and developmental analyis of the alarm reaction in the zebra danio, *Brachydanio rerio. Copeia* 1982:1–9.

WILLIAMS, D. D., and MOORE, K. A. 1985. The role of semiochemicals in benthic community relationships of the lotic amphipod *G. pulex pseudolimnaeus:* A laboratory analysis. *Oikos* 44:280–286.

WISENDEN, B. D. 2000. Scents of danger: The evolution of olfactory ornamentation in chemically mediated predator–prey interactions, pp. 365–386, *in* Y. Espmark, T. Admundsen and G. Rosenqvist (eds.). Signalling and Signal Design in Animal Communication. Tapir Publishers, Trondheim, Norway.

WISENDEN, B. D., CHIVERS, D. P., BROWN, G. E., and SMITH, R. J. F. 1995. The role of experience in risk assessment: Avoidance of areas chemically labelled with fathead minnow alarm pheromone by conspecifics and heterospecifics. *Ecoscience* 2:116–122.

WISENDEN, B. D., CLINE, A., and SPARKES, T. C. 1999. Survival benefit to antipredator behavior in *Gammarus minus* (Crustacea: Amphipoda) in response to injury-released chemical cues from conspecifics and heterospecifics. *Ethology* 105:407–414.

WUDKEVICH, K., WISENDEN, B. D., CHIVERS, D. P., and SMITH, R. J. F. 1997. Reactions of *Gammarus lacustris* to chemical stimuli from natural predators and injured conspecifics. *J. Chem. Ecol.* 23:1163–1173.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

MAMMALIAN EXOCRINE SECRETIONS XV. CONSTITUENTS OF SECRETION OF VENTRAL GLAND OF MALE DWARF HAMSTER, *Phodopus sungorus sungorus*

B. V. BURGER,[1,*] D. SMIT,[1] H. S. C. SPIES,[1] C. SCHMIDT,[2] U. SCHMIDT,[2] A. Y. TELITSINA,[3] and G. R. GRIERSON[4]

[1]*Laboratory for Ecological Chemistry*
Department of Chemistry, University of Stellenbosch
Stellenbosch 7600, South Africa

[2]*Zoological Institute, University of Bonn*
Poppelsdorfer Schloss, D-53115 Bonn, Germany

[3]*Institute of Animal Evolutionary Morphology and Ecology*
Russian Academy of Sciences
Leninsky 33, Moscow 117071, Russia

[4]*Rothmans International*
Stellenbosch 7600, South Africa

(Received January 3, 2000; accepted January 30, 2001)

Abstract—In a study aimed at the chemical characterization of constituents of the ventral gland secretion of the male dwarf hamster, *Phodopus sungorus sungorus*, 48 compounds, including saturated alcohols, saturated and unsaturated ketones, saturated and unsaturated straight-chain carboxylic acids, iso- and anteisocarboxylic acids, 3-phenylpropanoic acid, hydroxyesters, 2-piperidone, and some steroids were identified in the secretion. The position of the double bonds in γ-icosadienyl-γ-butyrolactone and γ-henicosadienyl-γ-butyrolactone, and the position of methylbranching in seven C_{16}–C_{21} saturated ketones could not be established. Several constituents with typically steroidal mass spectra also remained unidentified. The female dwarf hamster's ventral gland either does not produce secretion or produced so little secretion that it was impossible to collect enough material for analysis.

Key Words—*Phodopus sungorus sungorus*, dwarf hamster, mammalian semiochemicals, mammalian pheromones, exocrine secretion, ventral gland secretion.

*To whom correspondence should be addressed.

INTRODUCTION

The dwarf hamster, *Phodopus sungorus*, also known as the Djungarian striped or hairy-footed hamster, is a small rodent found on different types of steppes characterized by extreme temperature differences such as the dry steppes of Mongolia, Western Siberia, where air temperatures vary between $+30^{\circ}$C in summer and -40°C in winter (Heldmaier, 1975), and in the eastern parts of the Lake Baikal region (Pogosianz and Sokova, 1967), as well as on the steppes of Kazakhstan, Manchuria, and North China (Wynne-Edwards and Lisk, 1984). The dwarf hamster is unusually resistant to low temperatures (Veselofsky and Grundova, 1965). It was found (Heldmaier, 1975) that they tolerated severe cold stress, but were less able to withstand heat.

Phodopus belongs to the family Cricetinae, of which they are one of the smallest species with a body length between 67 and 102 mm (Flint, 1966). Two subspecies of the dwarf hamster are mentioned in the literature, namely *P. s. sungorus* (Pallas), which is found in the western regions of their habitat, and the eastern subspecies *P. s. campbelli* (Thomas). However, the majority of authors make no distinction between the subspecies. A few minor differences between the subspecies have been reported. *P. s. campbelli* is gregarious (Wynne-Edwards and Lisk, 1984), while *P. s. sungorus* appears to be more solitary (Reasner and Johnston, 1988). The seasonal pelage color change from dark grey in summer to white in winter is, for example, also quite pronounced in *P. s. sungorus* (Heldmaier, 1975).

Although their cold resistance is noteworthy, the most unusual characteristic of these hamsters is their highly photoperiodic nature. It seems that rather than hibernate, they have adapted strategies for survival of severe winter conditions by making use of the extreme seasonal changes in photoperiod, which are characteristic of their natural habitat. During short photoperiods ($\leq$8 hr of light), as experienced in the Siberian winter, a reduction in body weight occurs, pelage color changes dramatically, male testicle regression occurs and reproductive ability is diminished (Hoffmann, 1973), sexual development is slowed down (Brackmann, 1977; Yellon and Goldman, 1984), and a reduction in the ventral gland size is also observed (Sunderkötter et al., 1990).

According to Heisler (1984), the olfactory marking behavior in this hamster includes marking with feces, urine, and secretions from the ventral gland, as well as sand-bathing behavior, in which the animal rolls on its back, not necessarily only in sand. The marking behavior of both males and females in neutral areas, in areas where males had previously marked, and in areas where females had previously marked was investigated. The marking frequency was found to be low in both sexes and lower in females than in males. The hamsters were observed to mark the periphery of the test area more frequently than they marked in the nonperipheral areas, and while the males did not mark more frequently in an area that had already

been marked by a male, they did show an increased marking frequency in areas previously marked by females.

Feoktistova (1994) investigated the behavioral responses of adult, sexually experienced males of *P. sungorus* toward different olfactory cues, such as urine, bedding material, and integumentary skin gland secretions of conspecific males and diestrous females. Habituation studies showed that males were able to discriminate between the odors of males and females. The odors of urine, bedding material, and the secretion of the sacculi, located at the opening of the cheek pouches of conspecific males, elicited a high level of midventral gland marking, while the same odors of females resulted in anogenital marking. The low population density, nocturnal activity, and arid habitat of the dwarf hamster are expected to favor marking activity in these animals. *P. s. sungorus* has well developed glands, of which the ventral sebaceous gland (*glandula abdominales*) located along the axis body line before the genitals, is one of the most important. The gland becomes visible at an age of about 4 weeks, and it functions only during the reproductive period. The hamsters use the secretion of this gland for marking their home ranges.

In contrast to the large volume of literature on olfaction in other rodent species, no comprehensive chemical investigation of the constituents of the secretions of the dwarf hamster has so far been undertaken. The aim of the present study is to initiate chemical investigations into the nature of the exocrine secretions in hamsters, specifically in *P. s. sungorus*, in order to provide information that could lead to new avenues of investigation of chemical communication systems in hamsters. It is hoped, for example, that the compounds found in the secretions will generate ideas as to their possible origin and/or purpose, and will enable biologists to use them in behavioral studies. Using compounds present in the hamster secretions in research on rodent olfactory receptors and behavior, makes much more sense than using arbitrarily selected compounds totally unrelated to substances involved in the semiochemical communication of these animals.

METHODS AND MATERIALS

General. Dichloromethane (1 ml) (Merck, residue analysis grade), was concentrated to 10 μl, analyzed for impurities, and found to be pure enough for extraction of small quantities of semiochemicals. Syringes were cleaned with this solvent. Glassware used for the collection and extraction of samples was washed, rinsed with distilled water, heated in an annealing oven at 500°C for at least 30 min, and cooled immediately prior to use to remove all traces of adsorbed organic material.

Analytical Methods. Gas chromatographic analyses were carried out with a Carlo Erba 5300 gas chromatograph equipped with a flame ionization detector, Grob split-splitless injector, and glass columns manufactured by the Laboratory for Ecological Chemistry, University of Stellenbosch. Glass capillary columns, coated

with a 0.25-μm film of the apolar stationary phase PS-089-OH, which is a silanol-terminated 95% dimethyl–5%-diphenyl siloxane copolymer, were used throughout the study. Helium was used as carrier gas at a linear velocity of 28.6 cm/sec at 40°C. The detector and injector were used at 280°C and 220°C, respectively. Samples were injected in the split mode (split ratio 6 : 1) and an oven temperature of approximately 30°C. The oven was heated ballistically to 40°C and programmed at 2°/min from 40° to 260°C (hold). Gas chromatographic retention time comparison of the constituents of the secretion with the commercially available and synthesized reference compounds was done by coinjection of the reference compound and glandular extract to determine whether the component under investigation was enriched by the reference compound.

Quantitative gas chromatographic analyses were done with the same instrument and column, and data acquisition with Borwin Intuitive Chromatography Software (JMBS Developments, 38600 Fontaine, France) using hexadecanoic acid as external standard. Ventral gland secretion (4.8 mg) was collected, extracted with dichloromethane as described below, and the extract concentrated to 20 μl of which 1-μl quantities were injected for analysis.

A Carlo Erba QMD 1000 GC-MS system was used to record electron impact (EI) mass spectra (70 eV) employing the GC parameters specified above. The interface temperature was maintained at 250°C. The ion source temperature was 200°C, and the source pressure varied between *ca.* 2×10^{-5} torr at a column temperature of 40°C, decreasing to 1×10^{-5} torr towards the end of the temperature program at a column temperature of 260°C. A scan rate of 0.9 scan/sec, with an interval of 0.1 sec between scans, was employed. Chemical ionization (CI) mass spectra with methane as reactant gas were recorded on a Finnigan 4500 quadrupole GC-MS system. The interface temperature was 220°C, and spectra were recorded at 60 eV and at a source temperature of 150°C. Under these conditions the pressure in the source was approximately 7×10^{-5} torr. Spectra were recorded at a rate of 2 scans/sec. Accurate mass measurements of the synthetic compounds were done on an AMD 604 magnetic sector mass spectrometer. The spectra were recorded at an ionization potential of 70.7 eV and a source temperature of 192°C. The resolving power was 4000, and a scan rate of 1 scan/sec was used, with a recovery time of 0.5 sec. A sampling frequency of 100 kHz was used, and the source vacuum was 1.4×10^{-5} torr. In the case of samples introduced on the probe, the probe was heated from 20° to 350°C.

^{1}H and ^{13}C NMR spectra of synthesized compounds were obtained on a Varian VXR-300 spectrometer at 299.9 MHz and 75.42 MHz, respectively. The ^{13}C spectra were obtained by using a 45° pulse angle and a pulse repetition time of 0.8 sec. Chloroform-d_1 (Merck, 99.8% isotopic purity) was used as solvent and tetramethylsilane (TMS) as internal reference.

Determination of Double Bond Positions of Constituents. Dimethyl disulfide (DMDS) derivatives were prepared from the unsaturated constituents of

the secretion according to the procedure described by Vincenti et al. (1987). A dichloromethane extract of the secretion was concentrated in a Reacti-Vial using a slow stream of purified nitrogen. The residual material was dissolved in carbon disulfide (50 μl) and treated with an excess (5-μl) of a solution of iodine in ether (60 mg/ml) and 50 μl of dimethyl disulfide. The Reacti-Vial was sealed and the reaction mixture heated in an oven at 60°C for 40 hr; then the iodine was reduced with aqueous sodium thiosulfate solution (5%). Separation of the aqueous and organic layers was facilitated by centrifuging the reaction mixture at 3000 rpm. The organic layer was transferred to another vial and concentrated to 5 μl for GC-MS analysis.

Sample Collection and Preparation. Djungarian dwarf hamsters, *P. s. sungorus*, were bred from stock obtained from a breeding colony of the Zoological Institute at the University of Bonn. All adult animals were kept indoors in separate wire cages (36 × 25 × 23 cm) with an exercise wheel, except when males and females were put together for breeding purposes. The animals were kept at a constant temperature of 22°C in a laboratory free from artificial odors. To simulate summer conditions, the animals were exposed to natural light during daytime (Stellenbosch 33°50′ southern latitude) and, in addition, fluorescent lights were left switched on permanently resulting in a minimum light intensity of 200 lux during the nighttime. Each hamster was supplied with half a coconut shell that served as a hut into which it could withdraw into almost total darkness. To avoid contamination of the secretions with plant volatiles, shavings from old, dry poplar logs were provided for bedding material. When breeding, animals were also provided with cellulose tissues. All bedding and cellulose materials were renewed at weekly intervals. All hamsters were provided *ad libitum* with water and food containing sunflower seeds. Twice a week they were supplied with slices of apple, carrot, and lettuce. No problems were experienced with maintaining and breeding the hamsters under these conditions.

Teflon stoppers and lengths of stainless-steel wire (15 × 0.4 mm) were washed with detergent solution; rinsed with distilled water, methanol, and dichloromethane, and dried in an oven at 110°C. Using precleaned tweezers, the stainless-steel wires were inserted into the tapered end of the Teflon stoppers. The other ends of each of the wires were bent to form a small eyelet having a diameter of approximately 1 mm with which the secretion could be removed from the gland. Ventral gland secretion was collected individually from mature males in their summer state on a weekly basis. Although a hairless secretory area was clearly visible on females in this state, they did not produce enough substance for collection. The waxy secretion was collected by scooping it from the glandular area with a precleaned stainless-steel eyelet. The Teflon stoppers were inserted into the flared ends of small glass vials to provide an air-tight stopper for the vials The samples were stored at −20°C for future use.

For analysis, initially the collected secretion was scraped off the wire loop and transferred to a Reacti-Vial containing dichloromethane. However, a substantial

proportion of the sample was lost in this process. To minimize the loss of sample, the stainless steel eyelet containing the sample was therefore cut off and allowed to fall into a Reacti-Vial containing 30 μl of dichloromethane. The material was sonicated for 20 min, and then the light brown, turbid extract was centrifuged at 2500 rpm for 15 min in order to separate the extract into a clear dichloromethane extract and a turbid supernatant layer. The bottom layer was removed from underneath the supernatant layer with a 100-μl syringe and transferred to a clean Reacti-Vial in which the extract was concentrated in an inert atmosphere to a concentration suitable for GC and GC-MS analysis.

Reference Compounds. In addition to authentic commercially available reference compounds, the following compounds were synthesized for retention time and mass spectral comparison with constituents of the ventral secretion.

2-Heptadecanol (**17**); Figure 1 was prepared by $NaBH_4$ reduction of 2-heptadecanone. HR-MS: *m/z* $(M\text{-}H_2O)^+$ 238.267, calcd. for $C_{17}H_{34}$ 238.266. ^{13}C NMR ($CDCl_3$): $\delta = 68.20$ (d, C-2), 39.41 (t, C-3), 31.95 (t, C-15), 29.4–29.8 (t, C-6–C-14), 29.38 (t, C-5), 25.80 (t, C-4), 23.49 (q, C-1), 22.71 (t, C-16), 14.12 (q, C-17).

(*Z*)-6-Heptadecen-2-one, (*Z*)-8-heptadecen-2-one (**13**), (*Z*)-6-nonadecen-2-one, (*Z*)-8-nonadecen-2-one (**28**), (*Z*)-10-nonadecen-2-one, (*Z*)-12-nonadecen-2-one (**29**), and their *E* isomers were prepared by alkylation of ethyl acetoacetate with the appropriate (*E*)- and (*Z*)-alkenyl bromides, hydrolysis of the condensation product, and decarboxylation of the resulting β-ketoacid according to the following procedure described for the preparation of (*Z*)-8-nonadecen-2-one.

A solution of tetrabromomethane (41.5 mg, 0.125 mmol) in dry acetonitrile (230 μl) was added to a solution of (*Z*)-5-hexadecen-1-ol (30 mg, 0.125 mmol) (Pherobank, Wageningen, The Netherlands) and triphenylphosphine (32.8 mg, 0.125 mmol) in acetonitrile (230 μl) in a 3-ml Reacti-Vial at room temperature and the reaction mixture stirred magnetically at room temperature for 24 hr. The resulting bromide and traces of unchanged starting compounds were extracted with *n*-pentane from the acetonitrile by magnetically stirring the reaction mixture with the *n*-pentane (300 μl), centrifuging the mixture at 1000 rpm for a few minutes and removing the supernatant pentane layer with a 500-μl syringe. The extraction was repeated five times. The reaction product was purified by bulb-to-bulb distillation to give (*Z*)-1-bromohexadec-5-ene, 36 mg (95%) bp 102 (air bath)/7.5×10^{-4} mm Hg. The alkenyl bromide, containing less than 0.1% of the unchanged 5-hexadecen-1-ol as the only impurity (GC-MS), was used to alkylate ethyl acetoacetate. Ethyl acetoacetate (1.35 g, 10.4 mmol) was deprotonated by adding it to a solution of sodium ethoxide (10.4 mmol) in absolute ethanol (50 ml). The resulting anion was alkylated by magnetically stirring a sample (600 μl) of this solution in a 3-ml Reacti-Vial with (*Z*)-1-bromohexadec-5-ene (36 mg, 0.119 mmol) at 90°C for 11 hr during which time a precipitate of NaBr was formed. The ethanol was evaporated at room temperature by using a slow stream of purified

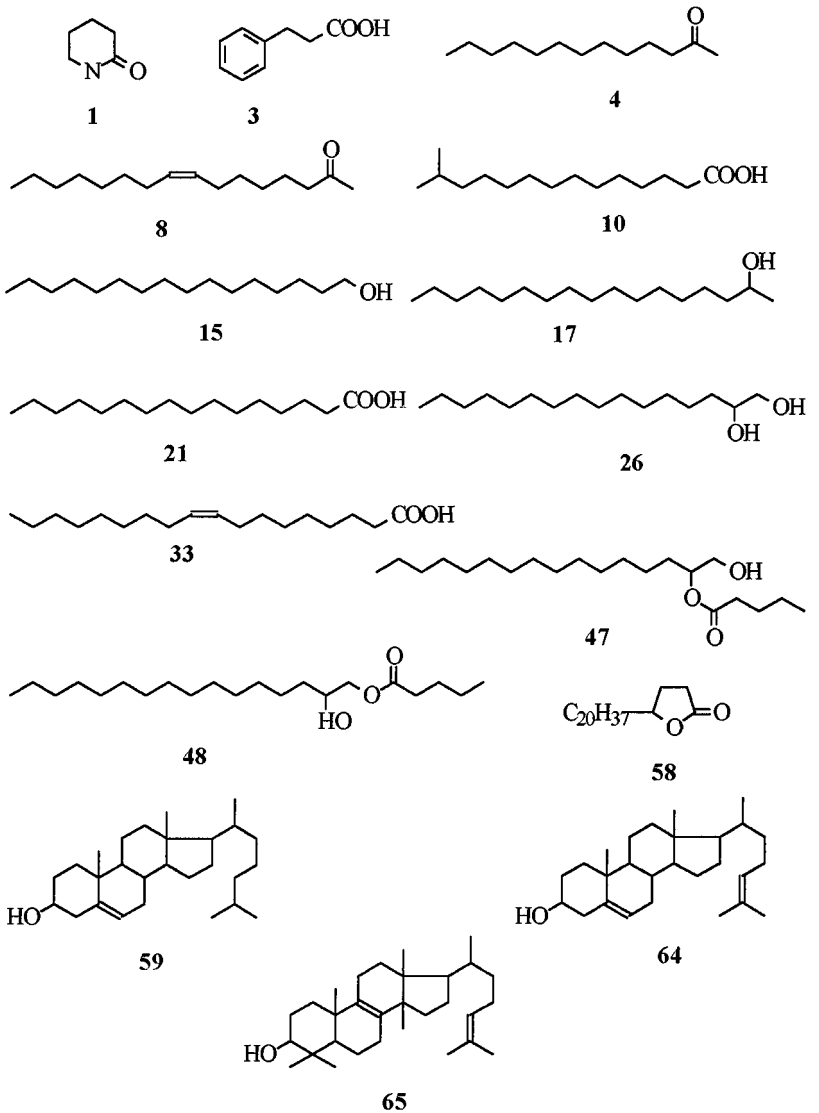


FIG. 1. Structures of representative examples of the compounds identified in the ventral secretion of the male dwarf hamster, *Phodopus sungorus sungorus.*

argon, after which the ethyl ester was hydrolyzed by stirring the residue in the Reacti-Vial with NaOH solution (1.5 M, 200 μl, 0.3 mmol) at room temperature for 24 hr. The unsaponified material and unchanged bromide were extracted twice with *n*-pentane (300 μl) by stirring the reaction product with the solvent, centrifuging the mixture at 1000 rpm, and removing the supernatant organic layer with a 500-μl syringe. The saponified material was acidified with H_2SO_4 (1.5 M) and the resulting ketoacid decarboxylated by heating the reaction mixture at 90°C until no further formation of gas bubbles in the Reacti-Vial (closed with a screw cap) could be observed. The organic material was extracted three times with *n*-pentane and washed free from H_2SO_4 with distilled water. Slow evaporation of the solvent gave (*Z*)-8-nonadecen-2-one (**28**) as a colorless oil (3.8 mg) containing 2.5% of the *E* isomer (GC-MS). ^{13}C NMR ($CDCl_3$): $\delta = 209.24$ (s, C-2), 130.22 (d, C-9), 129.48 (d, C-8), 43.78 (t, C-3), 31.93 (t, C-17), 29.85 (q, C-1), 29.77–28.83 (t, C-5, C-6, C-11–C-16) 27.25 (t, C-7)*, 27.03 (t, C-10)*, 23.79 (t, C-4), 22.69 (t, C-18), 14.11 (q, C-19) (* assignments interchangeable).

1-Hydroxyhexadec-2-yl pentanoate (**47**) and 2-Hydroxyhexadec-1-yl pentanoate (**48**) were prepared by the Al_2O_3-catalyzed reaction of 1,2-epoxyhexadecane with pentanoic acid as described by Burger et al. (1999a). The two isomers were formed in a ratio of 3 : 2. HR-MS (mixture of the two isomers): *m/z* M^+ 342.309, calcd. for $C_{21}H_{42}O_3$ 342.313. *Major component*: ^{13}C NMR ($CDCl_3$): $\delta = 174.37$(s, C-1), 75.50 (d, C-2′), 64.99 (t, C-1′), 34.30 (t, C-2), 31.95 (t, C-14′), 30.56 (t, C-3′), 29.4–29.7 (t, C-5′–C-13′), 27.16 (t, C-3), 25.33 (t, C-4′) 22.72 (t, C-15′), 22.28 (t, C-4), 14.13 (q, C-16′), 13.72 (q, C-5). *Minor component*: ^{13}C NMR ($CDCl_3$): $\delta = 174.05$ (s, C-1), 70.10 (d, C-2′), 68.58 (t, C-1′), 33.96 (t, C-2), 33.40 (t, C-3′), 31.95 (t, C-14′), 29.4–29.7 (t, C-5′–C-12′), 29.38 (t, C-13′), 27.05 (t, C-3), 25.39 (t, C-4′), 22.72 (t, C-15′), 22.28 (t, C-4), 14.13 (q, C-16′), 13.72 (q, C-5).

2-Piperidone (**1**). Cyclopentanone oxime was dried over phosphorus pentoxide and subjected to Beckmann rearrangement in concentrated H_2SO_4 to give 2-piperidone (δ-valerolactam) (Becker et al., 1973). HR-MS: *m/z* M^+ 99.066, calcd. for C_5H_9NO 99.068. ^{13}C NMR ($CDCl_3$): $\delta = 172.65$ (s, C-1), 42.20 (t, C-5), 31.51 (t, C-2), 22.28 (t, C-4), 20.88 (t, C-3).

RESULTS AND DISCUSSION

A typical total ion chromatogram of an extract of the ventral secretion of a male dwarf hamster is given in Figure 2 and the compounds identified in the secretion are listed in Table 1. Mass spectral data are given for only a few typical examples of the different compound classes present in the secretion, because the spectra of compounds within each class are quite similar and the spectral data of these typically long-chain compounds contain relatively little structural information. The structures of representative examples of each compound type are given

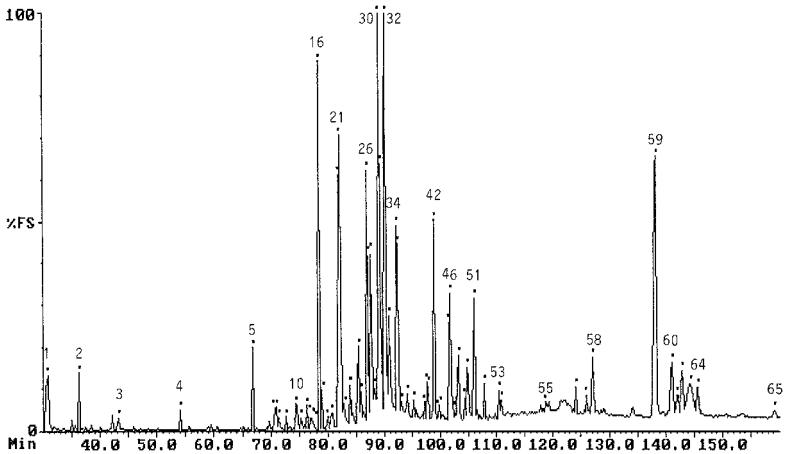


FIG. 2. Total ion chromatogram of an extract of the ventral secretion of a male dwarf hamster, *Phodopus sungorus sungorus.*

in Figure 1. Tentative identification of the constituents of the secretion was based on low-resolution EI mass spectra and CI mass spectral information obtained with methane as reactant gas. Final confirmation of many of the proposed structures was obtained by co-injection of an extract of the secretion with authentic synthetic material.

It is not possible to determine unequivocally the position of double bonds in unsaturated ketones from the low-resolution mass spectral data of their dimethyl disulfide (DMDS) adducts, because one CO and two CH_2 groups are isobaric, so that the DMDS derivative of one alken-2-one can give rise to two diagnostic ions with the same nominal masses (although having different accurate masses) as those from another isomeric DMDS derivative. Both the *E* and *Z* isomers of each of the pairs of alken-2-ones had therefore to be synthesized for retention-time comparison. There appeared to be two series of unsaturated ketones present in the secretion, one with the double bond in the 8 position and another one with the double bond equidistant from the methyl terminal end of the ketones. Although the position of the double bond of the unsaturated ketone **19** could not be determined, it is quite likely that it belongs to the first of these series and could therefore be (*Z*)-8-octadecen-2-one.

Although several presumably methyl-branched 2-alkanones having spectra similar to those of the unbranched ketones are present in the secretion, these compounds are present in such low concentrations that it was impossible to determine the position of the methyl branching from the available mass spectral data. The molecular masses of some of these ketones could be established from

TABLE 1. COMPOUNDS IDENTIFIED IN VENTRAL SECRETION OF DWARF HAMSTER

Peak (Figure 1)	Compound	EI Mass spectral data, *m/z* (%)	Quantity (ng/animal)
15	1-Hexadecanol[a,c]		0.04
17	2-Heptadecanol[a–c]		0.3
31	2-Nonadecanol[a–c]	266(0.4), 238(0.5), 227(0.4), 181(0.5), 168(0.5), 154(0.8), 140(1), 125(1), 111(7), 97(18), 83(19), 7(15), 69(18), 57(33), 55(30), 45(100), 43(47)	0.5
43	2-Henicosanol[a,d]		0.05
26	Hexadecane-1,2-diol[a–c]	227(15), 125(7), 117(6), 111(24), 97(58), 83(73), 71(38), 69(75), 61(22), 57(82), 55(88), 43(100), 41(75)	1.3
32	Heptadecane-1,2-diol[a–c]		7.1
4	2-Tridecanone[a,b,c,e]		0.1
5	2-Pentadecanone[a–d]		0.4
9	2-Hexadecanone[a–d]		0.05
16	2-Heptadecanone[a–d]	254(2), 239(1), 196(2), 194(2), 152(1), 138(1), 127(3), 96(7), 85(15), 71(44), 59(68), 58(100), 55(25), 43(97), 41(30)	0.2
23	2-Octadecanone[a,b,d,e]		0.3
30	2-Nonadecanone[a,d]		4.9
37	2-Icosanone[a,b,d]		0.1
42	2-Henicosanone[a,b,c]		1.2
52	2-Tricosanone[a,b,d]		0.2
6	Branched 2-hexadecanone[a,b,d]		0.1
12	Branched 2-heptadecanone[a,b,d]		0.2
20	Branched 2-octadecanone[a,b,d]		1.0
27	Branched 2-nonadecanone[a,b,d]	282(1), 224(0.1), 220(1), 96(5), 85(10), 71(38), 59(72), 58(100), 55(22), 43(100), 41(25)	0.3
35	Branched 2-icosanone[a,b,d]		2.2
39	Branched 2-henicosanone[a,b,d]		0.1
36	Branched 2-icosanone[a,b,d]		0.3
8	(*Z*)-8-Hexadecen-2-one[a,b,d]		0.06
13	(*Z*)-8-Heptadecen-2-one[a–d]		0.02
14	(*Z*)-10-Heptadecen-2-one[a,b,d]		0.05
19	(*Z*)-8-Octadecen-2-one[a,b, f]		0.09
22	(*Z*)-11-Octadecen-2-one[a,b,d]		0.07
28	(*Z*)-8-Nonadecen-2-one[a–d,g]	280(0.2), 265(0.1), 262(0.2), 222(1), 198(2), 184(1), 152(2), 135(4), 125(10), 111(8), 97(17), 96(15), 82(19), 81(18), 71(33), 58(25), 55(43), 43(100), 41(39)	0.7

TABLE 1. (*Continued*)

Peak (Figure 1)	Compound	EI Mass spectral data, *m/z* (%)	Quantity (ng/animal)
29	(*Z*)-12-Nonadecen-2-one[a−d,g]		0.6
40	(*Z*)-8-Henicosen-2-one[a,b,d,g]		0.2
41	(*Z*)-14-Henicosen-2-one[a,b,d,g]		0.2
21	Hexadecanoic acid[a−c]		4.9
34	Octadecanoic acid[a−d]		2.2
45	Icosanoic acid[a,c,d]	312(6), 269(3), 213(3), 185(4), 171(3), 157(1), 143(2), 129(20), 115(7), 97(18), 83(23), 73(65), 71(33), 61(20), 60(65), 57(69), 55(68), 43(100), 41(58)	0.5
53	Docosanoic acid[a−d]		0.2
55	Tetracosanoic acid[a,c,d]		0.1
10	13-Methyltetradecanoic acid[a−d]		0.3
18	14-Methylpentadecanoic acid[a,c,d]		0.1
24	15-Methylhexadecanoic acid[a−d]	270(4), 227(6), 125(4), 171(3), 157(1), 143(2), 129(16), 115(6), 97(15), 83(20), 73(61), 71(26), 69(35), 61(15), 60(60), 57(58), 55(59), 43(100), 41(67)	0.6
38	17-Methyloctadecanoic acid[a−d]		0.1
44	18-Methylnonadecanoic acid[a,c,d]		0.1
49	19-Methylicosanoic acid[a−d]		0.2
7	11-Methyltridecanoic acid[a−d]		0.2
25	14-Methylhexadecanoic acid[a−d]	270(4), 241(2), 227(3), 213(3), 185(4), 171(4), 157(3), 139(5), 129(16), 111(10), 97(23), 83(28), 73(55), 71(35), 69(44), 61(10), 60(55), 57(87), 55(98), 43(100), 41(86)	0.1
33	(*Z*)-9-Octadecenoic acid[a−e,g]	282(0.4), 264(2), 256(0.5), 227(0.7), 222(1), 213(1.5), 151(2), 137(3), 123(5), 111(10), 97(30), 83(42), 73(27), 69(65), 67(33), 60(25), 57(42), 55(100), 43(80), 41(88)	0.8
3	3-Phenylpropanoic acid[a,c,e]	150(27), 131(3), 104(44), 91(100), 79(10), 118(20), 77(24), 65(17), 51(20), 39(13)	0.2

(*Continued*)

TABLE 1. (*Continued*)

Peak (Figure 1)	Compound	EI Mass spectral data, *m/z* (%)	Quantity (ng/animal)
47	1-Hydroxyhexadec-2-yl pentanoate[a−c,e]		0.2
48	2-Hydroxyhexadec-1-yl pentanoate[a−c,e]		0.4
50	1-Hydroxyheptadec-2-yl pentanoate[a,b,e]	255(0.5), 227(2), 157(0.5), 116(8), 101(10), 85(100), 69(13), 57(48), 55(23), 48(48), 41(25)	0.5
51	2-Hydroxyheptadec-1-yl pentanoate[a,b,e]	323(0.5), 241(2), 145(5), 116(25), 101(45), 85(100), 69(20), 57(58), 55(24), 43(52), 41(33)	0.8
54	2-Hydroxyicos-1-yl acetate[a,c,e]		0.1
58	4-(Hexadecadien-1-yl)-butanolide[a,e,f,h]		0.8
62	4-(Heptadecadien-1-yl)-butanolide[a,e,f,h]	376(1), 358(0.5), 332(0.5), 221(1), 166(1), 151(2), 125(5), 111(16), 97(37), 85(100), 83(42), 69(50), 57(72), 55(75), 43(89), 41(53)	0.7
1	2-Piperidone[a−c,e]	99(100), 70(30), 58(7), 56(15), 55(38), 43(65), 42(78), 41(59), 30(89)	1.0
2	Unidentified[a,b]		0.3
59	Cholesterol[a−c,e]		5.6
56	Unidentified[a]		0.3
57	Unidentified[a]		0.4
60	Unidentified[a]		0.2
61	Unidentified[a]		1.0
63	Unidentified[a]		1.3
64	Desmosterol[a,c,e]		0.5
65	Lanosterol[a,e,i]		0.2
46	Unidentified[a,b]		0.9

[a] Low resolution EI mass spectral data.
[b] $Cl(CH_4)$ mass spectral data.
[c] Retention time comparison.
[d] Relative retention time interval comparison.
[e] Published data.
[f] Position of double bond uncertain.
[g] EI mass spectral data on DMDS derivative.
[h] Configuration of double bond uncertain.
[i] Tentative identification.

their $Cl(CH_4)$ mass spectra. The secretion contains unbranched as well as iso- and anteiso-branched fatty acids and it is, therefore, quite likely that most of the branched 2-alkanones could also be the iso ketones, while the unidentified ketone **36** could be 18-methyl-2-nondecanone. The retention-time increments observed

for the branched and unbranched carboxylic acids, for example, those between the C_{20} and C_{17} acids, correlate very well with the increments between the branched and unbranched ketones and therefore appear to confirm this assumption. Unfortunately, it was not possible to determine the position of the double bonds in the two γ-lactones **58** and **62**. As can be expected, their mass spectra do not contain the required structural information and their DMDS derivatives could not be found in the total ion chromatogram of a sample of the secretion subjected to DMDS derivatization, probably because the high masses of the derivatives precluded their elution from the capillary column.

According to its Cl(CH_4) mass spectrum, constituent **2** has a molecular mass of 166, and the compound apparently has an acetyl function. Its mass spectrum is similar to that of 1-acetylhydrindane, which also has a molecular mass of 166. However, the mass spectrum of this constituent has a prominent ion at *m/z* 109 (23%), which is relatively weak in the NBS library spectrum of 1-acetylhydrindane. Furthermore, the mass spectrum of the constituent **2** does not have an ion at *m/z* 148 which is present in the library spectrum. This constituent is, therefore, listed as unidentified in Table 1.

All of the constituents eluting in the high-molecular-weight range of the total ion chromatogram appear to be steroids. Some of these compounds remained unidentified in the present investigation and will be subjected to further scrutiny in future research on the dwarf hamster, because of their possible involvement in the chemical ecology of the animal.

Varying quantities of ventral secretion were collected from individual males. Table 1 contains quantitative data obtained from a rather large quantity (4.8 mg) of secretion collected from one male animal. These data are included merely to serve as a guideline for biologists planning behavioral studies on the dwarf hamster. The variation in the quantity of ventral secretion collected from individual animals, the probably incomplete extraction of, especially, the more polar compounds with a small volume of dichloromethane, and the fact that different samples of the secretion possibly contain different concentrations of moisture that is not detectable by FID are considered to be responsible for a larger factor of uncertainty than the use of an external standard and the unaccurate determination of the volume to which the sample was finally concentrated.

Carboxylic acids have been identified in many mammalian exocrine secretions. The longer-chain acids seem to be less common, especially in secretions investigated during the 1960s and 1970s, possibly because long-chain acids did not elute from some of the stationary phases and packed columns used during the earlier years of gas chromatography, or eluted as such broad peaks as to be almost undistinguishable from the baseline. Fatty acids were found in the anal sac secretions of many carnivores (Albone, 1984a) and in many other mammalian secretions. The preputial gland secretion of the musk rat, *Ondatra zibethica*, for example, contains a number of saturated, monounsaturated, and diunsaturated,

C_{12}–C_{20} fatty acids (Ritter et al., 1982). Fatty acids are also found in human vaginal secretions (Huggins and Preti, 1976) and in the interdigital secretion of the reindeer, *Rangifer tarandus* (Brundin et al., 1978). Many of the short-chain acids found in these secretions are iso- or anteiso-branched. 3-Phenylpropanoic acid **3** was identified, among others, in the anal sac secretion of the red fox, *Vulpes vulpes*, and the lion, *Panthera leo* (Albone and Eglinton, 1974), and in the urine of coyote, *Canis latrans* (Murphy et al., 1978).

Long-chain carboxylic acids (C_{15}–C_{25}) were found in the occipital secretion of the bactrian camel, *Camelus bactrianus*, together with some shorter-chain acids (Ayorinde et al., 1982). Long-chain fatty acids are particularly abundant in the interdigital secretions of bontebok, *Damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi*, containing the saturated unbranched C_6–C_8, C_{10}, C_{12}, C_{14}–C_{18}, and C_{20} fatty acids (Burger et al., 1999a). With the exception of the C_7, C_8, C_{10}, and C_{20} homologs, these acids are also present in the preorbital gland secretions of these two subspecies, which also contain the C_{19} acid (Burger et al., 1999b). The interdigital secretions of these two subspecies also contain oleic acid (*Z*9-C_{18}). The preorbital secretion of the grysbok, *Raphicerus melanotis*, contains the C_{14}–C_{18} and C_{20} fatty acids (Burger et al., 1996) and that of the steenbok, *R. campestris*, C_8, C_{10}, C_{12}, C_{14}–C_{16}, C_{18}, C_{20}, and unsaturated fatty acids (Burger et al., 1999c). Only the three C_{16}–C_{18} fatty acids are present in the preorbital secretion of the oribi, *Ourebia ourebi* (Mo et al., 1995) and the C_8, C_{10}, C_{12}, C_{14}–C_{16}, C_{18}, C_{20} fatty acids and several unsaturated analogs in the preorbital secretion of the grey duiker, *Sylvicapra grimmia* (Burger et al., 1990).

With a few exceptions, long-chain alcohols have not been found in many mammalian exocrine secretions. The identification of the C_{12}, C_{14}, C_{16}, and C_{18} alkanols in human vaginal secretions (Huggins and Preti, 1976) is one of these examples. Of the four long-chain C_{16}, C_{17}, C_{19}, and C_{21} alkanols (**15**, **17**, **31**, and **43**) present in the ventral secretion of the dwarf hamster, the C_{16} and C_{21} alkanols have been identified in the interdigital secretion of the bontebok and blesbok (Burger et al., 1999a), and the C_{16} alkanol in the preorbital secretion of the steenbok (Burger et al., 1999c), as well as in the tarsal volatiles of the reindeer, *Rangifer tarandus tarandus* (Andersson et al.,1975). Although the preorbital secretion of the grysbok contains at least 22 alkanols, alkenols, and alkadienols (Burger et al., 1996), the four alkanols found in the ventral secretion of the dwarf hamster are not among them.

Ketones are reasonably common in mammalian exocrine secretions, and Albone (1984b) has cited several examples of secretions containing a few short- to medium-chain ketones. Jemiolo et al. (1987) have found several saturated, unsaturated, and methyl-branched unsaturated ketones in the urine of the house mouse, *Mus musculus*. The ventral secretion of the dwarf hamster, however, seems to be unique in that it contains at least 25 alkan-2-ones and alken-2-ones from C_{13} to C_{23}. Only a few of the shorter-chain members of the group of ketones present in

the ventral secretion of the dwarf hamster were found in other animals, for example, 2-pentadecanone (**5**) and 2-heptadecanone (**16**) in the perineal secretion of the guinea pigs, *Cavia aperea* and *C. porcellus* (Wellington et al., 1979) and pentadecan-2-one in the dorsal secretion of the springbok, *Antidorcas marsupialis*, (Burger et al., 1981).

Unfortunately, the position of the double bonds in the two monounsaturated γ-lactones (**58** and **62**) could not be determined. One is immediately reminded of a similar compound, (*Z*)-6-dodecen-4-olide, that was identified as a component of the extract of the male tarsal hair tuft of the black-tailed deer, *Odocoileus hemionus columbianus* (Brownlee et al., 1969) and later shown to originate from the urine of the animal (Müller-Schwarze et al., 1978). The same compound was identified in the interdigital secretions of the bontebok and blesbok, secretions that also contain the δ-lactone, dodecan-5-olide (Burger et al., 1999a). The absolute configuration of this compound in the bontebok has not yet been established. Hexadecan-5-olide and its C_{17} and C_{18} homologs are present in the preorbital secretion of the bontebok and blesbok (Burger et al., 1999b). Dodecan-4-olide has been found in the occipital secretion of the bactrian camel (Ayorinde et al., 1982), the γ- and δ-lactones, pentan-4-olide, hexan-4-olide, and hexan-5-olide, have been identified in human urine (Zlatkis and Liebich, 1971); and the C_8–C_{12} γ-lactones in human skin lipids (Labows et al.,1979). The C_{15}–C_{18} and C_{20} alkan-4-olides and C_{16} alkan-5-olide were identified in the preorbital secretion of the grysbok (Burger et al., 1996) and the C_{16} and C_{18} alkan-4-olides in the preorbital secretion of the grey duiker (Burger et al., 1990).

Albone (1984c) discussed the possibility that 2-piperidone identified in the anal sac secretions of the dog, *Canis familiaris*, coyote, *C. latrans*, and mink, *Mustela vison*, could possibly be formed by the elimination of water from the precursor 5-aminovaleric acid that is formed by fermentation processes in the anal sac (Albone et al., 1976). 2-Piperidone has also been identified in human vaginal secretions (Huggins and Preti, 1976).

The ventral secretion of the dwarf hamster contains five long-chain hydroxyesters; **47**, **48**, **50**, **51**, and **54**. Although these hydroxyesters are not accompanied by long-chain epoxides in the ventral and other secretions, it is nevertheless accepted as a working hypothesis that they are formed through nucleophilic substitution of the corresponding epoxides by the corresponding carboxylic acids. The preorbital secretion of the grysbok contains 2-hydroxyoctadecan-1-yl acetate and 2-hydroxyicosan-1-yl acetate (Burger et al., 1996). The related steenbok's preorbital secretion contains 17 of these hydroxyesters, from 2-hydroxyheptadecan-1-yl acetate to 1-hydroxydocos-2-yl butanoate and 2-hydroxydocos-1-yl butanoate (Burger et al., 1999c), and the interdigital secretions of the bontebok and blesbok contain 12 hydroxyesters, from 2-hydroxyoctadecan-1-yl acetate to 2-hydroxydocosan-1-yl butanoate (Burger et al., 1999a). It is not clear whether these compounds have any semiochemical function because they are not very volatile.

Similarly it is not clear whether steroids such as the ubiquitous cholesterol, its derivative desmosterol, and lanosterol have a semiochemical function other than, possibly, serving as controlled-release carrier materials. Cholesterol and desmosterol have also been identified in the interdigital secretions of bontebok and blesbok (Burger et al., 1999a).

REFERENCES

ALBONE, E. S. 1984a. Mammalian Semiochemistry. The Investigation of Chemical Signals Between Mammals. Wiley-Interscience, Chichester, UK, p. 144.

ALBONE, E. S. 1984b. Mammalian Semiochemistry. The Investigation of Chemical Signals Between Mammals. Wiley-Interscience, Chichester, UK, pp. 99, 105, 176–177, 180.

ALBONE, E. S. 1984c. Mammalian Semiochemistry. The Investigation of Chemical Signals Between Mammals. Wiley-Interscience, Chichester, UK, pp. 148–149.

ALBONE, E. S., and EGLINTON, G. 1974. The anal sac secretion of the red fox (*Vulpes vulpes*); its chemistry and microbiology. A comparison with the anal sac secretion of the lion (*Panthera leo*). *Life Sci.* 14:387–400.

ALBONE, E. S., ROBINS, S. P., and PATEL, D. 1976. 5-Aminovaleric acid, a major free amino acid component of the anal sac secretion of the red fox, *Vulpes vulpes. Comp. Biochem. Physiol.* 55B:483–486.

ANDERSSON, G., ANDERSSON, K., BRUNDIN, A., and RAPPE, C. 1975. Volatile compounds from the tarsal scent gland of reindeer (*Rangifer tarandus*). *J. Chem. Ecol.* 1:275–281.

AYORINDE, F., WHEELER, J. W., WEMMER, C., and MURTAUGH, J. 1982. Volatile components of the occipital gland secretion of the bactrian camel, *Camelus bactrianus. J. Chem. Ecol.* 8:177–183.

BECKER, H., BERGER, W., DOMSCHKE, G., FANGHÄNEL, E., FAUST, J., FISCHER, M., GENTZ, F., GEWALD, K., GLUCH, R., MAYER, R., MÜLLER, K., PAVEL, D., SCHMIDT, H., SCHOLLBERG, K., SCHWETLICK, K., SEILER, E., and ZEPPENFELD, G. 1973. p. 599, *in* Organicum: Practical Handbook of Organic Chemistry, P. A. Ongley (ed.). Pergamon, Oxford.

BRACKMANN, M. 1977. Melatonin delays puberty in the Djungarian hamster. *Naturwissenschaften* 64:642–643.

BROWNLEE, R. G., SILVERSTEIN, R. M., MÜLLER-SCHWARZE, D., and SINGER, A. G. 1969. Isolation, identification and function of the chief component of the male tarsal scent in black-tailed deer. *Nature* 221:284–285.

BRUNDIN, A., ANDERSSON, G., ANDERSSON, K., MOSSING, T., and KÄLLQUIST, L. 1978. Short-chain aliphatic acids in the interdigital gland secretion of reindeer (*Rangifer tarandus*), and their discrimination by reindeer. *J. Chem. Ecol.* 4:613–622.

BURGER, B. V., LE ROUX, M., SPIES, H. S. C., TRUTER, V., and BIGALKE, R. C. 1981. Mammalian pheromone studies IV. Terpenoid compounds and hydroxyesters from the dorsal gland of the springbok, *Antidorcas marsupialis. Z. Naturforsch.* 36c:340–343.

BURGER, B. V. PRETORIUS, P. J., SPIES, H. S. C., BIGALKE, R. C., and GRIERSON, G. R. 1990. Mammalian pheromones VIII. Chemical characterization of preorbital gland secretion of grey duiker, *Sylvicapra grimmia. J. Chem. Ecol.* 16:397–416.

BURGER, B. V., TIEN, F.-C., LE ROUX, M., and MO, W.-P. 1996. Mammalian exocrine secretions X. Constituents of preorbital secretion of grysbok, *Raphicerus melanotis. J. Chem. Ecol.* 22:739–764.

BURGER, B. V., NELL, A. E., SPIES, H. S. C., LE ROUX, M., BIGALKE, R. C., and BRAND, P. A. J. 1999a. Mammalian exocrine secretions XII: Constituents of interdigital secretions of

bontebok, *Damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi. J. Chem. Ecol.* 25:2057–2084.

BURGER, B. V., NELL, A. E., SPIES, H. S. C., LE ROUX, M., and BIGALKE, R. C. 1999b. Mammalian exocrine secretions XIII: Constituents of preorbital secretions of bontebok, *Damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi. J. Chem. Ecol.* 25:2085–2097.

BURGER, B. V., GREYLING, J., and SPIES, H. S. C. 1999c. Mammalian exocrine secretions XIV: Constituents of preorbital secretion of steenbok, *Raphicerus campestris. J. Chem. Ecol.* 25:2099–2108.

FEOKTISTOVA, N. 1994. Sex discrimination by different odours in the dwarf hamsters, *Phodopus roborovskii* and *Ph. sungorus*. Proceedings, Chemical Signals in Vertebrates VII, Tübingen, Germany, p. 48.

FLINT, W. E. 1966. Die Zwerghamster der paläarktischen Fauna, Ziemsen Verlag, Wittenberg Lutherstadt, p. 7.

HEISLER, C. 1984. Zum Markierungsverhalten des Dshungarischen Zwerghamsters, *Phodopus sungorus. Biol. Rdsch.* 22:181–183.

HELDMAIER, G. 1975. Metabolic and thermoregulatory responses to heat and cold in the Djungarian hamster, *Phodopus sungorus. J. Comp. Physiol.* 102:115–122.

HOFFMANN, K. 1973. The influence of photoperiod and melatonin on testes size, body weight and pelage colour in the Djungarian hamster (*Phodopus sungorus*). *J. Comp. Physiol.* 85:267–282.

HUGGINS, G. R., and PRETI, G. 1976. Volatile constituents of human vaginal secretions. *Am. J. Obstet. Gynecol.* 126:129–136.

JEMIOLO, B., ANDREOLINI, F., WIESLER, D., and NOVOTNY, M. 1987. Variations in mouse (*Mus musculus*) urinary volatiles during different periods of pregnancy and lactation. *J. Chem. Ecol.* 13:1941–1956.

LABOWS, J. N., MCGINLEY, K. J., LEYDEN, J. J., and WEBSTER, G. F. 1979. Characteristic γ-lactone odor production of the genus, *Pityrosporum. Appl. Environ. Microbiol.* 38:412–415.

MO, W.-P., BURGER, B. V., LE ROUX, M., and SPIES, H. S. C. 1995. Mammalian exocrine secretions IX: Constituents of preorbital secretion of oribi, *Ourebia orebi. J. Chem. Ecol.* 21:1191–1215.

MÜLLER-SCHWARZE, D., DAVID, U., CLAESSON, A., SINGER, A. G., SILVERSTEIN, R. M., MÜLLER-SCHWARZE, C., VOLKMAN, N. J., ZEMANEK, K. F., and BUTLER, R. G. 1978. The deer-lactone: Source, chemical properties and responses of black-tailed deer. *J. Chem. Ecol.* 4:247–256.

MURPHY, E. L., FLATH, R. A., BLACK, D. R., MON, T. R., TERANISHI, R., TIMM, R. M., and HOWARD, W. E. 1978. Isolation, identification and biological activity assay of chemical fractions from estrus urine attractive to the coyote, pp. 66–77, *in* R. W. Bullard (ed.). Flavor Chemistry of Animal Foods. ACS Symposium Series 67, American Chemical Society, Washington, D.C.

POGOSIANZ, H. E., and SOKOVA, O. I. 1967. Maintaining and breeding of the Djungarian hamster under laboratory conditions. *Z. Versuchstierk.* 9:292–297.

REASNER, D. S., and JOHNSTON, R. E. 1988. Acceleration of reproductive development in female Djungarian hamsters by adult males. *Physiol. Behav.* 43:57–64.

RITTER, F. J., BRÜGGEMANN, I. E. M., GUT, J., and PERSOONS, C. J. 1982. Recent pheromone research in the Netherlands on muskrats and some insects, pp. 107–130, *in* B. A. Leonhardt and M. Beroza (eds.). Insect Pheromone Technology: Chemistry and Applications. ACS Symposium Series 190, American Chemical Society, Washington, D.C.

SUNDERKÖTTER, C. H., WITTKOWSKI, W., and HOFFMANN, K. 1990. Fenestrated capillaries in the ventral sebaceous gland of the Djungarian hamster, *Phodopus sungorus. Acta Anat.* 139:45–48.

VESELOFSKY, Z., and GRUNDOVA, S. 1965. Beitrag zur Kenntnis des Dsungar-Hamsters, *Phodopus sungorus. Z. Saeugetierkd.* 30:306–311.

VINCENTI, M., GUGLIELMETTI, G., CASSANI, G., and TONINI, C. 1987. Determination of double bond position in diunsaturated compounds by mass spectometry of dimethyl disulfide derivatives. *Anal. Chem.* 59:694–699.

WELLINGTON, J. L., BYRNE, K. J., PRETI, G., BEAUCHAMP, G. K., and SMITH, A. B. 1979. Perineal scent gland of wild and domestic guinea pigs. A comparative chemical and behavioural study. *J. Chem. Ecol.* 5:737–751.

WYNNE-EDWARDS, K. E., and LISK, R. D. 1984. Djungarian hamsters fail to conceive in the presence of multiple males. *Anim. Behav.* 32:626–628.

YELLON, S. M., and GOLDMAN, B. D. 1984. Photoperiod control of reproductive development in the male Djungarian hamster *Phodopus sungorus. Endocrinology* 114:664–670.

ZLATKIS, A., and LIEBICH, H. M. 1971. Profile of volatile metabolites in human urine. *Clin. Chem.* 17:592–594.

Journal of Chemical Ecology, Vol. 27, No. 6, 2001

MAMMALIAN EXOCRINE SECRETIONS XVI. CONSTITUENTS OF SECRETION OF SUPPLEMENTARY SACCULI OF DWARF HAMSTER, *Phodopus sungorus sungorus*

B. V. BURGER,[1,*] D. SMIT,[1] H. S. C. SPIES,[1] C. SCHMIDT,[2] U. SCHMIDT,[2] and A. Y. TELITSINA[3]

[1]*Laboratory for Ecological Chemistry*
Department of Chemistry, University of Stellenbosch
Stellenbosch 7600, South Africa

[2]*Zoological Institute, University of Bonn*
Poppelsdorfer Schloss, D-53115 Bonn, Germany

[3]*Institute of Animal Evolutionary Morphology and Ecology, Russian Academy of Sciences*
Leninsky 33, Moscow 117071, Russia

(Received January 3, 2000; accepted January 29, 2001)

Abstract—As a first step in a study of the role of the secretion of the supplementary sacculi (buccal secretion) of the dwarf hamster, *Phodopus sungorus sungorus*, almost complete chemical characterization of the secretion was achieved. The 35 compounds identified include carbon dioxide, hydrogen sulfide, a large number of carboxylic acids (representing the bulk of the organic volatile fraction of the secretion), phenol, 2-piperidone, indole, two long-chain hydroxyesters, cholestrol, desmosterol, and lanosterol. The position of the double bonds in γ-icosadienyl-γ-butyrolactone and γ-henicosadienyl-γ-butyrolactone could not be determined, and these two compounds remained only partially characterized. Large variations were found in the relative concentrations in which the short-chain carboxylic acids are present in the secretions of individual animals, and although this aspect was not investigated in sufficient detail in the present investigation, the difference in the carboxylic acid profiles of the secretions of individual animals could play a role in individual recognition in this animal.

Key Words—*Phodopus sungorus sungorus*, dwarf hamster, mammalian semiochemicals, mammalian pheromones, exocrine secretion, buccal gland secretion, supplementary sacculi.

*To whom correspondence should be addressed.

INTRODUCTION

Certain aspects of the biology of the dwarf hamster, *Phodopus sungorus sungorus*, were summarized in the previous article in the series on mammalian exocrine secretions (Burger et al., 2001). In addition to the ventral gland, both male and female dwarf hamsters produce a buccal secretion from the supplementary sacculi situated at the opening of their cheek pouches. Very little information is available on this subject. Functions attributed to this secretion include communication of information about sex, identity, female breeding condition, and even regulation of certain physiological functions in *P. s. campbelli* (Vasilieva and Feoktistova, 1994). These authors reported the results of a study on the role of the sacculi in the development of juveniles. It was found that surgical removal of the sacculi did not influence development of the pups when they could consume secretion from parents and littermates but that restriction or inability to consume secretion led to a significant increase in the pup mortality, with conditions such as nonspecific enterocolitis and disbacteriosis, and a significant delay in growth and reproductive development. An increase in adrenal gland weight occurred in such animals, and the effects of deprivation were found to be more pronounced in male pups.

The buccal secretion thus appears to play an important role in the survival and development of juvenile dwarf hamsters. Although polar involatile compounds could be responsible for at least some of the functions attributed to the secretion, it was decided to start with the investigation of the volatile organic fraction as a first step towards the complete chemical characterization of the secretion. We now report the identification of practically all its volatile organic constituents.

METHODS AND MATERIALS

General and Analytical Methods. The procedures and instrumentation used in the identification of the constituents of the buccal secretion of the hamster were described by Burger et al. (2001) in the previous paper in this series. In addition to the column coated with 95%-dimethyl–5%-diphenylsiloxane copolymer used for analysis of the ventral secretion of the male dwarf hamster, a glass capillary column (40 m $\times$ 0.3 mm) coated with 0.375 μm of OV-1701 was used for qualitative as well as quantitative analyses of the buccal secretion of male and female dwarf hamsters. The GC and GC-MS parameters used for analysis with the PS-089 column were also used for analyses on the OV-1701 column. These analyses were done on both columns using extracts of the buccal secretions of individual animals and the analyses were repeated using solventless sample introduction techniques. Small quantities of the secretion (*ca.* 0.2 mg) were, for example, introduced into the injector of the GC and the GC-MS instrument using a sample introduction

probe (Burger et al., 1990), and similar sample sizes were also introduced as a smear on the inside of a clean injector liner. When solventless sample introduction was used, the volatile material was cryotrapped on the column with dry ice. For quantitative analyses, the injector was operated in the splitless mode and the data acquisition and processing software specified in the previous paper in this series (Burger et al., 2001) were used.

Sample Collection and Preparation. The sticky, whitish secretion was collected separately from the supplementary sacculi of males and females in their summer state, i.e., with the day length exceeding 10 hr. Samples were collected on a weekly basis. Grasping a hamster firmly by the scruff of the neck caused the animal to open its mouth, and the waxy secretion was collected by scooping it from the glandular area with the device used for the collection of the animal's ventral secretion (Burger et al., 2001). The material was transferred from the wire loops to a Reacti-Vial containing dichloromethane using another piece of stainless-steel wire, after which the contents of the vial were thoroughly mixed using a thin glass rod. This caused the secretion to be spread out on the wall of the vial. The material in the vial was sonicated for 10 min, centrifuged for 15 min at 2500 rpm, and the dichloromethane extract containing the organic material transferred to a clean Reacti-Vial with a 100-μl syringe. The extract was concentrated in an inert atmosphere (Burger et al., 1999a) for GC and GC-MS analysis. Secretion intended for quantitative analyses was collected on a wire loop as described above. A small sample (*ca.* 0.2 mg) of the material was accurately weighed into a sample introduction probe (Burger et al., 1990) or a clean glass liner and immediately analyzed to avoid losing the more volatile constituents of the secretion. Hexadecanoic acid was used as external standard in the quantitative analyses.

Reference Compounds. Some of the compounds identified in the secretion are commercially available, while others were available from previous research projects. The following compounds were synthesized during the present study.

*3-Phenylpropanoic acid (**19**)*. Cinnamic acid (3.06 g, 20 mmol), dissolved in degassed methanol (25 ml), was hydrogenated at room temperature and atmospheric pressure in the presence of Pd on activated carbon (5%, 300 mg) until 1.02 equivalents of hydrogen had been taken up. The catalyst was filtered off and the methanol removed on a rotary evaporator. The product containing some residual catalyst was taken up in dichloromethane, the solution filtered through a layer of silica gel, and the solvent evaporated to give 3-phenylpropanoic acid (2.66g, 85.8%). ^{13}C NMR ($CDCl_3$): $\delta = 179.36$ (s, C-1), 140.14 (s, C-1′), 128.56 (d, C-3 and 5), 128.26 (d, C-2 and 6), 126.38 (d, C-4), 35.63 (t, C-2), 30.57 (t, C-3).

*2-Hydroxyoctadec-1-yl acetate (**31**)*. A mixture of the hydroxyester (**31**) and 1-hydroxyoctadec-1-yl acetate was synthesized by the Al_2O_3-catalyzed reaction of 1,2-epoxyoctadecane with acetic acid as described by Burger et al. (1999a). The title compound (**27**) and 1-hydroxyoctadec-1-yl acetate were formed in a ratio of 1 : 3 (GC-MS). HR-MS (mixture of the two isomers): m/z M^+ 255.262, calcd. for

$C_{20}H_{40}O_3$ 255.269. *Major component*: ^{13}C NMR ($CDCl_3$): $\delta = 171.48$ (s, C-1), 75.74 (d, C-2′), 64.82 (t, C-1′), 31.95 (t, C-16′), 30.51 (t, C-3′), 29.30–30.5 (t, C-5′– C-15′), 25.33 (t, C-4′), 22.71 (t, C-17′), 21.20 (q, C-2), 14.12 (q, C-18′). *Minor component* (**31**): ^{13}C NMR ($CDCl_3$): $\delta = 171.20$ (s, C-1), 69.99 (d, C-2′), 68.81 (t, C-1′), 33.37 (t, C-3′), 25.38 (t, C-4′) 29.30 (t, C-5′–C-15′), 31.95 (t,C-16′), 22.71 (t, C-17′), 20.89 (q, C-2), 14.12 (q, C-18′).

*2-Hydroxyicos-1-yl acetate (**32**)*. A mixture of the hydroxyester (**32**) and 1-hydroxyicos-2-yl acetate was prepared from 1,2-epoxyicosane as described above. The two isomers were present in the product in a ratio of 1 : 2 (GC-MS). HR-MS (mixture of the two isomers): *m/z* M^+ 356.332, calcd. for $C_{22}H_{44}O_3$ 356.329. *Major component:* ^{13}C NMR ($CDCl_3$): $\delta = 171.55$ (s, C-1), 75.75 (d, C-2′), 64.81 (t, C-1′), 31.95 (t, C-18′), 30.52 (t, C-3′), 29.3–30.5 (t, C-5′–C-17′), 25.33 (t, C-4′), 22.71 (t, C-19′), 21.22 (q, C-2), 14.13 (t, C-20′). *Minor component* (**32**): ^{13}C NMR ($CDCl_3$): $\delta = 171.27$ (s, C-1), 70.00 (d, C-2′), 68.80 (t, C-1′), 33.38 (t, C-3′), 31.95 (t, C-18′), 29.3–31.0 (t, C-5′–C-16′), 29.18 (t, C-17′), 25.39 (t, C-4′), 22.71 (t, C-19′), 20.90 (q, C-2), 14.13 (q, C-20′).

RESULTS AND DISCUSSION

A typical total ion chromatogram of an extract of the buccal secretion of a male dwarf hamster is given in Figure 1. Tentative identification of the constituents of the secretion was based on low-resolution mass spectral data, supported by information obtained by their chemical ionization mass spectra, generated with methane as reactant gas. Final confirmation of most of the proposed structures was obtained by coinjection of an extract of the secretion with authentic synthetic material.

Solventless sample introduction made it possible to identify even highly volatile constituents of the secretion such as carbon dioxide (which was to be expected in the material and is probably extracted from the animal's breath), hydrogen sulfide, and trimethylamine. The bulk of the volatile organic fraction of the secretion consists of short-chain carboxylic acids. These compounds together with the hydrogen sulfide and trimethyl amine are responsible for the unpleasant odor of the animal's breath.

A few diunsaturated compounds could not be fully characterized because dimethyl disulfide (DMDS) derivatization did not yield informative mass spectral data. This was probably due to the high molecular masses of the derivatives, which are expected to elute as flat peaks in the upper isothermal part of the chromatogram of the material subjected to DMDS derivatization. The interpretation of the mass spectra of long-chain hydroxyesters has been discussed by Burger et al. (1996). In the present study the identification of the hydroxyesters **31** and **32** was based

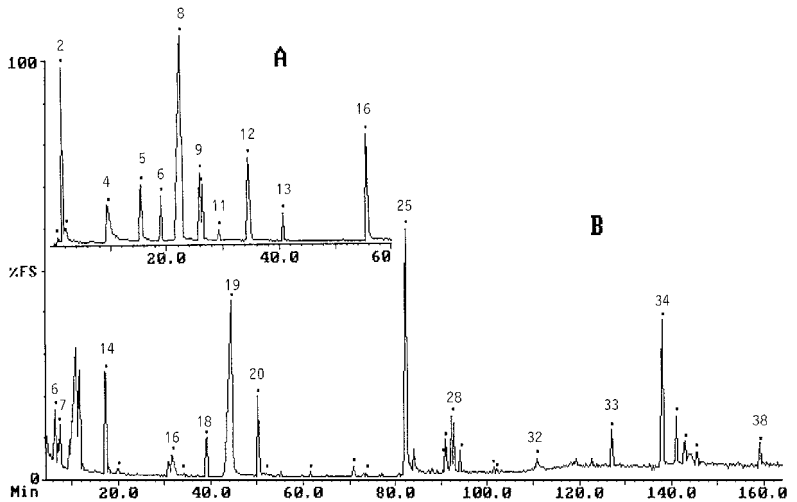


FIG. 1. Total ion chromatograms of the secretion of the supplementary sacculi (buccal secretion) of the dwarf hamster, *Phodopus sungorus sungorus*: (A) Analysis of unprocessed secretion on a relatively polar column (OV-1701) using solventless sample introduction; (B) Extract of the secretion analized on an apolar column (PS-089).

mainly on their coelution with the synthetic hydroxyesters because they are present in the secretion in such low concentrations that some of the less abundant ions were not visible in the high-molecular-weight range of their mass spectra. The two unsaturated γ-lactones, **33** and **36**, which are also present in the ventral secretion of the dwarf hamster (Burger et al., 2001), remained incompletely characterized in the present investigation. The compounds identified in the buccal secretion are listed in Table 1 together with information on the analytical techniques employed in their identification, and some quantitative data on the constituents present in male and female secretions. Several compounds identified in the animal's buccal secretion are also present in its ventral secretion. In addition to the structures of representative examples of these compounds given in the paper on the ventral secretion (Burger et al., 2001), the structures of some of the compounds that are not common to both secretions are given in Figure 2.

Typically about 1–4 mg of secretion could be collected from one animal, but some of the animals from our colony consistently produced almost no secretion. The quantitative determination of the volatile organic constituents of the secretion posed a problem because polar compounds such as the short-chain carboxylic acids were not quantitatively extracted with small quantities of dichloromethane from the mucoid material in which they are produced. The highly volatile constituents are lost during concentration of extracts when larger quantities of solvent are used.

TABLE 1. COMPOUNDS IDENTIFIED IN SUPPLEMENTARY SACCULI SECRETION (BUCCAL SECRETION) OF DWARF HAMSTER

Peak (Figure 1)	Compounds	EI Mass spectral data *m/z* (%)	Quantity (ng/animal)[a]	
			Male	Female
1	Carbon dioxide[b,c]	44(100), 28(7)	7	43
2	Hydrogen sulfide[b,c]	36(5), 35(3), 34(100), 33(38)	6	15
3	Trimethylamine[b,c]	59(46), 58(100), 57(8), 56(4), 43(5), 42(26), 41(5)	17	27
4	Ethanoic acid[b–d]	61(3), 60(100), 45(85), 43(92), 42(16)	569	682
5	Propanoic acid[b–d]	74(76), 73(5), 57(33), 55(19), 45(62)	30	376
6	2-Methylpropanoic acid[b–d]	88(7), 73(30), 55(8), 45(17), 43(100), 41(50)	65	163
7	Hexanal[b–d]	100(3), 99(4), 82(12), 72(19), 71(8), 67(11), 57(54), 56(78), 44(100), 43(60), 41(75)		9
8	Butanoic acid[b–d]	88(2), 73(30), 60(100), 55(9), 45(21), 43(21), 42(28), 41(25)	840	119
9	3-Methylbutanoic acid[b–d]	87(20), 74(4), 60(100), 45(30), 43(52), 41(40)	205	433
10	2-Methylbutanoic acid[b,d]	87(22), 74(100), 57(60), 45(19), 41(54)	140	143
11	Pentanoic acid[b–d]	87(3), 74(10), 73(36), 60(100), 57(12), 55(14), 45(19), 43(25), 41(28)	35	34
12	4-Methylpentanoic acid[b–d]	101(3), 87(6), 83(12), 74(48), 73(50), 60(35), 57(100), 55(57), 45(16), 43(40), 41(42), 39(20)	315	4
13	Phenol[b–d]	94(100), 66(44), 65(36), 55(15), 51(8), 50(10)	4	4
14	Hexanoic acid[b–d]	87(11), 73(40), 60(100), 56(11), 55(19), 45(20), 43(27), 41(36)	1	
15	1-Acetylimidazole[b–d]	110(6), 82(1), 68(100), 43(39), 41(9)		
16	2-Piperidone[b–d]	99(100), 70(30), 58(9), 56(20), 55(50), 43(78), 42(95), 41(69), 30(69)	367	290
17	1-Octyl acetate[b–d]	116(5), 112(7), 107(2), 101(5), 84(15), 83(17), 70(28), 69(20), 61(22), 57(12), 56(31), 55(30), 43(100), 41(27)		
18	Indole[b–e]	117(100), 90(49), 89(37), 63(22), 59(16), 51(7), 50(7)	18	3
19	3-Phenylpropanoic acid[b–e]	150(33), 105(18), 104(52), 91(100)	270	194
20	*m*-Hydroxyacetophenone[b–d]	136(38), 121(100), 93(38), 65(40), 53(10), 51(8), 43(18)		
21	Oxindole (1,3-Dihydro-2*H*-indol-2-one)[b–d]	133(100), 105(34), 104(90), 78(48), 77(22), 52(30), 51(33), 50(19)		

22	Hexadecane[b–d]	226(5), 155(3), 141(4), 127(6), 113(7), 99(9), 85(30), 71(52), 57(100), 43(81), 41(37)	1	3
23	Tetradecanoic acid[b–d]	228(7), 199(4), 185(14), 171(8), 157(6), 143(9), 129(28), 115(9), 101(8), 87(17), 73(87), 69(33), 60(97), 57(70), 55(80), 43(100), 41(78)	9	10
24	Octadecane[b–d]	155(1), 141(5), 127(6), 113(8), 99(9), 85(32), 71(50), 57(100), 43(80), 41(35)		3
25	Hexadecanoic acid[b–e]	256(7), 227(2), 213(8), 189(3), 185(7), 171(8), 157(9), 143(4), 129(27), 115(13), 101(8), 87(16), 73(96), 69(42), 60(99), 57(87), 55(89), 43(100), 41(75)	133	217
26	(*Z*)-9-Octadecenoic acid (Oleic acid)[b–e]	264(7), 185(3), 152(1), 151(1), 138(2), 137(2), 129(4), 125(5), 124(5), 123(6), 111(11), 97(30), 84(22), 83(36), 73(22), 69(55), 60(23), 57(41), 55(100), 43(68), 41(73)	32	30
27	(*E*)-9-Octadecenoic acid[b–e]	264(7), 185(3), 151(1), 138(2), 137(2), 125(3), 123(3), 111(10), 97(28), 84(20), 83(32), 73(20), 69(54), 60(25), 57(28), 55(100), 43(57), 41(65)	18	16
28	Octadecanoic acid[b–e]	284(4), 241(5), 199(3), 185(7), 171(4), 143(4), 129(20), 115(8), 101(7), 97(20), 87(12), 83(23), 73(68), 69(38), 60(73), 57(70), 55(80), 43(100), 41(70)	87	118
29	9,12-Octadienyl acetate[b,c, f,g]	308(0.1), 265(0.2), 248(0.3), 220(0.2), 149(4), 135(8), 121(15), 109(14), 107(10), 95(35), 81(54), 79(47), 67(88), 55(69), 43(100), 41(56)		
30	Icosanoic acid[b–d]	312(12), 284(2), 283(2), 269(10), 255(5), 227(3), 199(2), 185(3), 171(3), 157(2), 143(3), 129(11), 115(7), 101(5), 97(15), 87(8), 85(13), 83(18), 73(39), 69(26), 60(40), 57(53), 55(55), 43(100), 41(50)	1	4
31	2-Hydroxyoctadec-1-yl acetate[b,d]	225(3), 155(2), 125(2), 111(6), 103(10), 97(12), 83(15), 74(29), 71(12), 69(16), 57(27), 55(29), 43(100), 41(27)	1	
32	2-Hydroxyicos-1-yl acetate[b,d]	283(1), 111(6), 103(10), 97(13), 83(16), 74(28), 71(15), 69(17), 57(28), 55(25), 43(100), 41(23)		

(*Continued*)

TABLE 1. (*Continued*)

Peak (Figure 1)	Compounds	EI Mass spectral data *m/z* (%)	Quantity (ng/animal)[a] Male	Female
33	Tetracosadien-4-olide (γ-Icosadienyl-γ-butyrolactone)[b, f]	362(7), 251(6), 197(6), 155(8), 141(9), 125(7), 111(18), 97(38), 85(100), 83(43), 71(28), 69(63), 57(77), 55(88), 43(95), 41(73)	94	161
34	Cholesterol[b–d]	386(6), 368(5), 353(4), 326(1), 301(7), 275(10), 255(6), 231(4), 213(10), 199(5), 173(7), 159(16), 145(28), 133(17), 119(20), 107(17), 105(36), 91(40), 81(48), 79(37), 67(33), 57(54), 55(62), 43(100), 41(54)	143	93
35	Unidentified steroid[b]	384(2), 369(3), 351(4), 325(1), 300(4), 271(16), 253(7), 213(7), 173(6), 159(12), 145(19), 133(13), 119(15), 109(18), 107(23), 105(28), 94(28), 93(23), 91(32), 81(37), 69(100), 67(36), 55(70), 43(30), 41(75)	18	41
36	Pentacosadien-4-olide (γ-Henicosadienyl-γ-butyrolactone)[b, f]	376(2), 125(8), 111(19), 97(40), 85(95), 83(45), 71(35), 69(65), 57(80), 55(86), 43(100), 41(65)	97	299
37	Desmosterol[b–d]	385(3), 384(2), 370(7), 369(6), 352(4), 351(6), 329(3), 300(7), 271(33), 253(10), 231(8), 213(9), 191(4), 159(10), 145(13), 133(15), 119(23), 107(26), 105(32), 91(40), 81(41), 69(100), 67(42), 55(80), 43(48), 41(98)	19	53
38	Lanosterol[b–d]	426(2), 411(6), 393(4), 259(2), 229(3), 189(5), 187(6), 175(6), 173(5), 161(7), 159(8), 147(8), 145(8), 135(9), 133(9), 123(9), 121(13), 119(18), 109(27), 107(14), 105(16), 95(26), 93(16), 91(16), 83(13), 81(25), 79(15), 69(100), 55(6), 43(35), 41(60)	93	47

[a] Quantities lower than 1 ng/animal are not given.
[b] Low-resolution mass spectrum.
[c] Published data.
[d] Retention time comparison with authentic material.
[e] $Cl(CH_4)$ mass spectral data.
[f] Position and configuration of double bond(s) uncertain.
[g] Tentative identification.

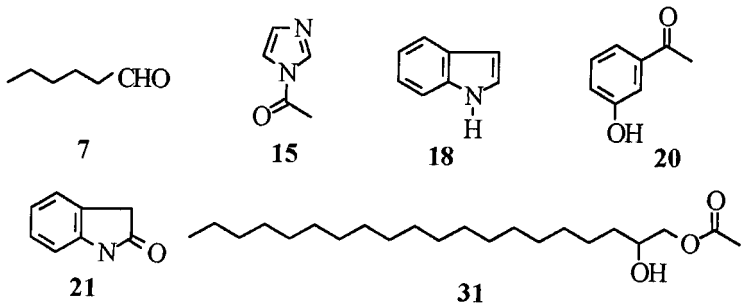


FIG. 2. Structures of some representative examples of the compounds identified in the buccal secretion of the dwarf hamster, *P. s. sungorus.*

Solventless sample introduction, on the other hand, discriminates to a certain extent against the heavy constituents of the secretion. Furthermore, the FID in GC analyses and the mass spectrometer in GC-MS analyses both have different quantitative responses to small and large molecules, while CO_2 and H_2S are not detected by FID. Ideally the volatile organic constituents present in the headspace gas of the secretion should be determined at temperatures corresponding to the hamster's body temperature in their summer and winter states. Furthermore, the extreme polarity differences between highly polar compounds such as ethanoic acid and the less polar long-chain constituents of the secretion, make it difficult to suggest an universal stationary phase for sample enrichment. Headspace analysis of the buccal secretion was not done in the present investigation. The quantitative data in Table 1 were obtained by combining the results of analyses using different capillary columns and sample introduction techniques in GC as well as GC-MS analyses. Although the quantitative results probably represent a relatively accurate picture of the quantitative composition of the secretion analyzed, the data are considered to be somewhat preliminary at this stage and are given merely as a guideline for biologists planning behavioral studies on the dwarf hamster. Nevertheless, the results do give quite a reliable picture of the ratio in which compounds within limited volatility ranges are present in the secretion. Some of the largest quantitative differences among the buccal secretions from individual animals were found among the short-chain carboxylic acids, and the role of these compounds in individual and sexual recognition in *P. sungorus* should be investigated in more detail.

The highly volatile compounds, carbon dioxide (**1**), hydrogen sulfide (**2**), and trimethylamine (**3**) were detected in the secretion because solventless sample introduction techniques were employed in GC and GC-MS analyses in addition to conventional analysis of extracts. These compounds are almost completely lost from an extract if the solvent has to be evaporated to concentrate an extract for analysis. The presence of carbon dioxide in the secretion is to be expected because it is

extracted from expired breath of the animal by the moisture-containing secretion. Hydrogen sulfide does not feature in reports on mammalian exocrine secretions, probably because it is not detected by FID. It will also not be registered in the total ion chromatogram if the mass spectra are scanned from a mass higher than *m/z* 34. Hydrogen sulfide, trimethylamine, and the short-chain fatty acids, of which almost all the unbranched, iso- and anteiso-branched members from C_2 to C_6 are present in the buccal secretion, are probably responsible for the obnoxious smell of the dwarf hamster's breath and the secretion. A mixture of similar volatile fatty acids has been identified in many mammalian secretions, such as, for example, the interdigital secretion of the reindeer (Brundin et al., 1978), the perineal scent gland of guinea pigs (Wellington et al., 1979), and human vaginal secretion (Huggins and Preti, 1976). The microbial production of trimethylamine, indole (**18**), other amines, organosulfur compounds, and fatty acids from amino acids in mammalian exocrine secretions has been reviewed and discussed by Albone (1984). The occurrence of long-chain saturated and unsaturated fatty acids, 2-piperidone (**16**), and 3-phenylpropanoic acid (**19**) in mammalian exocrine secretions was discussed briefly in the previous paper in this series (Burger et al., 2001).

Long-chain acetates are not common in mammalian secretions. Several have, however, been identified in the male abdominal scent gland of the jird, *Meriones tristrami* (Kagan et al., 1983), the preputial secretion of the mouse, *Mus musculus* (Spener et al., 1969), and the preorbital secretion of the male oribi, *Ourebia orebi* (Mo et al., 1995).

Phenol (**13**) and its derivatives, such as the three isomeric cresols, other alkyl-substituted phenols, and phenolic acids, are present in many mammalian secretions. Phenol and *p*-cresol were found in human vaginal secretions (Huggins and Preti, 1976); *p*-ethylphenol, *p*-propylphenol, and pyrocatechol in castoreum of the beaver, *Castor fiber*, (Lederer, 1946, 1949); phenol, *m*- and *p*-cresol, and *m*- and *p*-propylphenol in aged urine of some African Bovidae (Madubunyi et al., 1996); and phenol, *m*-cresol, *m*-ethylphenol, and *m*-propylphenol in the interdigital secretions of the bontebok, *Damaliscus dorcas dorcas*, and the blesbok, *D. d. phillipsi* (Burger et al., 1999a). The preorbital secretions of the antelope we have so far investigated do not contain phenols and phenolic compounds. As far as mammalian exocrine secretions are concerned, phenol and phenolic compounds are apparently found in glandular structures in which the conditions are favorable for survival of microorganisms and the accumulation of the secretions of the gland and/or the products of microbial activity.

Oxindole (**21**) as its steroid conjugate has been identified in the urine of children (Voeltler et al., 1971). Acetophenone has been identified in the urine of the red fox, *Vulpes vulpes* (Jorgenson et al., 1978), and *p*-hydroxyacetophenone in castoreum of the beaver (Lederer, 1946, 1949).

Hexanal (**7**) is a potent odor with a threshold value to the human nose slightly lower than that of limonene (Ohloff, 1978), which has been identified in human

breast milk (Stafford et al., 1976). It has also been found in interdigital and preorbital secretions of the bontebok, *Damaliscus dorcas dorcas* and blesbok, *D. d. phillipsi* (Burger et al., 1999a,b), and in the preorbital secretions of the steenbok, *Raphicerus campestris* (Burger et al., 1999c). In the interdigital secretions of the bontebok and blesbok, this aldehyde is accompanied by another nine saturated and unsaturated C_7-C_{10} aldehydes. The origin of aldehydes in mammalian secretions has not been established, but in addition to the possibility that they are produced by the exocrine glands of the mammals or by microbial action, short- to medium-chain aldehydes are also produced by the autoxidation of unsaturated lipids such as, for example, unsaturated triglycerides.

Unbranched short- to intermediate-chain alkanes have been identified in human effluvia (Ellin et al., 1974). The alkanes found in some mammalian exocrine secretions are mostly unbranched with intermediate to long chain lengths consisting of even as well as odd numbers of carbon atoms (Burger et al., 1990, 1999a–c). It has to be accepted that these compounds are produced by the glands or by microflora present in the glandular structures because there does not seem to be evidence that their presence can be ascribed to contamination of the secretions with, for example, petroleum products. The two γ-lactones (**33** and **36**) that are also present in the ventral secretion of the male dwarf hamster remained unidentified in this investigation. 2-Hydroxyicos-1-yl acetate (**32**) was also found in the male ventral secretion, but instead of 2-hydroxyoctadec-1-yl acetate (**31**), present in the buccal sectretion, the ventral secretion contains the 1-hydroxyhexadec-2-yl and 2-hydroxyhexadec-1-yl pentanoates and their hydroxyheptadecyl analoges. The presence of these γ-lactones, hydroxyesters, and the steroidal compounds cholesterol (**34**), desmosterol (**37**), and lanosterol (**38**) in the dwarf hamster was briefly referred to in the article on the ventral secretion of the animal (Burger et al., 2001).

REFERENCES

ALBONE, E. S. 1984. Mammalian Semiochemistry, The Investigation of Chemical Signals Between Mammals. Wiley-Interscience, Chichester, UK, pp. 135–162.

BRUNDIN, A., ANDERSSON, G., ANDERSSON, K., MOSSING, T., and KÄLLQUIST, L. 1978. Short-chain aliphatic acids in the interdigital gland secretion of reindeer (*Rangifer tarandus*), and their discrimination by reindeer. *J. Chem. Ecol*. 4:613–622.

BURGER, B. V., MUNRO, Z., SMIT, D., SCHMIDT, U., WU, C.-L., and TIEN, F.-C. 1990. Sample introduction in gas chromatography: Simple method for the solventless introduction of crude samples of biological origin. *J. Chromatogr*. 518:207–214.

BURGER, B. V., TIEN, F.-C., le ROUX, M., and MO, W.-P. 1996. Mammalian exocrine secretions X. Constituents of the preorbital secretion of grysbok, *Raphicerus melanotis. J. Chem. Ecol*. 22:739–764.

BURGER, B. V., NELL, A. E., SPIES, H. S. C., LE ROUX, M., BIGALKE, R. C., and BRAND, P. A. J. 1999a. Mammalian exocrine secretions XII: Constituents of interdigital secretions of

bontebok, *damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi. J. Chem. Ecol*. 25:2057–2084.

BURGER, B. V., NELL, A. E., SPIES, H. S. C., LE ROUX, M., and BIGALKE, R. C. 1999b. Mammalian exocrine secretions XIII: Constituents of preorbital secretions of bontebok, *Damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi. J. Chem. Ecol*. 25:2085–2097.

BURGER, B. V., GREYLING, J., and SPIES, H. S. C. 1999c. Mammalian exocrine secretions XIV: Constituents of preorbital secretion of steenbok, *Raphicerus campestris. J. Chem. Ecol.* 25:2099–2108.

BURGER, B. V., SMIT, D., SPIES, H. S. C., SCHMIDT, C., SCHMIDT, U., TELITSINA, A. Y., and GRIERSON, G. R. 2001. Mammalian exocrine secretions XV. Constituents of secretion of ventral gland of male dwarf hamster, *Phodopus sungorus sungorus*. *J. Chem. Ecol.* 27:1259–1276.

ELLIN, R. I., FARRAND, R. L., OBERST, F. W., CRAUSE, C. L., BILLUPS, N. B., KOON, W. S., MUSSELMAN, N. P., and SIDELL, F. R. 1974. An apparatus for the detection and quantitation of volatile human effluents. *J. Chromatogr*. 100:137–152.

HUGGINS, G. R., and PRETI, G. 1976. Volatile constituents of human vaginal secretions. *Am. J. Obstet. Gynecol*. 126:129–136.

JORGENSON, J. W., NOVOTNY, M., CARMACK, M., COPLAND, G. B., WILSON, S. R., KATONA, S., and WHITTEN, W. K. 1978. Chemical constituents in the urine of the red fox (*Vulpes vulpes L.*) during the winter season. *Science* 199:796–798.

KAGAN, R., IKAN, R., and HABER, O. 1983. Characterization of a sex pheromone in the jird *(Meriones tristrami). J. Chem. Ecol.* 9:775–783.

LEDERER, E. 1946. Chemistry and biochemistry of the scent glands of the beaver, *Castor fiber. Nature* 157:231–232.

LEDERER, E. 1949. Chemistry and biochemistry of some mammalian secretions and excretions. *J. Chem. Soc*. 1949:2115–2125.

MADUBUNYI, L. C., HASSANALI, A., OUMA, W., NYARANGO, D., and KABII, J. 1996. Chemoecological role of mammalian urine in host location by tsetse, *Glossina spp.(Diptera: Glossinidae). J. Chem. Ecol.* 22:1187–1199.

MO, W.-P., BURGER, B. V., LE ROUX, M., and SPIES, H. S. C. 1995. Mammalian exocrine secretions IX: Constituents of preorbital secretion of oribi, *Ourebia orebi. J. Chem. Ecol*. 21:1191–1215.

OHLOFF, G. 1987. Resent developments in the field of naturally occurring aroma compounds. *Fortsch. Chem. Organ. Naturst.* 35:431–527.

SPENER, F., MANGOLD, H. K., SANSONE, G., and HAMILTON, J. G. 1969. Long-chain alkyl acetates in the preputial gland of the mouse. *Biochim. Biophys. Acta* 192:516–521.

STAFFORD, M., HORNING, M. G., and ZLATKIS, A. 1976. Profiles of volatile metabolites in body fluids. *J. Chromatogr.* 126:495–502.

VASILIEVA, N. Y., and FEOKTISTOVA, N. Y. 1994. Unusual function of integumentary glandular complex in Djungarian hamsters *(Phodopus campbelli). Chemical Signals in Vertebrates V11*, *Tübingen*, Germany, Proceedings, p. 49.

VOELTLER, D., JUNG, G., BREITMAIER, E., KÖNIG, W., GUPTA, D., and BREITMAIER, G. 1971. Isolierung von Oxindol aus Urin von Kindern. *Z. Naturforsch.* 26b:1380–1381.

WELLINGTON, J. L., BYRNE, K. J., PRETI, G., BEAUCHAMP, G. K., and SMITH, A. B. 1979. Perineal scent gland of wild and domestic guinea pigs. A comparative chemical and behavioral study. *J. Chem. Ecol.* 5:737–751.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

EFFECTS OF GENOTYPE, NUTRIENT AVAILABILITY, AND DEFOLIATION ON ASPEN PHYTOCHEMISTRY AND INSECT PERFORMANCE

TOD L. OSIER* and RICHARD L. LINDROTH

Department of Entomology
University of Wisconsin—Madison
Madison, Wisconsin

(Received September 12, 2000; accepted March 7, 2001)

Abstract—Genetic and environmental variability, and their interactions, influence phytochemical composition and, in turn, herbivore performance. We evaluated the independent and interactive effects of plant genotype, nutrient availability, and defoliation on the foliar chemistry of quaking aspen (*Populus tremuloides*) and consequences for performance of gypsy moths (*Lymantria dispar*). Saplings of four genotypes were grown under two conditions of nutrient availability and subjected to three levels of artificial defoliation. Concentrations of all secondary and primary metabolites evaluated responded to at least one or more of the experimental treatments. Of the secondary metabolites, phenolic glycosides were affected strongly by genotype, less so by nutrient availability, and not induced by defoliation. Condensed tannins were strongly dependent upon genotype, soil nutrient availability, and their interaction, and, in contrast to phenolic glycosides, were induced by artificial defoliation. Of the primary metabolites, foliar nitrogen was affected by genotype and soil nutrient availability. Starch concentrations were affected by genotype, nutrient availability, defoliation and interactions among these factors. Foliar water content responded to genotype, nutrient availability, and defoliation, and the effect of nutrient availability depended on genotype. Herbivore performance on these plants was strongly influenced by plant genotype and soil nutrient availability, but much less so by defoliation. Although several of the compound types (condensed tannins, starch, and water) responded to defoliation, quantitative variation in these compounds did not contribute to substantive changes in herbivore performance. Rather, the primary source of variation in insect performance was due to plant genotype (phenolic glycoside levels), while nutrient availability (foliar nitrogen levels) was of secondary importance. These results suggest that genetic variation in aspen plays a major role in determining patterns of insect performance, whereas environmental variation, such as was tested, here is of negligible importance.

* To whom correspondence should be addressed. e-mail: osier@entomology.wisc.edu. Current address: Department of Biology, Fairfield University, Fairfield, Connecticut 06430.

0098-0331/01/0700-1289$19.50/0

Key Words—Plant–insect interactions, genotypic variability, fertilization, defoliation, quaking aspen, *Populus tremuloides*, gypsy moth, *Lymantria dispar*, phenolic glycosides, condensed tannins.

INTRODUCTION

Quantitative variation in both primary and secondary plant chemistry is largely responsible for patterns of herbivore performance and distribution across available hosts (Krischik and Denno, 1983; Schultz, 1988; Bernays and Chapman, 1994; Berenbaum, 1995). Variation in phytochemistry is due to plant genotype, environment, and interactions between genotype and environment (Karban, 1992). Numerous studies have evaluated the independent effects of genotype (e.g., Foulds and Grime, 1972; Bowers and Stamp, 1992; Orians et al., 1993; Hwang and Lindroth, 1997; Rousi et al., 1997; Han and Lincoln, 1997) and environmental factors such as nutrient availability (e.g., Larsson et al., 1986; Bryant et al., 1987a,b; Björkman et al., 1991) and herbivore damage (e.g., Baldwin, 1988; Karban, 1993; Julkunen-Tiitto et al., 1995) on plant chemistry and plant–herbivore associations. However, the relative importance of, and interactions among, genetic and environmental factors remains poorly understood (Karban, 1992; Stiling and Rossi, 1996).

We used an experimental system including quaking (=trembling) aspen (*Populus tremuloides*) and the gypsy moth (*Lymantria dispar*) to test the effects of plant genotype, two environmental factors (nutrient availability and foliar damage), and their interactions on phytochemistry and herbivore performance. Quaking aspen is adapted to a variety of habitats (Mitton and Grant, 1996) and is the most widely distributed tree species in North America (Dickmann and Stuart, 1983). Quaking aspen has high genetic variability; variation can be observed for leaf and bark morphology, leaf phenology, and growth rate (Barnes, 1969; Dickmann and Stuart, 1983; Perala, 1990; Mitton and Grant, 1996). Moreover, clones vary in susceptibility to disease and herbivores (Barnes, 1969; Dickmann and Stuart, 1983; Perala, 1990). Across its range, quaking aspen is attacked by over 100 species of insects, a number of which are prone to population outbreaks (Perala, 1990). One such insect is the gypsy moth, which causes significant, but not uniform, defoliation to aspen during outbreaks in the Great Lakes region of the United States.

This system is well suited to studies of genotype $\times$ environment interactions because variation in aspen phytochemistry is known to have both genetic and environmental components (Lindroth and Hwang, 1996) and because induced responses have been documented for *Populus* species (Nef, 1988; Mattson and Palmer, 1988; Havill and Raffa, 1999). Variation in phytochemistry among clones has consistently been found to impact herbivores, and high concentrations of phenolic glycosides have strong and typically negative effects on herbivore development (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997, 1998) and

fecundity (Osier et al., 2000). Although less well studied than genotypic effects, environmental factors also alter phytochemistry and herbivore performance on aspen. For example, availability of resources (nutrients, CO_2, light) affects aspen phytochemistry and herbivore performance (Bryant et al., 1987b; Lindroth et al., 1993; Kinney et al., 1997; Hemming and Lindroth, 1999; Agrell et al., 2000). Induced chemical responses in aspen are less well understood. Previous research has documented rapidly induced responses in secondary chemistry as a result of foliar damage. Responses have included slight increases in phenolic glycoside content (Lindroth and Kinney, 1998) and increases in condensed tannin (Clausen et al., 1989; Roth et al., 1998) and total phenolic (Mattson and Palmer, 1988) concentrations. The demonstrated impact of such induced chemical changes on insect performance, however, has been small (Lindroth and Kinney, 1998) to nonexistent (Roth et al., 1998). Varied responses among studies with aspen suggest that genetic or environmental factors may modulate the induction response.

METHODS AND MATERIALS

Experimental Design. The experiment was a completely randomized design with four aspen genotypes, two levels of soil nutrients, and three levels of defoliation. Each combination of aspen genotype, nutrient availability, and defoliation (24 treatment combinations) was replicated with four independent saplings in a fully factorial design (a total of 96 saplings).

Aspen Clonal Material, Propagation and Growth Conditions. The four experimental genotypes represent a range of low to high levels of constitutive resistance (Hwang and Lindroth, 1997, 1998). Responses of the genotypes to nutrient availability and damage were unknown. The genotypes originated from root material collected from several sites in south-central Wisconsin (Hwang and Lindroth, 1997). Genotypes A, B, C, and D correspond to Wau 1 (Waushara County), Dan 1 and 2 (Dane County), and Sau 3 (Sauk County), respectively, of Hwang and Lindroth (1997). Root material for use in this study was obtained from saplings maintained for several years in a common garden on the University of Wisconsin–Madison campus.

The four aspen genotypes were propagated from root cuttings (as in Hwang and Lindroth, 1997) in summer 1996. Suckers were planted in 1-liter pots and grown outside until leaf drop in autumn 1996. Saplings were bare-rooted and overwintered in moist peat moss at 4°C. In the spring of 1997, saplings were potted individually in 16-liter pots containing a 7:3 mixture of sand and local field soil (silt loam). To manipulate nutrient availability, Osmocote 8–9 month slow release fertilizer (18:6:12 N-P-K + micronutrients) was added at a rate of 3.5 g/liter to high-nutrient pots; low nutrient pots received no fertilizer. Saplings were watered and maintained throughout the summer of 1997. In the spring of

1998, high-nutrient plants were treated (top dressed) for a second time with the same dose of fertilizer as used previously.

Artificial Damage. Saplings were defoliated May 14, 1998, to coincide with the period when attack by outbreaking herbivores, such as young gypsy moth or forest tent caterpillars, would be expected. The goals of our simulated herbivore feeding were to remove leaf area to produce carbon stress (mimicking leaf area removed by feeding) and to maximize damage along the cut edge [assuming the cue for induction would come from the remaining damaged portion of the leaf (Mattson and Palmer, 1988)]. To accommodate both goals, leaves were cut across the mid-rib near the base of the leaf. This procedure removed 90% of the area of each leaf damaged, yet produced a long cut edge. Herbivore damage was simulated using hair-thinning shears, which produce a more ragged cut than regular shears. The damage treatment had three levels: 0, 25, and 75% of the leaves on a plant damaged. Within each branch, leaf plastochron index 1, 2, 3, or 4 was chosen randomly as the starting point. In the 25% damage treatment, every fourth leaf from the starting point was damaged, and in the 75% damage treatment, every fourth leaf from the starting point was skipped.

Although studies have shown that mechanically damaged foliage is not always of identical quality to herbivore-damaged foliage (Baldwin, 1990; Hartley and Lawton, 1991), we chose to artificially damage our saplings for several reasons. First, artificial damage can be inflicted more uniformly across genotype and nutrient treatments than can damage by free-feeding insects. For example, we knew from previous studies that the well-defended genotype used in this study (genotype D) is extremely unpalatable to herbivores (Hwang and Lindroth, 1997, 1998) and that it would not be possible to inflict a uniform amount of insect damage among the replicates of this genotype. Second, free-feeding insects would likely feed selectively on the best foliage, leaving the poorer foliage for insect bioassays and chemistry collections. Such experimental artifacts would falsely indicate induction. Finally, a recent study with hybrid poplar (Havill and Raffa, 1999) showed that artificial damage and damage from feeding herbivores generated foliage of statistically similar quality for gypsy moths. Moreover, it showed that genotypes that responded to any induction stimulus (artificial damage or gypsy moth damage) were likely to respond similarly to others, that is, no genotype $\times$ elicitor type interaction was found.

Rationale for Magnitude of Treatments Applied. For the test of genotypic variation, we chose genotypes that span the known range of resistance against foliar-feeding Lepidoptera such as the gypsy moth, forest tent caterpillar, big poplar sphinx moth, and Canadian tiger swallowtail. Thus, our aspen genotypes represented a range of low (genotype A), moderate (genotypes B and C), and high (genotype D) levels of phenolic glycosides (Hwang and Lindroth, 1997). Likewise, for our nutrient treatment we applied levels that span the range from very poor growth (our low nutrient level) to optimal growth without overfertilization (our

high nutrient level) (Hemming and Lindroth, 1999). The nutrient levels we used produced 2.5-fold variation in size after one year (based on an index of height $\times$ basal diameter^2). Foliage of low-nutrient plants was more yellow, although not chlorotic, when compared to the deep green foliage of high-nutrient plants (Osier, personal observation). For the defoliation experiment, we used levels that span the range of defoliation experienced by aspen in the field (control, minimal damage without substantial carbon stress, and severe damage) as recommended by Neuvonen and Haukioja (1985). *Populus* species can withstand 25% defoliation (our low level) with no growth inhibition, whereas $\geq$75% defoliation (our high level) results in carbon stress and growth loss (Hodson, 1981; Bassman et al., 1982; Reichenbacker et al., 1996).

Insect Bioassays. To match the phenology of the larvae and foliage of the experimental saplings, bioassays began on May 24 (approximately four weeks after budbreak and 10 days after defoliation). At this time, the foliage was of appropriate age and toughness (fully expanded and nearly fully mature) for fourth stadium gypsy moths. Fourth instars were used in bioassays because responses to phytochemistry of this developmental stage are representative of much of the larval developmental period (Lindroth and Bloomer, 1991; Hwang and Lindroth, 1997; Hemming and Lindroth, 1999). Due to quarantine restrictions, bioassays were conducted using excised foliage in Percival growth chambers within the University of Wisconsin Biotron. Growth chambers were maintained at a 15L:9D photoperiod and 25/10°C to simulate early summer conditions in Madison, Wisconsin (National Climatic Data Center).

Gypsy moth egg masses were provided by USDA-APHIS (Otis Air National Guard Base, Massachusetts). Egg masses were surface sterilized in a solution of 0.1% sodium hypochlorite with 1% Tween 80 (Sigma, St. Louis, Missouri) as a surfactant. All larvae were reared on aspen foliage known to contain low concentrations of plant secondary compounds until the end of the third stadium. Upon molting into the fourth stadium, larvae were assigned randomly among the treatments. To reduce the potential of bias due to loss of experimental cells, multiple larvae (subsamples) were reared on foliage from each of the four replicate saplings per treatment combination: three larvae were reared per sapling for genotypes A, B, and C, and six larvae per sapling for genotype D (more larvae were used per sapling for genotype D because of anticipated mortality due to high levels of constitutive resistance). Because the sapling served as the experimental unit, average performance of each group of larvae reared on foliage from a particular sapling was used for statistical analysis. To control for the effects of leaf age in insect bioassays and chemistry collections, we collected foliage from only the initial leaf flush (which comprises >95% of available leaves in the spring) and avoided new leaves at indeterminately growing shoot tips. Foliage was removed in a haphazard manner from the remaining undamaged foliage located on the upper third of each sapling. To maintain leaf turgor and freshness, floral waterpiks were

used and foliage was changed at least every three days; treatment of foliage in this way has been shown to maintain foliar concentrations of aspen compounds important for insect feeding (Lindroth, unpublished data).

Larvae for use in the bioassays were restricted to females because with the experimental design employed (relatively low replication and minimal subsampling), there was a high probability of losing experimental cells due to nonuniform distribution of gender across replicates (i.e., all males or females on a sapling). Gender of newly molted fourth-instar larvae was determined by use of known weight distributions from previous studies. This approach was highly successful, as $>95\%$ of the larvae used in the experiment were females. At the conclusion of each bioassay, gender was definitively determined by inspection of the genital pores of the fifth stadium larvae. Males were removed from the study.

Final larval mass, stadium duration, and consumption were recorded for the fourth stadium. Herbivore relative growth rate (RGR) and efficiency of conversion of ingested food to biomass (ECI) were calculated as in Waldbauer (1968). Calculation of relative growth rate was modified to use initial biomass rather than average biomass as the relative term (Farrar et al., 1989).

Chemical Analyses. Foliage was collected from experimental saplings on May 29, 1998, two weeks after defoliation and approximately midway through the insect bioassays. Fifteen leaves were collected per sapling by snipping leaves cleanly at the petioles; removing leaves in this way has been shown not to induce a response from the ramet (Mattson and Palmer, 1988). After removal, leaves were transported to the laboratory in plastic bags on ice, flash-frozen in liquid nitrogen, freeze-dried in a cooled specimen chamber (-10°C), ground through 40 mesh in a Wiley mill, and stored at -20°C until analysis. Treatment of aspen leaf material in this manner preserves labile compounds such as phenolic glycosides (Lindroth and Koss, 1996).

Percent water was determined gravimetrically, by the ratio of leaf dry mass to fresh mass. To measure nitrogen levels, Kjeldahl acid digestions were conducted using the method of Parkinson and Allen (1975), followed by nitrogen quantification using the micro-Nesslerization method of Lang (1958). Glycine *p*-toluene-sulfonic acid (5.665% nitrogen) was similarly digested and served as the standard. Condensed tannins were exhaustively extracted from leaf tissue in 70% acetone at 4°C (with 10 mM ascorbic acid as an antioxidant). To quantify condensed tannins in the extract, we used the butanol–HCl method of Porter et al. (1986). As the standard, we used condensed tannins purified from aspen by the method of Hagerman and Butler (1980). Concentrations of phenolic glycosides were determined by high performance thin-layer chromatography as described by Lindroth et al. (1993). Salicortin and tremulacin purified from aspen leaves served as standards. Starch was determined by the enzymatic method of Schoeneberger et al. (unpublished method).

Statistical Analyses. The effects of aspen genotype, soil nutrient availability, defoliation, and their interactions on aspen phytochemistry and insect performance data were analyzed using fixed-effects analysis of variance [PROC MIXED, Version 8 (SAS Institute, 1989)] with a completely randomized design. Critical α was 5% for this study. When subsampling was used (i.e., multiple insects reared per sapling), a mean was generated so that the unit of replication was the sapling ($N = 4$). We did not use analysis of covariance for the analysis of herbivore performance parameters, contrary to the recommendation of Raubenheimer and Simpson (1992). Because of the subsampled nature of this design, variation in initial larval weights among saplings was very low after generating means within saplings. Therefore, ANCOVA was not used, as specified by the model fitting guidelines of Littell et al. (1996).

To relate gypsy moth performance to quantitative variation in aspen phytochemistry, we used stepwise multiple regression [PROC REG, Version 8 (SAS Institute, 1989)]. Stepwise regression in SAS uses a combination of forward selection ($\alpha = 0.10$) and backward elimination ($\alpha = 0.10$) to fit a model. We used group means for each defoliation × genotype × nutrient combination ($N = 24$). To illustrate the relative proportion of variance explained by individual independent variables (phytochemicals), the contributions of individual coefficients of determination (partial R^2) to the total (R^2) are described when applicable. This is a valid approach in multiple regression when independent variables (phytochemical concentrations) in the model are not intercorrelated (Sokal and Rohlf, 1995). This was the case for the phytochemicals related with insect relative growth rate and final mass in this study (Table 1 and Table 3). To investigate relationships among phytochemicals and between constitutive and induced levels of plant secondary compounds, we used correlation analyses [PROC CORR, Version 8 (SAS Institute, 1989)].

TABLE 1. CORRELATION MATRIX OF RELATIONSHIPS AMONG ASPEN PHYTOCHEMICALS FROM 24 COMBINATIONS OF PLANT GENOTYPE, NUTRIENT AVAILABILITY, AND DEFOLIATION[a]

	Phenolic glycosides		Condensed tannins		Nitrogen		Starch	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Condensed tannins	−0.283	0.180						
Nitrogen	−0.105	0.625	−0.770	<0.001				
Starch	−0.374	0.072	0.165	0.440	−0.268	0.203		
Water	−0.649	<0.001	−0.410	0.046	0.700	<0.001	0.164	0.442

[a] Pearson product–moment correlations are based on the mean values for each treatment.

RESULTS

Phytochemistry. Concentrations of both secondary and primary metabolites were universally affected by plant genotype and soil nutrient availability and in some cases were influenced by defoliation (Figures 1 and 2). Significant interactions among the factors were generally uncommon. Foliar concentrations of plant secondary metabolites (phenolic glycosides and condensed tannins) responded differently to the treatments (Figure 1). Phenolic glycoside concentrations were highly variable among genotypes and were slightly higher under conditions of high nutrient availability, but were unaffected by defoliation (Figure 1). Phenolic

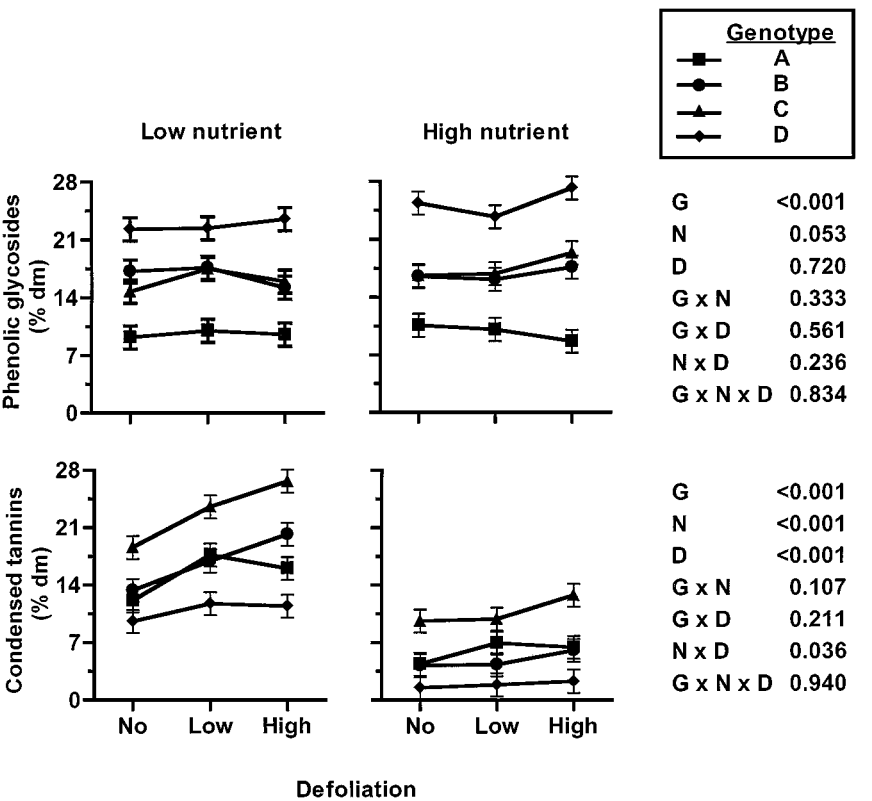


FIG. 1. Norm of reaction plots for phenolic glycoside and condensed tannin concentrations of aspen foliage in relation to nutrient availability and defoliation. *P* values indicate the results of three-way ANOVA: genotype (G) $df = 3$; nutrient availability (N) $df = 1$; defoliation (D) $df = 2$; G × N $df = 3$; G × D $df = 6$; N × D $df = 2$; G × N D $df = 6$. Each line represents a single aspen genotype. Vertical lines represent 1 standard error (calculated based on the pooled variance) of the mean.

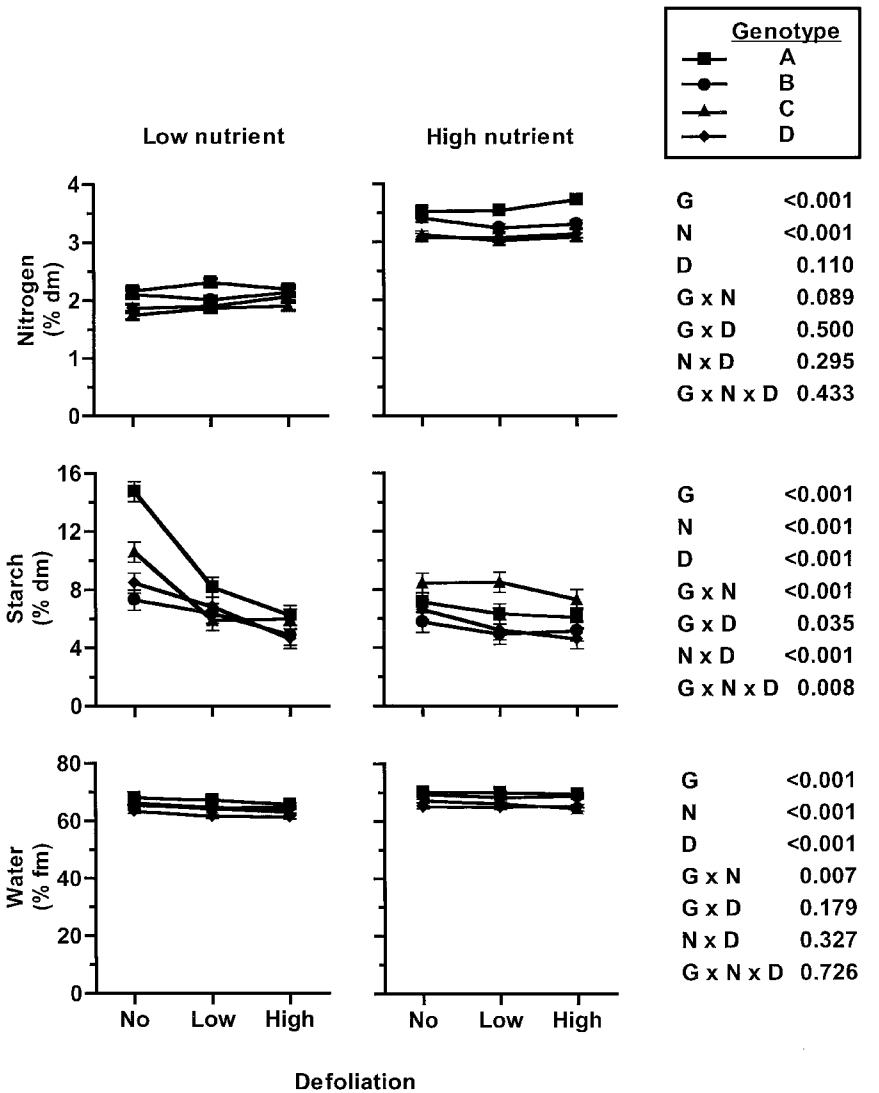


FIG. 2. Norm of reaction plots for foliar nitrogen, starch, and water concentrations of aspen foliage in relation to nutrient availability and defoliation. Format as in Figure 1.

glycoside concentrations were strongly dependent upon aspen genotype, which accounted for 93% of the variation explained (Figure 3). In contrast, concentrations of condensed tannins were strongly affected by all three treatments (Figure 1). Tannin concentrations averaged 1.8-fold higher under low nutrient availability and

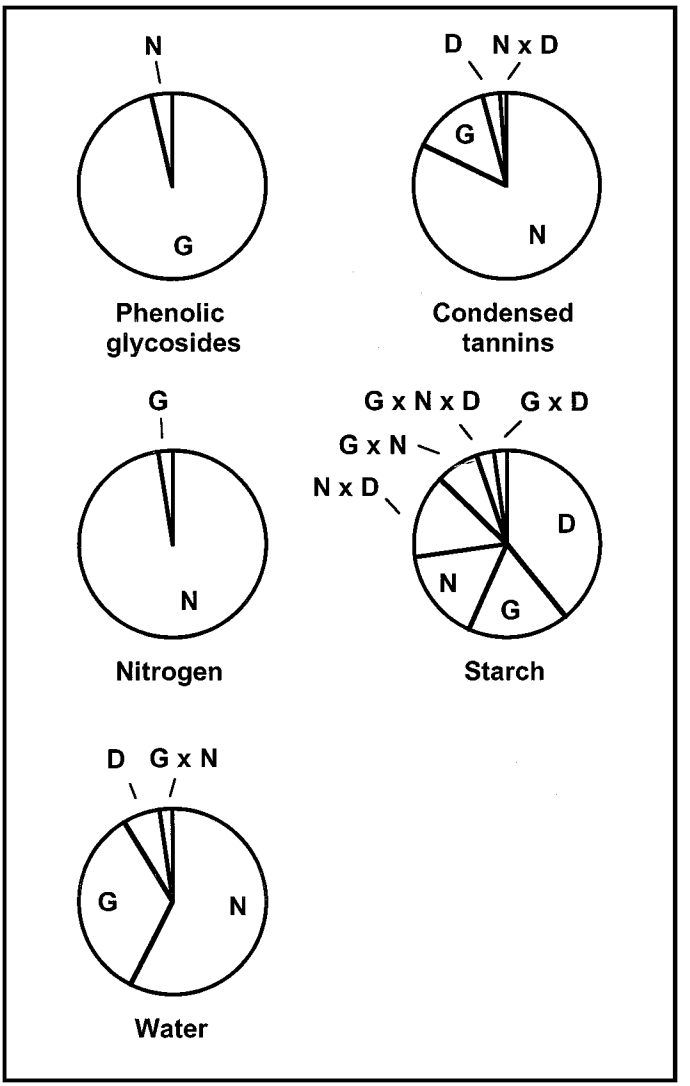


FIG. 3. Proportion of "explained variation" in relation to aspen genotype (G), nutrient availability (N), defoliation (D), and their interactions for phytochemical variables. For each variable, experimental treatments and interactions are ranked in decreasing order (clockwise from 12 o'clock) of the proportion of variation explained. Proportion of "explained variation" was calculated as: mean square for each treatment/total mean square explained. For the sake of clarity, only variation due to significant parameters is shown (in all cases variation due to nonsignificant factors was $<1\%$ of the total).

TABLE 2. CORRELATIONS BETWEEN INDUCED LEVELS OF CONDENSED TANNINS AND CONSTITUTIVE RESISTANCE (CONDENSED TANNINS, PHENOLIC GLYCOSIDES, OR THEIR COMBINATION) UNDER LOW AND HIGH NUTRIENT AVAILABILITY[a]

	Induced levels of tannins under the experimental treatments							
	Low damage, Low nutrient		Low damage, High nutrient		High damage, Low nutrient		High damage, High nutrient	
Constitutive levels	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Condensed tannins	0.566	0.435	−0.132	0.867	0.914	0.086	0.973	0.027
Phenolic glycosides	0.968	0.032	−0.682	0.318	−0.311	0.689	−0.621	0.379
Combined	0.572	0.428	−0.826	0.174	0.328	0.672	−0.087	0.913

[a] Pearson product–moment correlations are based on a mean value per treatment ($N = 4$ clones).

were induced by defoliation. In addition, the effect of defoliation was influenced by nutrient availability; condensed tannins were more strongly induced under conditions of low nutrient availability. Soil nutrient availability accounted for the greatest amount of the variation (82%) among treatments, whereas plant genotype, defoliation, and the nutrient × defoliation interaction accounted for only 13, 3, and 1% of the variation, respectively (Figure 3). Under conditions of low damage, induced levels of condensed tannins related positively to phenolic glycosides (low nutrient availability) (Table 2). Under conditions of severe damage, however, induced levels of tannins related positively to constitutive levels of tannins (Table 2).

Similar to the secondary metabolites, patterns of responses of primary metabolites varied among the treatments (Figure 2). Foliar nitrogen was minimally variable among the genotypes and increased 1.4-fold with nutrient addition, but was not affected by defoliation (Figures 2 and 3). Foliar concentrations of starch were variable among genotypes and reduced by nutrient addition and defoliation. Although all factors interacted significantly, the magnitude of the defoliation effect was reduced dramatically by high nutrient availability for some genotypes (Figures 2 and 3). Concentrations of foliar water were significantly affected by plant genotype, soil nutrient availability, and defoliation, but the magnitude of variation in response to these treatments was small compared to that of the other phytochemicals measured (Figure 1).

Insect Performance. Plant genotype and nutrient availability affected all gypsy moth performance parameters, whereas defoliation affected only herbivore relative growth rate (Figures 4 and 5). Insect relative growth rate was strongly affected by aspen genotype, which accounted for the greatest proportion (78%) of the variation (Figures 4 and 6). Nutrient availability, although highly significant, had less of an effect on growth rate than did plant genotype (Figures 4 and 6). Larvae grew faster feeding on foliage from the high-nutrient treatment, and this effect depended upon plant genotype (Figure 4). Relative growth rates were weakly

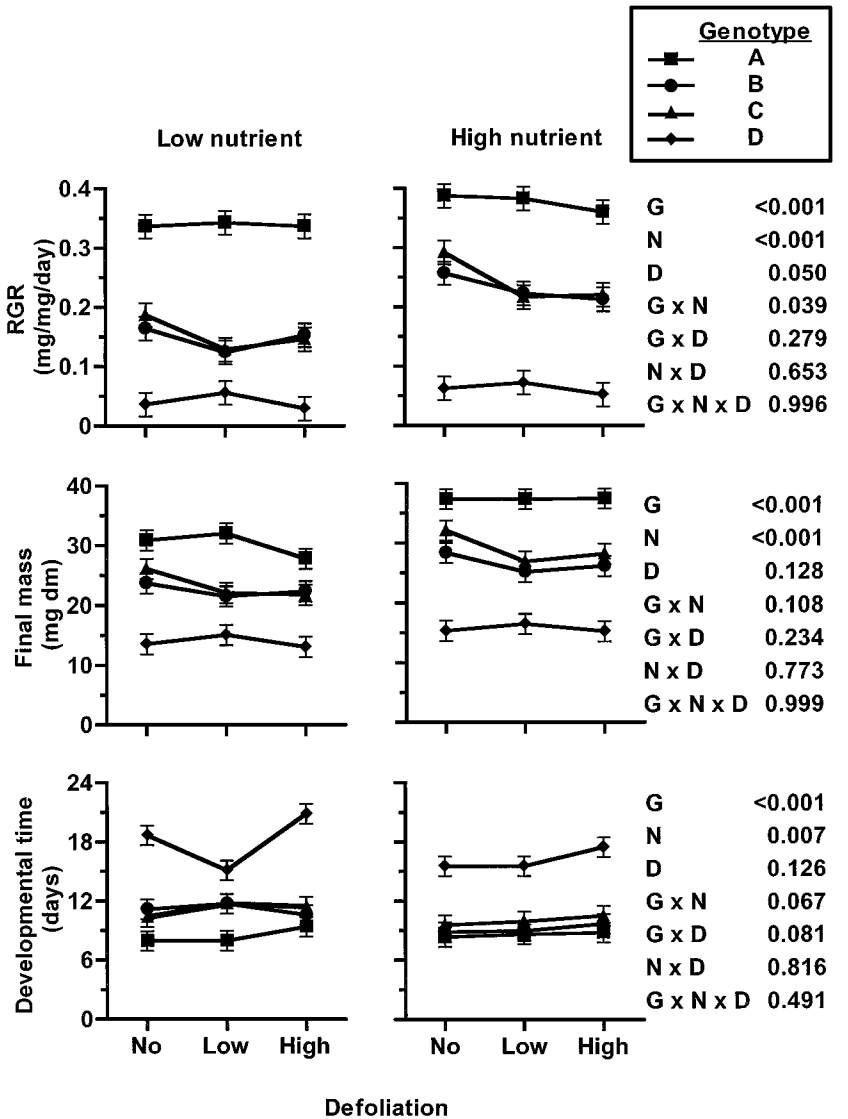


FIG. 4. Norm of reaction plots for gypsy moth relative growth rate, final mass, and developmental time in relation to nutrient availability and defoliation. Format as in Figure 1.

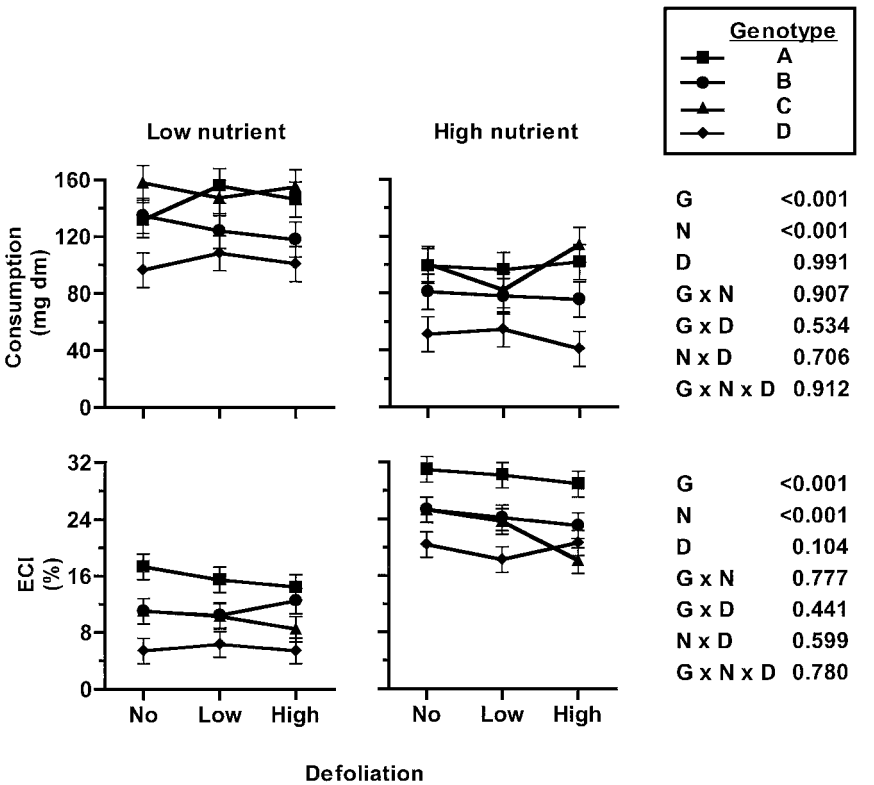


FIG. 5. Norm of reaction plots for gypsy moth consumption and utilization efficiency in relation to nutrient availability and defoliation. Format as in Figure 1.

affected by the defoliation treatments; insects feeding on defoliated plants grew more slowly than did those feeding on undamaged plants (Figure 4). Treatment effects on the dry mass of newly molted fifth-stadium larvae and on developmental time paralleled those of relative growth rate, except that a defoliation effect was not observed (Figures 4 and 6). Food consumption by larvae varied among the aspen genotypes and increased an average of 1.6-fold under low nutrient availability. Consumption was not, however, affected by defoliation treatment (Figures 5 and 6). Similarly, the efficiency of conversion of ingested food to biomass (ECI) was moderately affected by aspen genotype, enhanced by high nutrient availability, and unaffected by defoliation (Figures 5 and 6).

Relationship of Herbivore Performance to Phytochemistry. Stepwise regressions indicate that gypsy moth performance was related to concentrations of a number of phytochemicals; these correlations accounted for a large proportion of

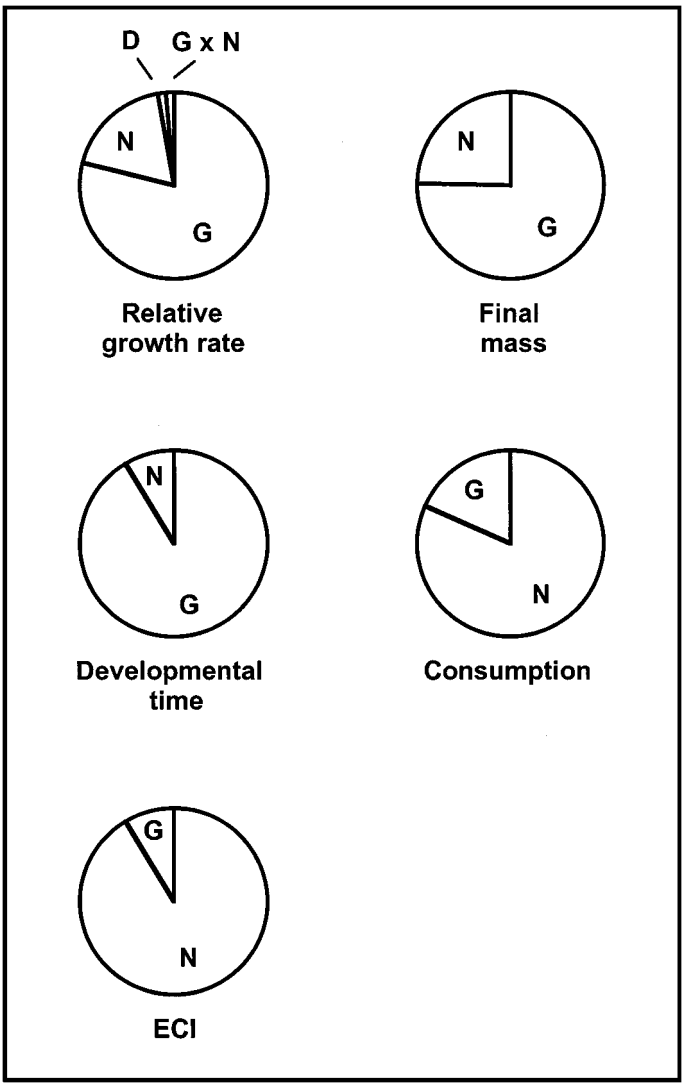


FIG. 6. Proportion of "explained variation" in relation to aspen genotype (G), nutrient availability (N), defoliation (D), and their interactions for insect performance variables. For each variable, experimental treatments and interactions are ranked in decreasing order (clockwise from 12 o'clock) of the proportion of variation explained. Proportion of "explained variation" was calculated as: mean square for each treatment/total mean square explained. For the sake of clarity, only variation due to significant parameters is shown (in all cases variation due to non-significant factors was $<1\%$ of the total).

TABLE 3. PHYTOCHEMICAL COMPONENTS ACCOUNTING FOR VARIATION IN GYPSY MOTH PERFORMANCE[a]

	Regression model			Partial regression components		
Parameter	Equation	R^2	P	Variable	R^2	P
Relative growth rate	$Y = 0.27 - 0.02(PG) + 0.07(N) + 0.01(S)$	0.957	<0.001	PG	0.818	<0.001
				N	0.128	<0.001
				S	0.012	0.030
Final dry mass	$Y = 33.34 - 1.19(PG) + 4.39(N)$	0.931	<0.001	PG	0.782	<0.001
				N	0.149	<0.001
Developmental time	$Y = 74.18 + 0.19(PG) - 0.97(W) - 0.18(CT)$	0.834	<0.001	PG	0.731	<0.001
				W	0.063	<0.020
				CT	0.040	<0.039
Consumption	$Y = 177.31 + 2.28(CT) - 2.70(PG) - 18.79(N)$	0.905	<0.001	CT	0.781	<0.001
				PG	0.083	0.002
				N	0.041	0.008
ECI	$Y = -92.10 + 7.59(N) + 1.35(W)$	0.964	<0.001	N	0.872	<0.001
				W	0.092	<0.001

Partial regression components for developmental time, consumption and ECI should be interpreted with caution due to intercorrelation of independent variables (see *Statistical Analyses* section).

[a] Stepwise multiple regressions, $\alpha = 0.10$ was used as the criterion for acceptance to, or rejection from, the model. CT = condensed tannins, N = nitrogen, PG = phenolic glycosides, S = starch, W = water.

the total variation in herbivore performance (Table 3). Relative growth rates were related negatively to foliar phenolic glycoside concentrations and positively to foliar nitrogen and starch concentrations (Table 3, Figure 7). Quantitative variation in these three types of compounds explained 96% of the among-treatment variation in relative growth rate (Table 3). Variation in phenolic glycoside concentrations explained a much greater proportion of the total variation in relative growth rate (82%) than did nitrogen (13%) or starch (1%). As was the case for relative growth rate, insect final mass was related negatively to phenolic glycoside concentrations and positively to nitrogen concentrations, explaining a total of 93% of variation (Table 3). Developmental time of gypsy moths was positively related to phenolic glycosides and negatively related to water and condensed tannin concentrations. These three foliar constituents accounted for a total of 83% of the variation in developmental time, and phenolic glycosides appeared most important (Table 3). Insect consumption was related positively to concentrations of condensed tannins and negatively to concentrations of phenolic glycosides and nitrogen. These three variables explained a total of 90% of the variation in insect consumption (Table 3). Two variables, nitrogen and water, explained 96% of the variation in insect growth efficiency (ECI; Table 3).

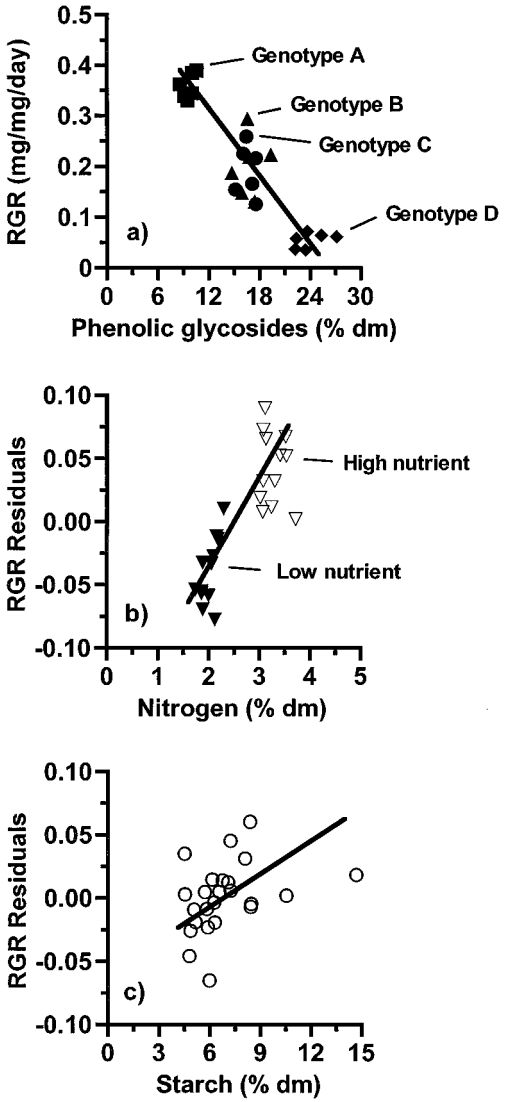


FIG. 7. Relationship of gypsy moth growth to concentrations of phytochemicals implicated as important by stepwise regression analyses. (a) The relationship of relative growth rate to phenolic glycoside concentrations; (b) the variation in relative growth rate unexplained by phenolic glycosides (residuals) plotted against foliar nitrogen concentrations; and (c) the variation in relative growth rate unexplained by both phenolic glycosides and nitrogen plotted against foliar starch concentrations.

To highlight how variation in phytochemical concentrations related to experimental treatments and likely determined patterns of herbivore performance, we present a series of figures illustrating results of the regression analyses for a single performance parameter (Figure 7). Insect relative growth rate was strongly and negatively related to phenolic glycoside concentrations. Variation in phenolic glycoside concentrations (along the *x* axis) is largely associated with aspen genotype (Figure 7a). A plot of the residuals (unexplained variation) from the first regression analysis versus nitrogen concentrations reveals a strong positive relationship (Figure 7b). Data points along the *x* axis are no longer grouped by genotype, but by nutrient availability, suggesting that soil nutrient availability is driving much of the variation in nitrogen concentrations. Finally, the remaining residuals are weakly and positively related to starch concentrations (Figure 7c). Variation in starch was affected by all treatments and their interactions, so position along the *x* axis is not clearly dominated by one or a combination of these.

DISCUSSION

Phytochemistry. Plant genotype, soil nutrient availability, and defoliation treatments affected phytochemical concentrations, although the magnitude and direction of responses to these treatments differed among chemical constituents. Aspen genotype was responsible for most of the variation in phenolic glycoside concentrations, while nutrient availability had a secondary effect. Nutrient addition resulted in a slight, but significant, increase in concentrations of phenolic glycosides. Such a response is not consistent with the predictions of the carbon–nutrient balance hypothesis (Bryant et al., 1983), growth–differentiation balance hypothesis (Herms and Mattson, 1992), or the protein competition model of phenolic allocation (Jones and Hartley, 1999). That phenolic glycosides responded positively to nutrient addition was unexpected given that in previous studies concentrations either responded as would be predicted (Hemming and Lindroth, 1999) or not at all (Kinney et al., 1997).

Condensed tannins responded most strongly to the nutrient treatment and were less influenced by plant genotype, defoliation, and the defoliation by nutrient interaction. As predicted by the carbon–nutrient balance hypothesis (Bryant et al., 1983), the growth–differentiation balance hypothesis (Herms and Mattson, 1992), and the protein competition model (Jones and Hartley, 1999), concentrations of condensed tannins were markedly lower under conditions of high nutrient availability. The short-term induction response of condensed tannins in our saplings is consistent with that of other studies using quaking aspen (Roth et al., 1998; Lindroth and Kinney, 1998). Furthermore, the induction response was ameliorated by the addition of nutrients. Such a pattern suggests a passive response due to altered carbon–nutrient balance, as demonstrated by Hunter and Schultz (1995)

for *Quercus prinus*, rather than an active defensive response on the part of the plant. Although the responses of tannins to nutrient addition accord with the predictions of the carbon–nutrient balance hypothesis, other phytochemical evidence suggests that the induction response is indeed an active response by the plant to defoliation, rather than a passive response driven by nutrient loss due to defoliation. The defoliated saplings appear to be under carbon (rather than nutrient) stress, indicated by the very low starch concentrations in these plants (as compared to undefoliated plants). The low carbon–nutrient ratios in the defoliated plants would predict low levels of tannins, rather than the dramatic increases observed.

The overall weak response of phenolic glycosides to environmental factors (both nutrient availability and defoliation) is consistent with other studies of aspen and is a striking contrast to the strong response of condensed tannins (Lindroth et al., 1993; Kinney and Lindroth, 1997; Roth et al., 1998; Hemming and Lindroth, 1999; Agrell et al., 2000). Although differences in environmental plasticity between phenolic glycosides and condensed tannins were striking, different suites of compounds within a plant can behave differently in response to environmental variability (Koricheva et al., 1998; Keinänen et al., 1999).

Although physiological responses to environmental conditions are important in determining allocation to phenolic glycosides and condensed tannins, allocation was found be largely genetically determined. The question arises as to what factors contribute to the maintenance, at the population level, of polymorphisms in concentrations of these secondary compounds. Polymorphisms in defensive allocation are likely driven, in part, by costs of production and storage (sensu Herms and Mattson, 1992). This appears to be the case with phenolic glycosides in quaking aspen, which are produced at a cost to growth (Osier and Lindroth, unpublished data). For condensed tannins, however, no ecological cost of production has been observed.

Trade-offs between constitutive defense and inducibility are considered as evidence that defenses are costly, although few such relationships have been demonstrated (Karban and Baldwin, 1997). In contrast to predictions, we found positive relationships between damage-induced changes in condensed tannins and constitutive levels of secondary metabolites under several nutrient–defoliation regimes. This is especially true for plants that were severely defoliated, as plants that had the highest constitutive levels of tannins also exhibited the strongest induced response. Our results differ from two other studies that reported negative correlations between induced and constitutive levels of phenolics in poplars. Mattson and Palmer (1988) reported a negative relationship between induced and constitutive levels of total phenolics in aspen clones with high levels of total phenolics, although a strong negative relationship was not observed across the entire range of genotypes used in that study. Nef (1988) found a negative relationship between induced and constitutive levels of total phenolics for three poplar hybrids. Both of these studies suggest that a negative relationship between induced and constitutive resistance

should be expected in aspen, but our results are strongly contradictory. One difference between our work and that of other researchers is that previous studies relied upon the method of Folin and Denis (1912), which measures not only "total phenolics," but also other compounds (e.g., reducing sugars) not quantified in our study.

Foliar nitrogen varied primarily in response to nutrient availability. Responses accorded well with predictions of the carbon–nutrient balance hypothesis (Bryant et al., 1983), as has been shown previously for aspen (Kinney and Lindroth, 1997; Hemming and Lindroth, 1999). Also similar to previous work (Hwang and Lindroth, 1997, 1998; Osier et al., 2000), foliar nitrogen varied among genotypes, although not dramatically so. Foliar starch concentrations decreased as a result of both nutrient addition and defoliation, as predicted by the carbon–nutrient balance hypothesis (Bryant et al., 1983; Tuomi et al., 1988). Moreover, the effect of defoliation was dependent upon nutrient availability; plants stressed by low nutrient availability experienced the greatest decrease in starch concentrations. Foliar water concentration was affected by plant genotype, soil nutrient availability, defoliation, and the interaction of genotype and nutrient availability. Although foliar water is important for herbivores (Scriber and Slansky, 1981), the variation in this study was small and unlikely to affect herbivore performance.

Herbivore Performance. Gypsy moth relative growth rates were most strongly affected by aspen genotype, less so by nutrient availability, and only minimally by defoliation and the interaction of genotype and nutrient availability. That plant genotype had a large effect on relative growth rates was not surprising, given that differences among aspen genotypes have been found to have similar effects in previous studies (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997, 1998; Osier et al., 2000). Although the effect of genotype was expected to be strong, its magnitude far exceeded that of the environmental treatments. As expected, insects performed better on fertilized plants. The pattern for herbivore final dry mass and developmental time closely followed the pattern for herbivore relative growth rate, except there was no defoliation main effect and the effect of soil nutrient availability did not depend upon genotype.

Phenolic glycoside concentrations were implicated as the primary determinant of host quality; insects feeding on foliage from saplings containing high concentrations of phenolic glycosides took longer to develop and attained a lower final mass, as has been found previously (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997, 1998; Osier et al., 2000). Studies in which purified phenolic glycosides were added to either foliage or artificial diet confirm the role of phenolic glycosides in altering host quality (Lindroth and Hemming, 1990; Hemming and Lindroth, 1995). In addition to phenolic glycosides, variation in nitrogen concentrations (due primarily to nutrient treatment) provided explanatory power for herbivore growth rate and final mass. This result is not surprising given the variation in nitrogen levels observed and that such variation is an important

determinant of insect performance (Mattson, 1980; Scriber and Slansky, 1981; Slansky, 1993).

Food consumption by gypsy moths was most dramatically influenced by nutrient availability. As is commonly observed (Mattson, 1980), larvae fed in a compensatory manner on the poorer quality foliage in the low-nutrient treatment. Although little evidence exists for aspen condensed tannins functioning as feeding deterrents or toxins to insects (Lindroth and Hwang, 1996), high concentrations of these compounds may have diluted nutrients and led to compensatory feeding. This response has been found previously (Osier et al., 2000) for condensed tannins in aspen and is similar to the response of insects to indigestible dietary components such as cellulose (Slansky, 1993). Larvae fed less on foliage containing high levels of phenolic glycosides and nitrogen, likely due to their feeding deterrent and nutritive properties, respectively. Although less important than nutrient availability, the magnitude of the genotype effect on consumption was similar to that observed previously for insects reared on aspen (Hwang and Lindroth, 1997, 1998). Insects fed foliage grown under low nutrient availability were much less efficient in converting food to body mass than were insects fed foliage from high-nutrient plants. As expected, the larvae were most efficient at converting ingested food to biomass when foliage contained high concentrations of nitrogen and water (Mattson, 1980; Scriber and Slansky, 1981; Slansky, 1993). Surprisingly, and in contrast to earlier research (Hwang and Lindroth, 1997), phenolic glycosides were not implicated as determinants of gypsy moth growth efficiency in this study. The absence of relationships may, in part, be a statistical artifact. Foliar water concentrations related strongly and negatively to those of phenolic glycosides (Table 1), and water may have served as a proxy for phenolic glycosides in the regression model.

The lack of a substantial damage-induced change in food quality was surprising given the importance of induced responses in many other systems (Karban and Baldwin, 1997). Absence of an effective induction response may explain why aspen forests are susceptible to outbreaks of spring-feeding insects such as gypsy moths, forest tent caterpillars (*Malacosoma disstria*), and large aspen tortrix (*Choristoneura conflictana*). Even though the defoliation treatment did not appear to markedly affect gypsy moth performance, we note that the aspen saplings did respond with a rapid induction of condensed tannins. This response may confer resistance against other types of enemies such as browsing mammals or pathogens at the site of wounding. Additionally, the possibility exists that extended duration of defoliation or repeated defoliation may alter the induction response observed (Karban and Baldwin, 1997). Other unpublished work with aspen, however, suggests that repeated defoliation (D. Parry, personal communication) or defoliation in the previous year (Osier and Lindroth, unpublished data) produces responses similar to those in this study.

Relative Importance of Plant Genotype and Environment. Results from other studies that compare the relative roles of plant genotype and environmental

conditions have implicated either genotype (Abrahamson et al., 1988; Hakulinen et al., 1995; Horner and Abrahamson, 1999) or environment (Orians and Fritz, 1996; Rossi and Stiling, 1998) as primary causes of variation important for herbivores. Studies with *Salix sericea* (another member of the Salicaceae) found results very different than ours; genotype was of relatively little importance compared to soil nutrient availability in determining the abundance of a suite of herbivores in the field (Orians and Fritz, 1996). Employing an experimental design similar to ours, a study with *Betula pendula* determined that fertilization explained most of the variation in insect performance and plant genotype explained less (Mutikainen et al., 2000). As with our study, however, defoliation (a delayed induced response) was relatively unimportant for insect feeding.

In addition to highlighting the importance of plant genotypic variation, our study revealed few interactions between genotype and environment for phytochemicals important for insect herbivores. The paucity of interactions between genotype and environment in our study is not surprising, given that such interactions have rarely been found to play a major role in other systems (Houle and Simard, 1996; Stiling and Rossi, 1996; but see also Horner and Abrahamson, 1999).

Because the relative strength of each treatment applied is important when attempting to rank treatment types, we endeavored to apply levels of each treatment that were biologically realistic and of comparable strength to the other treatments (see Methods and Materials). Even when environmental treatments were pushed nearly to extremes, environment and genotype $\times$ environment interactions were markedly less important than genotype in this study. Our results suggest that patterns of insect performance among aspen clones in the field are likely due to genetic variability rather than to environmental heterogeneity in nutrient availability or defoliation.

Acknowledgments—This research was supported by USDA NRI Competitive Grant 95-37302-1810 and UW Hatch Project 3931 to R.L.L. We thank Michelle Arnold, Brian Kopper, Sarah Wood, and Patrick Koss for technical assistance. Lynn Hummel and Laura Van Slyke provided invaluable assistance at the Walnut Street greenhouses. Thanks to Jen Klug for comments on the manuscript, Rick Nordheim for statistical advice, and Gary Bernon for providing gypsy moth eggs.

REFERENCES

ABRAHAMSON, W. G., ANDERSON, S. S., and MCCREA, K. D. 1988. Effects of manipulation of plant carbon nutrient balance on tall goldenrod resistance to a gallmaking herbivore. *Oecologia* 77:302–306.

AGRELL, J., MCDONALD, E. P., and LINDROTH, R. L. 2000. Effects of CO_2 and light on tree phytochemistry and insect performance. *Oikos* 88:259–272.

BALDWIN, I. T. 1988. Short-term damage-induced increases in tobacco alkaloids protect plants. *Oecologia* 75:367–370.

BALDWIN, I. T. 1990. Herbivory simulations in ecological research. *Tree* 5:91–93.

BARNES, B. V. 1969. Natural variation and delineation of clones of *Populus tremuloides* and *P. grandidentata* in northern lower Michigan. *Silvae Genet.* 18:130–142.

BASSMAN, J., MYERS, W., DICKMANN, D. I., and WILSON, L. 1982. Effects of simulated insect damage on early growth of nursery-grown hybrid poplars in northern Wisconsin, USA. *Can. J. For. Res.* 12:1–9.

BERENBAUM, M. R. 1995. The chemistry of defense—theory and practice. *Proc. Natl. Acad. Sci. U.S.A* 92:2–8.

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York.

BJÖRKMAN, C., LARSSON, S., and GREF, R. 1991. Effects of nitrogen-fertilization on pine needle chemistry and sawfly performance. *Oecologia* 86:202–209.

BOWERS, M. D., and STAMP, N. E. 1992. Chemical variation within and between individuals of *Plantago lanceolata* (Plantaginaceae). *J. Chem. Ecol.* 18:985–995.

BRYANT, J. P., CHAPIN, F. S. I., and KLEIN, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

BRYANT, J. P., CHAPIN, F. S. I., REICHARDT, P. B., and CLAUSEN, T. P. 1987a. Response of winter chemical defense in Alaska paper birch and green alder to manipulation of plant carbon/nutrient balance. *Oecologia* 72:510–514.

BRYANT, J. P., CLAUSEN, T. P., REICHARDT, P. B., MCCARTHY, M. C., and WERNER, R. A. 1987b. Effect of nitrogen-fertilization upon the secondary chemistry and nutritional-value of quaking aspen (*Populus tremuloides* Michx) leaves for the large aspen tortrix (*Choristoneura conflictana* Walker). *Oecologia* 73:513–517.

CLAUSEN, T. P., REICHARDT, P. B., BRYANT, J. P., WERNER, R. A., POST, K., and FRISBY, K. 1989. Chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores. *J. Chem. Ecol.* 15:2335–2346.

DICKMANN, D. I., and STUART, K. W. 1983. The Culture of Poplars in Eastern North America. Michigan State University, East Lansing, Michigan.

FARRAR, R. R., BARBOUR, J. D., and KENNEDY, G. G. 1989. Quantifying food consumption and growth in insects. *Ann. Entomol. Soc. Am.* 82:593–598.

FOLIN, O., and DENIS, W. 1912. On phosphotungstic–phosphomolybdic compounds and color reagents. *J. Biol. Chem.* 12:239–243.

FOULDS, W., and GRIME, J. P. 1972. The response of cyanogenic and acyanogenic phenotypes of *Trifolium repens* to soil moisture supply. *Heredity* 28:181–187.

HAGERMAN, A. E., and BUTLER, L. G. 1980. Condensed tannin purification and characterization of tannin-associated proteins. *J. Agric. Food Chem.* 28:947–952.

HAKULINEN, J., JULKUNEN-TIITTO, R., and TAHVANAINEN, J. 1995. Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism. *Trees—Struct. Funct.* 9:235–240.

HAN, K., and LINCOLN, D. E. 1997. The impact of plasticity and maternal effect on the evolution of leaf resin production in *Diplacus aurantiacus. Evol. Ecol.* 11:471–484.

HARTLEY, S. E., and LAWTON, J. H. 1991. Biochemical aspects and significance of the rapidly induced accumulation of phenolics in birch foliage, pp. 105–132, *in* D. W. Tallamy and M. J. Raupp (eds.). Phytochemical Induction by Herbivores. Wiley, New York.

HAVILL, N. P., and RAFFA, K. F. 1999. Effects of elicitation treatment and genotypic variation on induced resistance in *Populus*: Impacts on gypsy moth (Lepidoptera: Lymantriidae) development and feeding behavior. *Oecologia* 120:295–303.

HEMMING, J. D. C., and LINDROTH, R. L. 1995. Intraspecific variation in aspen phytochemistry: Effects on performance of gypsy moths and forest tent caterpillars. *Oecologia* 103:79–88.

HEMMING, J. D. C., and LINDROTH, R. L. 1999. Effects of light and nutrient availability on aspen: Growth, phytochemistry, and insect performance. *J. Chem. Ecol.* 25:1687–1714.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: To grow or defend. *Q. Rev. Biol.* 67:283–335.

HODSON, A. C. 1981. The response of aspen (*Populus tremuloides*) to artificial defoliation. *Great Lakes Entomol.* 14:167–169.

HORNER, J. D., and ABRAHAMSON, W. G. 1999. Influence of Plant genotype and early season water deficits on oviposition preference and offspring performance in *Eurosta solidaginis* (Diptera: Tephritidae). *Am. Midl. Nat.* 142:162–172.

HOULE, G., and SIMARD, G. 1996. Additive effects of genotype, nutrient availability and type of tissue damage on the compensatory response of *Salix planifolia* ssp. *planifolia* to simulated herbivory. *Oecologia* 107:373–378.

HUNTER, M. D., and SCHULTZ, J. C. 1995. Fertilization mitigates chemical induction and herbivore responses within damaged oak trees. *Ecology* 76:1226–1232.

HWANG, S.-Y., and LINDROTH, R. L. 1997. Clonal variation in foliar chemistry of aspen: Effects on gypsy moths and forest tent caterpillars. *Oecologia* 111:99–108.

HWANG, S.-Y., and LINDROTH, R. L. 1998. Consequences of clonal variation in aspen phytochemistry for late season herbivores. *Ecoscience* 5:508–516.

JONES, C. G., and HARTLEY, S. E. 1999. A protein competition model of phenolic allocation. *Oikos* 86:27–44.

JULKUNEN-TIITTO, R., BRYANT, J. P., KUROPAT, P., and ROININEN, H. 1995. Slight tissue wounding fails to induce consistent chemical defense in 3 willow (*Salix* spp.) clones. *Oecologia* 101:467–471.

KARBAN, R. 1992. Plant variation: Its effects on populations of herbivorous insects, pp. 195–215, *in* R. S. FRITZ and E. L. SIMMS (eds.). Plant Resistance to Herbivores and Pathogens. University of Chicago Press, Chicago.

KARBAN, R. 1993. Costs and benefits of induced resistance and plant density for a native shrub, *Gossypium thurberi. Ecology* 74:9–19.

KARBBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago.

KEINÄNEN, M., JULKUNEN-TIITTO, R., MUTIKAINEN, P., WALLS, M., OVASKA, J., and VAPAAVUORI, E. 1999. Trade-offs in phenolic metabolism of silver birch: Effects of fertilization, defoliation, and genotype. *Ecology* 80:1970–1986.

KINNEY, K. K., and LINDROTH, R. L. 1997. Responses of three deciduous tree species to atmospheric CO_2 and soil NO_3^- availability. *Can. J. For. Res.* 27:1–10.

KINNEY, K. K., LINDROTH, R. L., JUNG, S. M., and NORDHEIM, E. V. 1997. Effects of CO_2 and soil NO_3^- availability on deciduous trees: Phytochemistry and insect performance. *Ecology* 78:215–230.

KORICHEVA, J., LARSSON, S., HAUKIOJA, E., and KEINÄNEN, M. 1998. Regulation of woody plant secondary metabolism by resource availability: Hypothesis testing by means of meta-analysis. *Oikos* 83:212–226.

KRISCHIK, V. A., and DENNO, R. F. 1983. Individual, population, and geographic patterns in plant defense, pp. 463–512, *in* R. F. Denno and M. S. McClure (eds.). Variable Plants and Herbivores in Natural and Managed Systems. Academic Press, New York.

LANG, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. *Anal. Chem.* 30:1692–1694.

LARSSON, S., WIREN, A., LUNDGREN, L., and ERICSSON, T. 1986. Effects of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* and susceptibility to *Galerucella lineola* (Coleoptera). *Oikos* 47:205–210.

LINDROTH, R. L., and BLOOMER, M. S. 1991. Biochemical ecology of the forest tent caterpillar: Responses to dietary protein and phenolic glycosides. *Oecologia* 86:408–413.

LINDROTH, R. L., and HEMMING, J. D. C. 1990. Responses of the gypsy moth (Lepidoptera: Lymantriidae) to tremulacin, an aspen phenolic glycoside. *Environ. Entomol.* 19:842–847.

LINDROTH, R. L., and HWANG, S.-Y. 1996. Diversity, redundancy, and multiplicity in chemical defense systems of aspen, pp. 25–56, *in* J. T. Romeo, J. A. Saunders, and P. Barbosa (eds.). Phytochemical Diversity and Redundancy in Ecological Interactions. Plenum Press, New York.

LINDROTH, R. L., and KINNEY, K. K. 1998. Consequences of enriched atmospheric CO_2 and defoliation: Chemistry and gypsy moth performance. *J. Chem. Ecol.* 24:1677–1695.

LINDROTH, R. L., and KOSS, P. A. 1996. Preservation of Salicaceae leaves for phytochemical analyses: Further assessment. *J. Chem. Ecol.* 22:765–771.

LINDROTH, R. L., KINNEY, K. K., and PLATZ, C. L. 1993. Responses of deciduous trees to elevated atmospheric CO_2: Productivity, phytochemistry, and insect performance. *Ecology* 74:763–777.

LITTELL, R. C., MILLIKEN, G. A., STROUP, W. W., and WOLFINGER, R. D. 1996. SAS System for Mixed Models. SAS Institute Inc., Cary, North Carolina.

MATTSON, W. J. 1980. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. Syst.* 11:119–161.

MATTSON, W. J., and PALMER, S. R. 1988. Changes in levels of foliar minerals and phenolics in trembling aspen, *Populus tremuloides*, in response to artificial defoliation, pp. 157–169, *in* W. J. Mattson, J. C. Levieux, and B. Dagan (eds.). Mechanisms of Woody Plant Defences Against Insects: Search for Pattern. Springer-Verlag, New York.

MITTON, J. B., and GRANT, M. C. 1996. Genetic variation and the natural history of quaking aspen. *Bioscience* 46:25–31.

MUTIKAINEN, P., WALLS, M., OVASKA, J., KEINÄNEN, M., JULKUNEN-TIITTO, R., and VAPAAVUORI, E. 2000. Herbivore resistance in *Betula pendula*: Effect of fertilization, defoliation, and plant genotype. *Ecology* 81:49–65.

NEF, L. 1988. Interactions between the leaf miner, *Phyllocnistis suffusella*, and poplars, pp. 239–251, in W. J. Mattson, J. C. Levieux, and B. Dagan (eds.). Mechanisms of Woody Plant Defences Against Insects: Search for Pattern. Springer-Verlag, New York.

NEUVONEN, S., and HAUKIOJA, E. 1985. How to study induced plant resistance. *Oecologia* 66:456–457.

ORIANS, C. M., and FRITZ, R. S. 1996. Genetic and soil–nutrient effects on the abundance of herbivores on willow. *Oecologia* 105:388–396.

ORIANS, C. M., FRITZ, R. S., and CLAUSEN, T. P. 1993. The genetic basis for variation in the concentration of phenolic glycosides in *Salix sericea*: Clonal variation and sex-based differences. *Biochem. Syst. Ecol.* 21:535–542.

OSIER, T. L., HWANG, S. Y., and LINDROTH, R. L. 2000. Effects of phytochemical variation in quaking aspen *Populus tremuloides* clones on gypsy moth *Lymantria dispar* performance in the field and laboratory. *Ecol. Entomol.* 25:197–207.

PARKINSON, J. A., and ALLEN, S. E. 1975. A wet oxidation procedure suitable for the determination of nitrogen and mineral nutrients in biological material. *Commun. Soil Sci. Plant Anal.* 6:1–11.

PERALA, D. A. 1990. *Populus tremuloides* Michx. quaking aspen, *in* R. M. Burns and B. H. Honkala (eds.). Silvics of North America. United States Department of Agriculture Forest Service, Washington, D.C.

PORTER, L. J., HRSTICH, L. N., and CHAN, B. G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 25:223–230.

RAUBENHEIMER, D., and SIMPSON, S. J. 1992. Analysis of covariance: An alternative to nutritional indices. *Entomol. Exp. Appl.* 62:221–231.

REICHENBACKER, R. R., SCHULTZ, R. C., and HART, E. R. 1996. Artificial defoliation effect on *Populus* growth, biomass production, and total nonstructural carbohydrate concentration. *Environ. Entomol.* 25:632–642.

ROSSI, A. M., and STILING, P. 1998. The interactions of plant clone and abiotic factors on a gall–making midge. *Oecologia* 116:170–176.

ROTH, S. K., LINDROTH, R. L., VOLIN, J. C., and KRUGER, E. L. 1998. Enriched atmospheric CO_2 and defoliation: Effects on tree chemistry and insect performance. *Global Change Biol.* 4:419–430.

ROUSI, M., TAHVANAINEN, J., HENTTONEN, H., HERMS, D. A., and UOTILA, I. 1997. Clonal variation in susceptibility of white birches (*Betula* spp.) to mammalian and insect herbivores. *For. Sci.* 43:396–402.

SAS INSTITUTE. 1989. SAS User's Guide: Statistics, Versions 8. SAS Institute Inc., Cary, North Carolina.

SCHULTZ, J. C. 1988. Many factors influence the evolution of herbivore diets, but plant chemistry is central. *Ecology* 69:896–897.

SCRIBER, J. M., and SLANSKY, F. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol.* 26:183–211.

SLANSKY, F. 1993. Nutritional ecology: the fundamental quest for nutrients, pp. 29–91, *in* N. E. Stamp and T. M. Casey (eds.). Caterpillars: Ecological and Evolutionary Constraints on Foraging. Chapman and Hall, New York.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry, 3rd ed. Freeman and Company, New York.

STILING, P., and ROSSI, A. M. 1996. Complex effects of genotype and environment on insect herbivores and their enemies. *Ecology* 77:2212–2218.

TUOMI, J., NIEMELÄ, P., CHAPIN, F. S. I., BRYANT J. P., and SIREN, S. 1988. Defensive responses of trees in relation to their carbon/nutrient balance, pp. 57–72, *in* W. J. Mattson, J. C. Levieux, and B. Dagan (eds.). Mechanisms of Woody Plant Defences Against Insects: Search for Pattern. Springer-Verlag, New York.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

IMPORTANCE OF BACTERIAL DECOMPOSITION AND CARRION SUBSTRATE TO FORAGING BROWN TREESNAKES

S. M. JOJOLA-ELVERUM, J. A. SHIVIK, and L. CLARK*

United States Department of Agriculture
Animal and Plant Health Inspection Service, Wildlife Services
National Wildlife Research Center
4101 LaPorte Ave., Fort Collins, Colorado 80521

(Received October 12, 2000; accepted March 6, 2001)

Abstract—Brown treesnakes are an invasive species to the island of Guam that have caused extensive ecological and economic damage. Efforts to control the snake population have included trapping using live mouse lures, but for logistical and economic reasons a synthetic lure is needed. When searching for live food, brown treesnakes use both visual and odor cues. However, when searching for carrion, odor cues are sufficient. Attempts to develop synthetic lures based on chemical reconstruction of the complex carrion odor have not succeeded. We provide evidence that a microbial–substrate interaction is important for bait take by brown treesnakes. Microbial cultures taken from mouse carrion indicate that *Enterobacter agglomerans* is the predominant bacterium, and field tests suggest that this organism may be important to odor production that attracts brown treesnakes. This information may prove useful in the development of microbial-based biological reactors that could be formulated to produce a continuous stream of odor of sufficient complexity so as to be attractive to foraging snakes.

Key Words—Bacteria, bait, *Boiga irregularis*, brown treesnake, carcass, carrion, decomposition, *Enterobacter agglomerans*, lure, odor, rot

INTRODUCTION

The brown treesnake (*Boiga irregularis*) was accidentally introduced to Guam in the 1940s or early 1950s (Savidge, 1987; Rodda et al., 1992). Since then the island's snake population has increased dramatically, at times reaching densities of 50–100 snakes/ha (Rodda et al., 1992). The ecological and economic effect of the

* To whom correspondence should be addressed.

0098-0331/01/0700-1315$19.50/0

snake has been devastating, ranging from power outages to extirpation of avifauna and herpetofauna (Savidge, 1984, 1987; Fritts et al., 1987; Conry, 1988; Engbring and Fritts, 1988; Rodda and Fritz, 1992a). Guam's importance as a shipping hub makes the spread of brown treesnakes from Guam a threat to other sensitive Pacific Island ecosystems (e.g., Fritts, 1988). As part of a containment policy, the U.S. Department of Agriculture employs a variety of methods to reduce the likelihood that brown treesnakes will be exported inadvertently from Guam. These methods include trapping, search dogs, and hand removal of snakes from fences during spotlight searches (U.S. Department of Agriculture, 1996).

Trapping is a highly effective method for removing snakes from an area (Rodda and Fritts, 1992b; U.S. Department of Agriculture, 1996; Engeman et al., 1998). However, since live mice are used as lures, this method involves substantial logistical effort in field and laboratory maintenance, which in turn limits the number of traps that can be employed, especially for large-scale operations (Fritts et al., 1989; Shivik and Clark, 1999a). As a consequence, considerable research effort has been invested in searching for artificial lures that would reduce reliance on live mice, and hence reduce the labor needed to carry out an effective control operation using traps.

For live prey, brown treesnakes attend to both visual and olfactory cues (Chiszar et al., 1988, 1993, 1997a; Fritts et al., 1989; Shivik, 1998; Shivik and Clark, 1999a). Presentation of a combination of cues from these modalities is necessary to produce the most intense investigatory and foraging behavior in brown treesnakes (Chiszar et al., 1997b; Shivik, 1998; Lindberg et al., 2000). Somewhat surprisingly, brown treesnakes also actively forage for carrion in the field, and the carrion's effectiveness for capture is only slightly less than that seen for live mice (Shivik, 1998; Shivik and Clark, 1997, 1999a,b; Shivik et al., 2000). However, most notable was the observation that chemosensory cues were predominantly critical to brown treesnakes when foraging for carrion. Thus, the potential for the synthesis of an inanimate chemical lure exists. Unfortunately, attempts to reconstruct an effective chemical lure based on the chemical analysis of mouse carrion has proved difficult and ineffective because of the chemical complexity of the carrion odor and the snakes' lack of a reaction to imperfect chemosensory cues (Clark, 1997; Shivik, 1998; Shivik and Clark, 1999a). In this study, we set out to better understand the source and nature of the attractive odor of carrion that would be helpful in the development of a bioreactor that would generate salient odors. Because the odor of carrion decomposition is largely a result of a substrate–bacterial interaction, we hypothesized that the most attractive odors to brown treesnakes would be generated from the surface of mice. We reasoned that knowledge about the appropriate nutrient substrate and the bacteria responsible for decomposition would be helpful in guiding us to construct biological (microbial) reactors.

METHODS AND MATERIALS

We conducted experiments in the Conventional Weapons Storage Area on Andersen Air Base near Tarague and Haputo beaches, Guam, in May and June 2000. The disappearance of dead neonatal mice (DNM) from PVC tubes (i.e., bait take) was used as an index of acceptance of carrion by snakes. Analysis of over 1000 hr of video tape indicates that brown treesnakes are almost exclusively the sole vertebrate responsible for the disappearance of baits (Clark, personal observation).

Prior to an experiment PVC tubes (10.1 × 30.5 cm) were cleaned with mild chlorine bleach solution (1:100 chlorine–water) and rinsed with water. Patches of vegetation where PVC tubes were placed were systematically divided by a grid of roads. These patches of vegetation were approximately 6–12 ha, depending upon the spacing of roads (Savarie et al., 2001). Following cleansing, PVC tubes were suspended from strings at heights of 1.5–2 m in shrubs and trees and linearly placed along the forest edge adjacent to roads at 20-m intervals. When multiple transects were set out on the same day, the minimum distance between transects was 50 m, separated by road. Previous studies showed that the probability of snakes traveling this or longer distances in a given night was low (Savarie et al., 2001), hence transects could be considered independent experimental units.

Each transect consisted of $k \cdot r$ PVC tubes, where k was the number of treatment levels and r was the number of blocks within a transect. The number of blocks within transects was constant across all experiments ($r = 10$). The k treatment levels were randomly ordered within each block, r, such that each of the treatment levels was represented once within each block. We used transect as the statistical experimental unit. For each treatment level, k, the dependent variable was calculated as the proportion of baits missing, i.e., the number of baits missing divided by $r = 10$. Thus, the number of replicates for comparisons among treatment levels, k, for any given experiment corresponded to the number of transects, N.

Our experiments addressed three broad biological questions. In the first set of experiments we evaluated how the stage of decomposition of dead mice affected bait take by snakes. In a second set of experiments we evaluated how different parts of decomposing mice influenced bait take by snakes. In a third set of experiments we evaluated whether microbial degradation of carrion affected bait take by snakes.

Set 1

Experiment 1: Importance of Decomposition of Carrion for Lure Attractiveness. The objective of this experiment was to determine the attractiveness of fresh (<24 hr old) whole dead neonatal mice relative to 1-day old (24–48 hr) whole dead neonatal mice. In the latter case, the mice were aged under natural conditions.

Subsequently, both sets of mice were placed in the field. This experiment consisted of two treatment levels ($k = 2$) with $r = 10$ blocks per transect ($N = 4$), for a total of 20 PVC tubes per transect ($k \cdot r$). PVC tubes were checked for presence or absence of baits after 24 hr. Following the experiment, we removed, cleaned, and placed PVC tubes in a new, isolated edge of forest in preparation for monitoring the next transect in the experiment. Patterns of bait take were analyzed using a *t* test, where treatment was the between-measures factor and the proportion of baits taken was the response variable (StatSoft, 1994).

Set 2

Experiment 2: Whole vs. Reconstituted Mouse Lures. The objective of this experiment was to determine whether the modification of carrion was important for the attractiveness of the lure. This experiment consisted of two treatment levels ($k = 2$) with $r = 10$ blocks per transect ($N = 7$), for a total of 20 PVC tubes per transect ($k \cdot r$). For one treatment level we ground whole dead neonatal mice ($N = 10$) in a blender and rolled the ground meat and fur into balls (DMG). The second treatment level, the control, consisted of unaltered whole dead neonatal mice (DNM) ($N = 10$). PVC tubes were checked for presence or absence of baits after 24 and 48 hr. Placement, scoring, and cleaning of PVC tubes were similar to experiment 1. Bait take was analyzed using a two-way repeated-measures analysis of variance, where treatment was the between-measures factor, day was the within-measures (i.e., repeated) effect, and the proportional cumulative bait take was the response metric (Winer 1971, p. 520). Tukey's HSD test was used to determine the post-hoc differences among means for the main effect, and simple orthogonal contrasts were used to compare group means for the interaction term (StatSoft, 1994).

Experiment 3: Attractiveness of Mouse Parts and Tofu-wrapped Pelts. Grinding and combining mouse parts substantially reduced the attractiveness of baits to foraging brown treesnakes (experiment 2). Therefore, the objective of this experiment was to determine whether dissected, intact mouse parts were attractive baits. To separate pelts from carcasses (C), we thawed dead neonatal mice and carefully skinned them using dissecting scissors. We removed pelts at the wrist, ankle, tail bone, and rostrum; then wrapped the pelt around a piece of tofu (P_{tof}). The tofu served as a moisture source in the field to prevent the pelt from desiccating. All three bait types (C, P_{tof}, DNM) were placed into separate plastic cups, sealed, and kept cold until transported to the field. The methods for bait placement, assigning treatment levels within transects, monitoring PVC tubes, and analysis were identical to those described in experiment 2. This experiment consisted of three treatment levels ($k = 3$) with $r = 10$ blocks per transect ($N = 6$), for a total of 30 PVC tubes per transect ($k \cdot r$).

Experiment 4: Attractiveness of Mouse Parts and Cotton-wrapped Pelts. The objective of this experiment was to determine the attractiveness of pelts with moistened cotton as a moisture source (P_{cot}) and pelts without a moisture source (P) relative to DNM. The methods for bait placement, assigning treatment levels within transects, monitoring PVC tubes, and analysis were identical to those described above. This experiment consisted of three treatment levels ($k = 3$) with $r = 10$ blocks per transect ($N = 5$), for a total of 30 PVC tubes per transect ($k \cdot r$).

Set 3

Experiment 5: Attractiveness of Baits Treated with Alcohol. Because we found that pelts were taken at the same rate as DNM and that skinned carcasses were taken at lower rates (experiment 3), we limited further testing on the source of the lure odor cue to pelts in comparison to DNM. In this set of experiments, we treated pelts in one of two ways, working under the hypothesis that surface bacteria may be responsible for generating the odors to which snakes were attracted. In the first series of tests, pelts were prepared as described in experiment 3. No special treatment was afforded to nontreated pelts wrapped around tofu (P_{nt}). A second set of pelts (P_{alc}) was treated using alcohol. Briefly, pelts and tofu were separately soaked in 70% ethyl alcohol for 5 min prior to being wrapped together. Treated baits were placed in disinfected plastic cups, sealed for transport to the field, then placed into PVC tubes sprayed with 70% ethyl alcohol. PVC tubes and baits were again sprayed after bait placement to ensure a sanitary local environment, after which natural conditions were allowed to prevail. The methods for bait placement, assigning treatment levels within transects, monitoring PVC tubes, and analysis were identical to those described in experiment 2. This experiment consisted of three treatment levels ($k = 3$) with $r = 10$ blocks per transect ($N = 6$), for a total of 30 PVC tubes per transect ($k \cdot r$).

Experiment 6: Attractiveness of Baits Treated with Bleach. In a second set of experiments, we treated pelts with a commercial chlorine bleach (7:3, P_{chl}) and repeated the above experiment. The methods for bait placement, assigning treatment levels within transects, monitoring PVC tubes, and analysis were identical to those described above. This experiment consisted of three treatment levels ($k = 3$) with $r = 10$ blocks per transect ($N = 6$), for a total of 30 PVC tubes per transect ($k \cdot r$).

We carried out two types of field laboratory microbial assays to evaluate the efficacy of the chemical treatment technique and to provide a presumptive identification of the primary bacteria cultured from the surface of mouse pelts. In the lab, we placed newly prepared baits (P_{alc}, P_{chl}, P_{nt}, DNM; $N = 10$ per treatment type) onto nutrient agar plates and incubated the baits at 35°C for 24 and 48 hr, scoring the plates for presence of bacterial growth. From the field, a sample of

these same bait types was removed after the second day's evaluation, taken to the laboratory, placed on nutrient agar, incubated, and checked for bacterial growth as above. The rationale for this second evaluation was to determine if uneaten baits were more or less likely to foster bacterial growth. Finally, we streaked nutrient agar plates with swabs taken directly from the surface of DNM. Colonies of bacteria were isolated and tested for presumptive identification using Enterotube II and Oxi/Ferm tubes (BBL, Becton Dickinson).

Experiment 7: Attractiveness of Denatured Baits. The objective of this experiment was to determine the attractiveness of P_{nt}, of pelts that were boiled (P_b), and of intact DNM that were boiled (DNM_b) relative to DNM. Whole DNM and pelts were boiled for approximately 20 min to remove surface bacteria. As in previous experiments pelts were wrapped around tofu that served as a moisture source. This experiment served mainly as a pilot study for future experimentation and was one in which we assessed a nonchemical avenue of treatment while acknowledging the change in the nature of the proteins. Thus, only a single transect was used with $N = 10$ baits per treatment level (DNM, DNM_b, P_{nt}, P_b; 40 PVC tubes). The methods for bait placement, assigning treatment levels within the transect, and monitoring PVC tubes were identical to experiment 2. Comparison of treatments to DNM was done using a binomial test.

RESULTS

Set 1

Experiment 1: Importance of Carrion Decomposition for Lure Attractiveness. Carrion decomposed for two days was more attractive to brown treesnakes. There was a 90% increase in the rate at which 2-day-old whole mouse carrion was removed from PVC tubes relative to 1-day-old mouse carrion (Figure 1).

Set 2

Experiment 2: Whole vs. Reconstituted Mouse Lures. Whole mouse carrion was more attractive as a bait than reconstituted ground mouse (Figure 2A). DMG was 20% as attractive to brown treesnakes as DNM. As a function of decomposition over two days (Figure 2B), there was little increase in attractiveness for DMG, and a large increase in attractiveness for DNM.

Experiment 3: Attractiveness of Mouse Parts and Tofu-wrapped Pelts. Snakes removed whole dead mice and mouse parts from PVC tubes at different rates (Figure 3A). The cumulative proportional bait take of skinned carcasses was 49% lower than that observed for whole dead mice. However, the cumulative bait take of whole dead mice and pelts was similar. The bait take of pelts and skinned carcasses also was similar. The day effect, where the biological substrate presumably

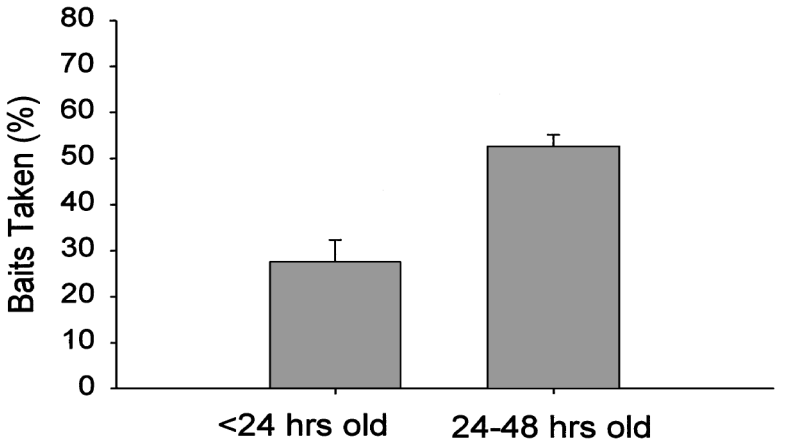


FIG. 1. Bait take (mean ± SEM) by brown treesnakes on Guam in experiment 1 for whole dead neonatal mice as a function of bait decomposition ($t = 21.429$, $df = 6$, $P = 0.004$). Percentage estimates were calculated using transect as the experimental unit ($N = 4$) with 10 observations per bait type per transect.

becomes more decomposed on day 2 vs. day 1, indicated that bait take varied as a function of time over two days for the treatments (Figure 3B). On the first day all bait types had similar rates of take. However, by the second day the cumulative bait take was much higher for the whole dead mouse relative to skinned carcasses. Bait take for pelts on the second day was intermediate between carcasses and whole dead mice. These observations suggest that the second-day effect is partly a function of changes taking place on the surface of the mouse, i.e., skin.

Experiment 4: Attractiveness of Mouse Parts and Cotton-wrapped Pelts. Bait take was the same for all bait types (Figure 4A). Bait take did not vary across days as a function of treatment (Figure 4B). Thus, bait take was not substantially affected by the presence or absence of the moisture source, at least for the time frame of these experiments.

Set 3

Experiment 5: Attractiveness of Baits Treated with Alcohol. Alcohol treatment diminished the attractiveness of pelts (Figure 5A). The cumulative bait take for alcohol-treated pelts was 50% lower relative to whole dead mice. The cumulative bait take for whole dead mice and nontreated pelts and that of nontreated and treated pelts were similar. The magnitude of the day effect was lowest for the alcohol-treated pelts and was proportionally similar for the whole dead mouse and nontreated pelts (Figure 5B). These observations suggest that treating the bait's

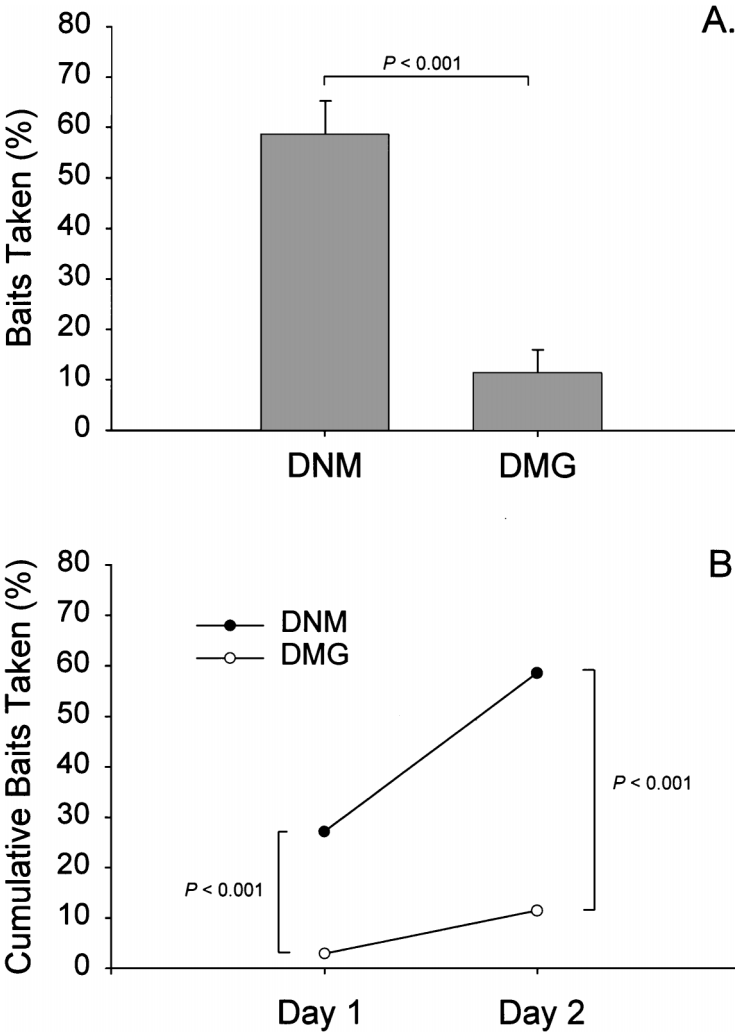


FIG. 2. (A) Bait take (mean ± SEM) by brown treesnakes on Guam in experiment 2 for whole dead neonatal mice (DNM) and reconstituted ground mice (DMG) ($F = 54.348$, $df = 1,12$, $P < 0.001$). Tukey's HSD comparisons and associated probability values are indicated by horizontal lines. (B) Profiles for the cumulative mean (±SEM) take of DNM and DMG baits as a function of time ($F = 6.508$, $df = 1,12$, $P = 0.025$). Simple orthogonal planned contrasts and their associated probabilities are indicated by vertical lines. Percentage estimates were calculated using transect as the experimental unit ($N = 7$) with 10 observations per bait type per transect.

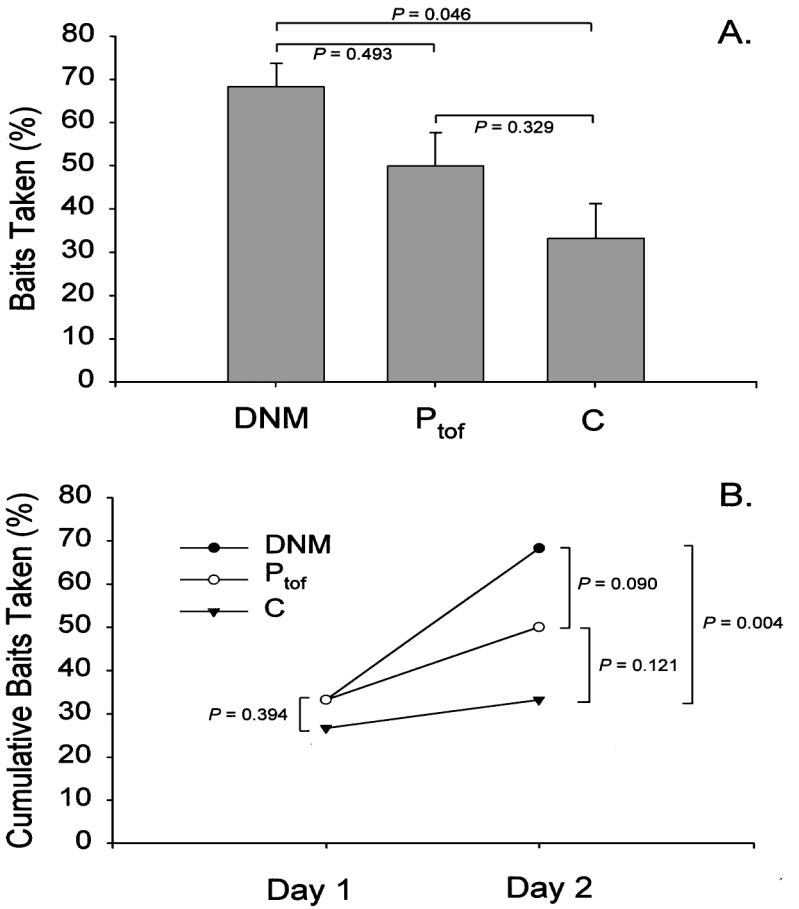


FIG. 3. (A) Bait take (mean ± SEM) by brown treesnakes on Guam in experiment 3 for whole dead neonatal mice (DNM), mouse pelts wrapped around tofu (P_{tof}), and mouse carcasses without skin (C) ($F = 3.499$, $df = 2,15$, $P = 0.057$). Tofu served as a moisture source to prevent desiccation of the pelt. Tukey's HSD comparisons and associated probability values are indicated by horizontal lines. (B) Profiles for the cumulative mean (±SEM) bait take of DNM, P_{tof}, and C baits as a function of time ($F = 5.777$, $df = 2,15$, $P = 0.014$). Simple orthogonal planned contrasts and their associated probabilities are indicated by vertical lines. Percentage estimates were calculated using transect as the experimental unit ($N = 6$) with 10 observations per bait type per transect.

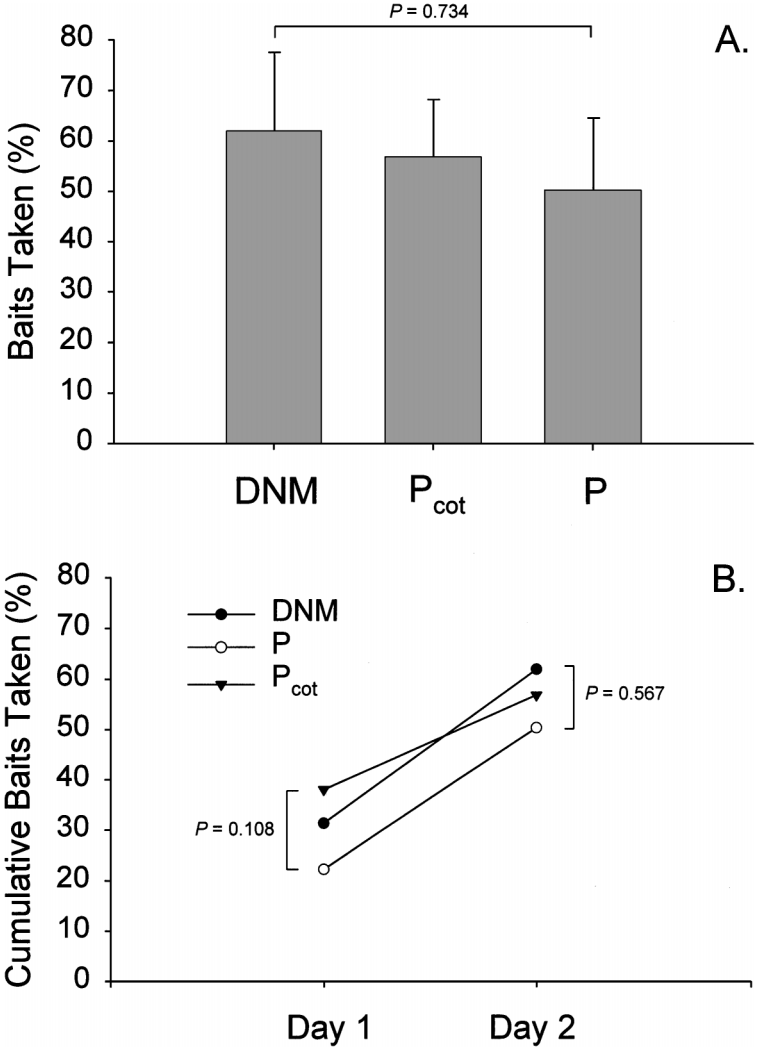


FIG. 4. (A) Bait take (mean ± SEM) by brown treesnakes on Guam in experiment 4 for whole dead neonatal mice (DNM), mouse pelts wrapped around moistened cotton (P_{cot}), and mouse pelts (P) ($F = 0.416$, $df = 2,12$, $P = 0.669$). (B) Profiles for the cumulative mean (±SEM) bait take of DNM, P_{cot}, and P baits as a function of time ($F = 0.375$, $df = 2,12$, $P = 0.695$). Percentage estimates were calculated using transect as the experimental unit ($N = 5$) with 10 observations per bait type per transect.

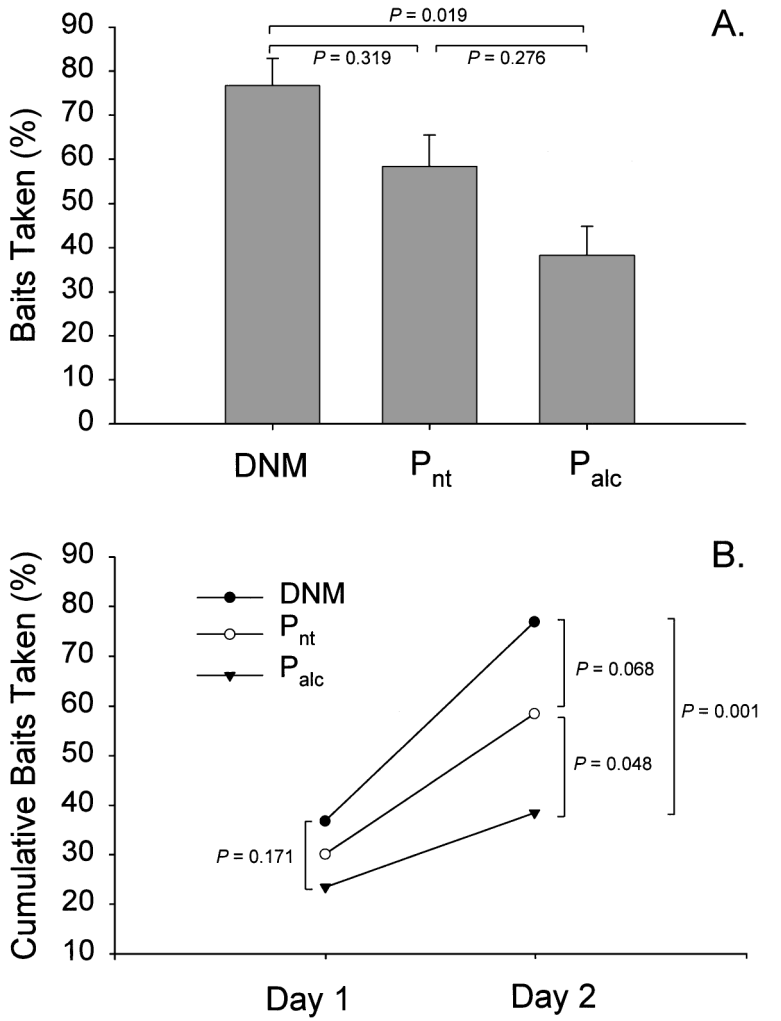


FIG. 5. (A) Bait take (mean ± SEM) by brown treesnakes on Guam in experiment 5 for whole dead neonatal mice (DNM), nontreated mouse pelts wrapped around tofu (P_{nt}), and alcohol-treated mouse pelts wrapped around tofu (P_{alc}) ($F = 4.807$, $df = 2,15$, $P = 0.024$). Tofu was used to preserve moisture content of pelts. Tukey's HSD comparisons and associated probability values are indicated by horizontal lines. (B) Profiles for the cumulative mean (±SEM) bait take of DNM, P_{nt}, and P_{alc} baits as a function of time ($F = 4.643$, $df = 2,15$, $P = 0.027$). Simple orthogonal planned contrasts and their associated probabilities are indicated by vertical lines. Percentage estimates were calculated using transect as the experimental unit ($N = 6$) with 10 observations per bait type per transect.

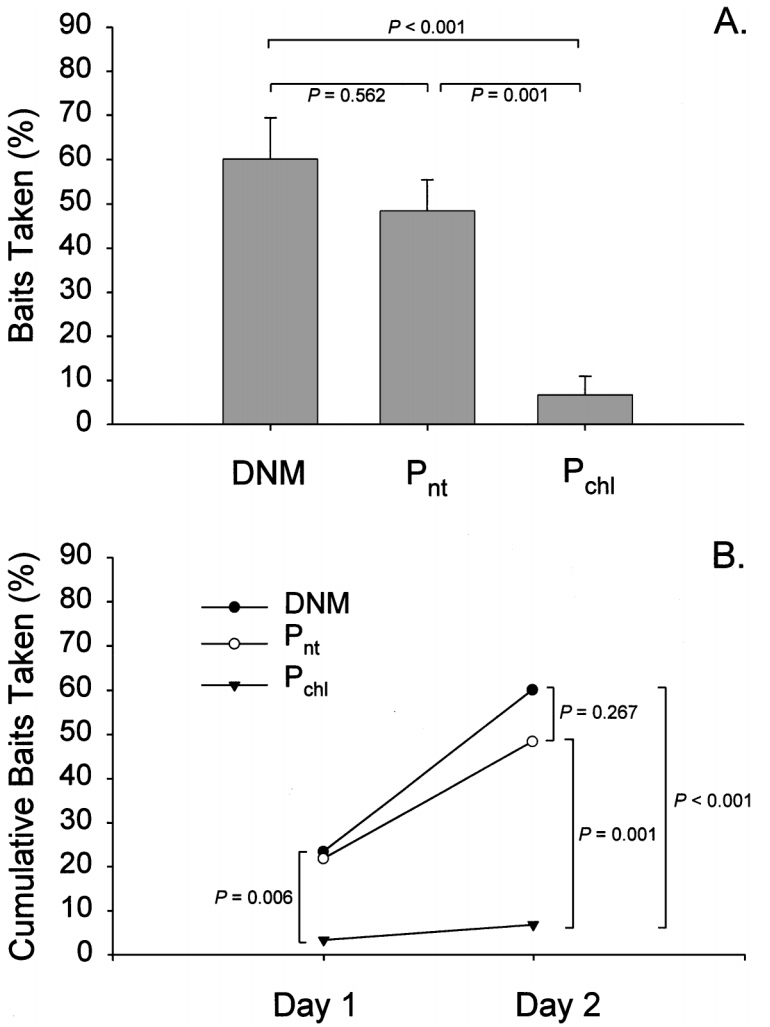


FIG. 6. (A) Bait take (mean ± SEM) by brown treesnakes on Guam in experiment 6 for whole dead neonatal mice (DNM), nontreated mouse pelts wrapped around tofu (P_{nt}), and bleach-treated mouse pelts wrapped around tofu (P_{chl}) ($F = 18.727$, $df = 2,15$, $P < 0.001$). Tofu was used to preserve moisture content of pelts. Tukey's HSD comparisons and associated probability values are indicated by horizontal lines. (B) Profiles for the cumulative mean (±SEM) bait take of DNM, P_{nt}, and P_{chl} baits as a function of time ($F = 4.877$, $df = 2,15$, $P = 0.023$). Simple orthogonal planned contrasts and their associated probabilities are indicated by vertical lines. Percentage estimates were calculated using transect as the experimental unit ($N = 6$) with 10 observations per bait type per transect.

surface decreases the attractiveness of the bait, either by chemical extraction of critical volatiles or by the antimicrobial effects of the alcohol wash.

Experiment 6: Attractiveness of Baits Treated with Bleach. Treatment of pelts with bleach strongly decreased the attractiveness of the bait (Figure 6A). Pelts treated with bleach were taken at 11% of the rate of whole dead mice, and at 14% of the rate of nontreated pelts. This strong main treatment effect held across days (Figure 6B). The source of the strong interaction effect was owing to the proportional cumulative increase in bait take for the whole dead mouse and non-treated pelts in contrast to the lack of increase in bait take for the bleach treated pelts. These observations indicate that bleach treatment exerted a stronger negative effect on bait take than an alcohol wash and that this action either by chemical extraction or antimicrobial properties eliminated important cues for foraging brown treesnakes that originated from the surface of the baits.

Both alcohol and bleach treatments of the pelts decreased microbial growth (Table 1). However, treatment with alcohol apparently did not eliminate all colony forming units because there was noticeable bacterial growth on agar plates after 48 hr. Bleach was a more effective disinfectant because none of the cultures showed evidence of any colony forming units for up to six days, after which some cultures showed signs of fungal growth.

From the field, a sample of unconsumed baits left in PVC tubes after two to three days was taken back to the laboratory and placed onto nutrient agar plates and incubated for 24 hr. All samples of whole dead mice and non-chemically-treated pelts yielded substantial bacterial growth after 24 hr of incubation (Table 1). The alcohol- and bleach-treated baits did not yield appreciable microbial growth after incubation. Of course, the microbial contamination of baits that were consumed is

TABLE 1. POSITIVE CULTURES FOR BAIT TREATMENTS PREPARED FOR BROWN TREE SNAKES ON GUAM IN MAY AND JUNE 2000

Treatment	Laboratory assays[a]		Field recovery assays at 24 hr[b]
	24 hr	48 hr	
DNM	10	10	10
P_{nt}	10	10	10
P_{alc}	3[d]	7	1[d]
P_{chl}	0[d]	0[d]	0[d]
Control[c]	0[d]	0[d]	0[d]

[a] The number of agar plates out of 10 that showed signs of microbial growth. Freshly prepared baits were placed on nutrient agar cultures and checked at 24- and 48-hr intervals.
[b] The number of agar plates out of 10 that showed signs of microbial growth for bait treatments recovered from the field after >72 hr and checked after 24 hr of incubation on a nutrient agar culture plate.
[c] The negative control consisted of nutrient agar plate.
[d] Within-column comparisons of treatments to DNM ($P < 0.05$, binomial test).

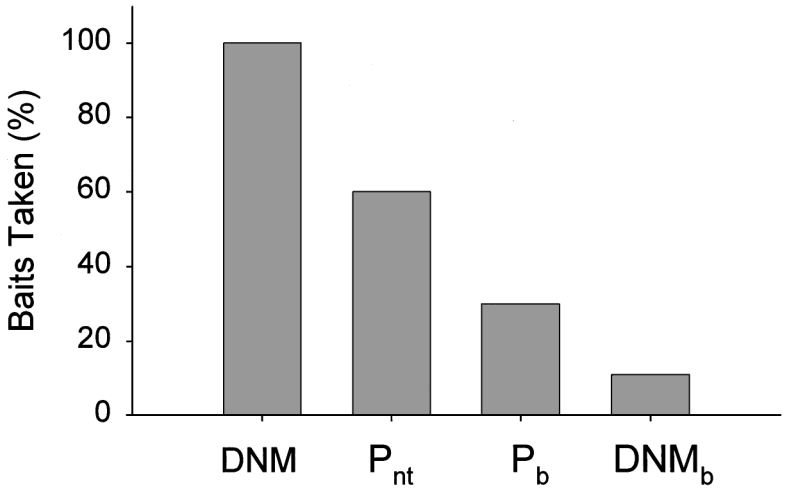


FIG. 7. Bait take by brown treesnakes on Guam for a single transect in experiment 7 for whole dead neonatal mice (DNM), nontreated mouse pelts wrapped around tofu (P_{nt}), boiled mouse pelts wrapped around tofu (P_b), and boiled whole mice (DNM_b). Tofu was used to preserve moisture content of pelts. Estimates were based on 10 observations per treatment type for this single transect. All treatments (P_{nt}, P_b, DNM_b) were different from DNM ($P < 0.05$, binomial test).

unknown. However, when the bait take rates of alcohol- and bleach-treated baits are considered in the context of the laboratory microbial culture experiments, the bait take rates are concordant with the degree of microbial contamination. That is to say, there is sufficient inferential evidence to suggest that the rate of bait take is dependent on the degree of microbial growth on the surface of the bait. Isolates from the cultures indicate that the predominant bacterial growth originating from the surface of dead mice is *Enterobacter agglomerans*.

Experiment 7: Attractiveness of Denatured Baits. Treating the mouse substrate by boiling also affects the attractiveness of baits (Figure 7), although there may also be effects owing to the general denaturation of the mouse parts.

DISCUSSION

Future effective snake control programs on Guam will use both traps and toxic bait delivery. For traps, the current lure used is a live mouse contained within a cage inside the trap. Such traps are reasonably effective in depopulating modest areas of brown treesnakes (Engeman et al., 1998). One drawback with this method of control is the expense and effort of maintaining live mouse colonies in the lab

and maintaining the live mouse lures in the field (Clark et al., unpublished results). The use of toxic baits also can be effective at depopulating modest areas of forest of snakes. Savarie et al. (2001) employed whole dead neonatal mice laced with acetaminophen as the lure/bait-toxicant delivery system in achieving near zero survivorship of snakes in 6-ha experimental plots. Despite this success there still is a need to develop a lure/bait system that does not rely on the killing of mice.

The successful artificial lure/bait system must provide snakes with long-distance cues that act to attract the snake to the vicinity of the substrate and short-distance cues that will promote ingestion. These cues are not necessarily the same (Clark and Kimball, unpublished results). The difficulty in constructing artificial lure and bait systems has been the uncertainty of what these cues might be. Presentation of simple chemical components of mouse carrion have yielded variable, but generally poor, success as attractants (Shivik and Clark, 1999a; Clark and Kimball, unpublished results). Even reconstruction of mouse carrion odor based on chemical analysis of the volatiles produced during rotting have failed to successfully attract snakes (Shivik, 1998). Clearly, brown treesnakes, despite their anecdotal reputation for eating anything, are quite discriminating foragers. One possible solution in the development of a realistic mimic of complex carrion odor is to develop a system that includes the odor-producing agents and the appropriate nutrient substrate for those agents. This study is a critical first step in that process.

Throughout the experiments we found a concordance between factors relating to microbial decomposition of the mouse and the rate at which baits were taken by snakes: baits left in the field for two days were taken at higher rates, and chemically treated (alcohol or bleach) baits were taken at lower rates. These chemicals act as disinfectants, but they may also act as extractive solvents, removing critical volatiles. While we infer that the critical attractive volatiles are products of microbial metabolism, and our experimental evidence is consistent with this view, these chemicals may also have removed critical volatiles by extraction. Experiments using antibiotics to suppress bacterial growth would be useful in determining which hypothesis is responsible for the taking of baits by snakes. It is unlikely that chemical treatment resulted in residues that acted as repellents to snakes. The positive controls, DNM, were in PVC tubes that were cleansed with bleach, and taking of bait was consistently higher in those treatments.

We also found that the nature of the bait substrate was important. Mouse carcasses without skin and reconstituted ground-up mice were not good baits. Whole mice and pelts were good baits. These observations make sense in the context of the cues a foraging brown treesnake is likely to encounter. Typically, the initial odors generated from carrion will be the result of aerobic decomposition of the surface of the animal. Thus, snakes should be more attentive to these types of cues.

Future development of artificial lure/bait systems may include the cell culture of mouse skin as the nutrient substrate that could be embedded into agar blocks

and inoculated with appropriate bacteria. We suggest *Enterobacter agglomerans* as an initial odor-producing agent, because it is the dominant bacterium isolated from mouse skin, although other bacteria could contribute to the complex odor of carrion. These bioreactor systems could potentially act both as lure and bait systems. Such systems would greatly reduce the need for large numbers of live and dead mice for snake control, thus making progress toward the goal of using artificial lure/bait systems to achieve effective snake control. Future work in our laboratory is directed at addressing these issues.

Acknowledgments—We thank K. G. Dyer for assistance in the field. We appreciate the continued efforts and help from H. Hirsh and others at Andersen Air Force Base, Guam. Comments from R. L. Bruggers, E. Campbell, M. W. Fall, T. H. Fritts, G. H. Rodda, M. E. Tobin, and D. S. Vice were helpful. This research was conducted with funds provided to the National Wildlife Research Center by the U.S. Department of Defense under Legacy Project Number DO822, "Field Evaluation of Chemical Methods for Brown Treesnake Management," and the sustained support of the Legacy Program is greatly appreciated. All experiments were performed in compliance with Institutional Animal Care and Use Guidelines.

REFERENCES

CHISZAR, D., KANDLER, K., and SMITH, H. M. 1988. Stimulus control of predatory attack in the brown treesnake (*Boiga irregularis*) 1. Effects of visual cues arising from prey. *Snake* 20:151–155.

CHISZAR, D., DUNN, T. M., and SMITH, J. M. 1993. Response of brown treesnakes (*Boiga irregularis*) to human blood. *J. Chem. Ecol*. 19:91–96.

CHISZAR, D., LUKAS, W., and SMITH, H. M. 1997a. Response to rodent saliva by two species of rodentiophagous snakes. *J. Chem. Ecol*. 23:829–836.

CHISZAR, D., RODDA, G. H., and SMITH, H. M. 1997b. Experiments on chemical control of behavior in brown treesnakes, pp. 121–128, *in* J. R. Mason (ed.). Repellents in Wildlife Management: Proceedings of a Symposium. USDA, National Wildlife Research Center, Fort Collins, Colorado.

CLARK, L. 1997. Responsiveness of brown treesnakes to odors, pp. 129–137, *in* J. R. Mason (ed.). Repellents in Wildlife Management: Proceedings of a Symposium. USDA, National Wildlife Research Center, Fort Collins, Colorado.

CONRY, P. J. 1988. High nest predation by brown treesnakes on Guam. *Condor* 90:478–482.

ENGBRING, J., and FRITTS, T. H. 1988. Demise of an insular avifauna: The brown treesnake on Guam. *Trans. West. Sect. Wildl. Soc*. 24:31–37.

ENGEMAN, R. M., LINNELL, M. A., POCHOP, P. A., and GAMBOA, J. 1998. Substantial reductions of brown treesnake (*Boiga irregularis*) populations in blocks of land on Guam through operational trapping. *Int. Biodeterior. Biodeg.* 42:167–171.

FRITTS, T. H. 1988. The brown treesnake, *Boiga irregularis*, a threat to Pacific Islands. *U.S. Fish Wildl. Serv. Biol. Rep*. 88:31.

FRITTS, T. H., SCOTT, N. J., JR., and SAVIDGE, J. A. 1987. Activity of the arboreal brown treesnake (*Boiga irregularis*) on Guam as determined by electrical outages. *Snake* 19:51–58.

FRITTS, T. H., CHISZAR, D., SCOTT, N. J., JR., and SMITH, B. E. 1989. Trapping *Boiga irregularis* on Guam using bird odors. *J. Herpetol*. 23:189–192.

LINDBERG, A. C., SHIVIK, J. A., and CLARK, L. 2000. Mechanical mouse lure for brown treesnakes. *Copeia* 3:886–888.

RODDA, G. H., and FRITTS, T. H. 1992a. The impact of the introduction of the colubrid snake *Boiga irregularis* on Guam's lizards. *J. Herpetol*. 26:166–174.

RODDA, G. H., and FRITTS, T. H. 1992b. Sampling techniques for an arboreal snake, *Boiga irregularis*. *Micronesica* 25:23–40.

RODDA, G. H., FRITTS, T. H., and CONRY, P. J. 1992. Origin and population growth of the brown treesnake, *Boiga irregularis*, on Guam. *Pac. Sci*. 46:46–57.

SAVARIE, P. J., SHIVIK, J. A., WHITE, G. C., HURLEY, J. C., and CLARK, L. 2001. Use of acetaminophen for large scale control of brown treesnakes. *J. Wildl. Manage.* 65:356–365.

SAVIDGE, J. A. 1984. Guam: Paradise lost for wildlife. *Biol. Conserv*. 30:305–317.

SAVIDGE, J. A. 1987. Extinction of an island forest avifauna by an introduced snake. *Ecology* 68: 660–668.

SHIVIK, J. A. 1998. Brown treesnake response to visual and olfactory cues. *J. Wildl. Manage*. 62: 105–111.

SHIVIK, J. A., and CLARK, L. 1997. Carrion seeking in brown treesnakes: Importance of olfactory and visual cues. *J. Exp. Zool*. 279:549–553.

SHIVIK, J. A., and CLARK, L. 1999a. The development of chemosensory attractants for brown treesnakes, pp. 649–654, *in* R. E. Johnston, D. Müller-Schwarze, and P. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Plenum Press, New York.

SHIVIK, J. A., and CLARK, L. 1999b. Ontogenetic shifts in carrion attractiveness to brown treesnakes (*Boiga irregularis*). *J. Herpetol*. 33:334–336.

SHIVIK, J. A., WRIGHT, W. G., and CLARK, L. 2000. Seasonal variability in brown treesnake (*Boiga irregularis*) response to lures. *Can. J. Zool*. 78:79–84.

StatSoft. 1994. Statistica, v 4.3. StatSoft, Inc., Tulsa, Okalahoma.

U.S. DEPARTMENT OF AGRICULTURE. 1996. Brown treesnake control activities on Guam. Environmental assessment. U.S. Department of Agriculture, Washington D.C.

WINER, B. J. 1971. Statistical Principles in Experimental Design, 2nd ed. McGraw-Hill, New York.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

EFFECTS OF PREDATOR CHEMICAL CUES AND BEHAVIORAL BIORHYTHMS ON FORAGING ACTIVITY OF TERRESTRIAL SALAMANDERS

JOHN C. MAERZ,* NOVA L. PANEBIANCO, and DALE M. MADISON

Department of Biological Sciences
State University of New York at Binghamton
Binghamton, New York 13902-6000

(Received November 8, 2000; accepted March 1, 2001)

Abstract—Red-backed salamanders, *Plethodon cinereus*, show a variety of alarm responses to chemical cues from eastern garter snakes, *Thamnophis sirtalis*. We measured the foraging activity of red-backed salamanders exposed to water soiled by a garter snake (fed *P. cinereus*) or to unsoiled water. Salamanders exposed to snake-soiled water showed less foraging activity than salamanders exposed to unsoiled water; therefore, predators could have nonlethal effects on salamander populations. Our results also show additional factors influenced salamander foraging activity. Salamander foraging activity and responsiveness to chemical cues do not appear to have been affected by sex or food deprivation. Salamander foraging activity does appear to have been influenced by activity biorhythms. Foraging activity of animals in both treatments showed a bimodal periodicity that is consistent with natural activity patterns controlled by internal biorhythms. Exposure to snake-soiled water significantly reduced foraging activity during periods of peak foraging activity, but had a subtler effect on foraging activity during natural lulls in activity. We suggest that both activity biorhythms and exposure to chemical cues are important factors affecting salamander foraging behavior.

Key Words—Biorhythm, chemical cues, *Plethodon cinereus*, foraging, salamander.

INTRODUCTION

Animals can significantly reduce predation risk by responding to alarm cues in the environment. Many animals show a variety of behavioral responses to chemical

* To whom correspondence should be addressed at current address: Department of Natural Resources, Fernow Hall, Cornell University, Ithaca, NY 14853. e-mail: jcm63@cornell.edu

0098-0331/01/0700-1333$19.50/0

alarm cues or predator chemical cues, including avoidance of, or reduced activity within, areas that contain cues (Kats and Dill, 1998). Although there are clear benefits to behavioral responses to predator cues, there can also be costs (Lima, 1998). For example, avoiding particular habitats or reducing foraging activity can compromise an animal's foraging success (reviewed by Lima, 1998; Lima and Dill, 1990). Understanding the potential costs of prey responses to predator cues is important because those costs determine the "nonlethal" effects of predators on prey populations (Laurila and Kujasalo, 1999; Laurila et al., 1998; Relyea, 2000; Relyea and Werner, 1999; see also review by Lima, 1998) and whole communities (Peacor and Werner, 2000; Persson, 1999; Turner, 1997; Turner and Mittelbach, 1990; Werner and Anholt, 1996). Lima (1998) suggests that the nonlethal effects of predators on prey populations might be less conspicuous, but far more important, than direct predation, and therefore, require increased attention.

Terrestrial salamanders within the genus *Plethodon* show several defensive responses to a variety of chemical cues that include combinations of predator-specific chemical cues and chemical alarm cues from conspecifics. Independent sources of chemical cues include the damaged tissues of conspecifics (Chivers et al., 1997), and chemicals released by predators such as the ring-necked snake (*Diadophus punctatus*) (Cupp, 1994) and the eastern garter snake (*Thamnophis sirtalis*) (Madison et al., 1999a,b; McDarby et al., 1999). Under certain conditions, salamanders only respond to combinations of predator chemical cues and chemical alarm cues. For example, *P. cinereus* responses to chemical cues from snakes depend on the time of day and the diet of the snake (Madison et al., 1999a,b; Murray and Jenkins, 1999). *P. cinereus* respond to chemical cues from snakes fed *P. cinereus* throughout the evening, but *P. cinereus* stop responding to the chemical cues of snakes fed alternative prey shortly after dusk (Madison et al., 1999b; Madison, Maerz, and Sullivan, unpublished data). *Plethodon* show variable responses to exposure to alarm chemical cues including avoiding substrates that contain the cues (Chivers et al., 1997; Cupp, 1994; Madison et al., 1999a,b; McDarby et al., 1999; Murray and Jenkins, 1999), changing their activity level (Madison et al., 1999a), or delaying emergence from refugia (Sullivan, Maerz, and Madison; unpublished data). Whether there are costs associated with these responses has not been addressed.

We investigated whether exposure to chemical cues from an eastern garter snake, *T. sirtalis*, would affect the foraging behavior of adult *P. cinereus*. *P. cinereus* foraging success is positively correlated with activity level outside retreats (Fraser, 1976; Jaeger, 1978, 1980, 1990). For fish and larval amphibians, activity level is positively correlated with predation risk (Godin and Smith, 1988; Skelly, 1994), so they generally reduce activity in response to alarm cues (Kats and Dill, 1998). *Plethodon* predators such as *T. sirtalis* and *D. punctatus* are more likely to attack a moving salamander (Maerz, personal observation), and terrestrial salamanders use immobility as their primary response to encounters with predators (Arnold, 1982;

Dodd, 1989; Ducey and Brodie, 1983). Therefore, we predicted that *P. cinereus* will reduce foraging activity when exposed to snake-soiled water. We also predicted that those salamanders exposed to snake-soiled water would pursue prey from shorter distances.

METHODS AND MATERIALS

Collection of Snake Odor. We collected adult *T. sirtalis* (∼400 mm snout–posterior vent length) from a mixed deciduous forest in Broome County, New York. We housed the snakes in individual 38-liter aquaria and fed them earthworms (*Lumbricus* sp.) twice weekly. We fasted an adult snake for 10 days prior to the start of trials, and then fed the snake two adult male *P. cinereus*. After the snake had eaten the salamanders, we transferred the snake to a clean 38-liter aquarium lined with dry paper towels. A second 38-liter aquarium lined with dry paper towels was set up for the unsoiled water (control). After four days, we removed the snake from the first aquarium, transferred the paper towels from each aquarium to individual plastic jars, and added 400 ml of distilled water to each jar 12 hr prior to the first set of trials. Jars were kept sealed between trials to reduce exposure to air and slow any potential aging of the sample. In order to reduce stimulus variation, the same solution was used for all trials. Prior research shows that *P. cinereus* responses are the same to chemical cues from different *T. sirtalis* provided those snakes are fed the same diet (Madison et al., 1999b); therefore, the results of our study, which uses chemical cues from a single snake, can be generalized to *P. cinereus* responses to other *T. sirtalis*.

Collection and Maintenance of Salamanders. We collected 24 adult male and 24 adult female *P. cinereus* from the State University of New York Nature Preserve, Broome County, New York. Salamanders were housed individually in 30-cm × 16-cm clear plastic boxes lined with 5 cm of moist soil and covered with a glass lid. We maintained salamanders in a controlled environment room at 17.5°C on a 12L:12D photoperiod, checked them every two days, moistened their soil as needed, and fed them five crickets (∼6 mm in length) weekly. After 60 days, we discontinued feeding and initiated trials.

Experimental Protocol. We could not test all 48 salamanders simultaneously, so we randomly assigned salamanders to consexual pairs for testing and subsequent statistical comparison. Within a pair, one salamander was randomly assigned to the snake-soiled water treatment and the other to the unsoiled water treatment. We tested partners sequentially on a preassigned trial date, randomizing the order of testing within each pair. Although not part of our initial design, pairs of salamanders were tested in blocks 4–6, 10–12, or 25–27 days after they were last fed. On the test day, we added 8 ml of the appropriate water treatment evenly across the soil surface of the salamander's box, and after allowing 2–3 min for the solution

to settle into the soil, we placed five 0.25-in. crickets in the box, covered the box with a glass lid, and videotaped the trial for 30 min. We conducted all trials during the dark portion of the photocycle with just enough incandescent light to record the trial with a 0.8 lux video camera.

P. cinereus foraging behavior is slow and deliberate. Salamanders usually orient toward prey from a resting position and then move toward prey in a series of discrete actions. Salamanders will often pause motionless between movements toward prey. From the videotapes, we measured the number of actions per minute and pursuit distance. "Actions" were defined as any single bout of continuous movement, which ranged from a reorientation of a salamander's head to several strides. We discriminated between "foraging actions" that were oriented toward a prey, and "nonforaging actions" that were oriented in all other directions. We defined pursuit as one or more actions culminating in a strike at prey, so pursuit distance was the interval between the salamander and its prey when pursuit began. Two independent observers scored all trials, and their scores were compared for consistency. A third observer reviewed any discrepancies between the scores of the two independent observers.

RESULTS

Only 18 of 24 salamander pairs could be included in our analyses. For four pairs, one or both salamanders managed to push into small crevices in the soil just prior to the application of treatment. Two other pairs were excluded because neither salamander moved during the entire 30-min observation period.

The proportion of salamanders exposed to snake-soiled water that attempted a strike was significantly less than that of salamanders exposed to unsoiled water (chi-square test: $\chi^2 = 6.78$, $df = 1$, $P = 0.009$). Half (9 of 18) the salamanders exposed to snake-soiled water struck at prey, while nearly all salamanders (17 of 18) exposed to unsoiled water struck at prey.

Overall, salamanders exposed to snake-soiled water made fewer foraging actions per minute than did their partners (Wilcoxon matched pairs test: $T = 12$, $N = 18$, $P = 0.001$; Figure 1). For different food-deprivation levels, foraging activity of unsoiled-water-exposed salamanders was greater within 4- to 6- and 10- to 12-day food-deprivation groups (Table 1). This trend was not significant for the 25- to 27-day group, but salamanders exposed to snake-soiled water showed reduced foraging activity compared to partners in four of six pairs.

The difference in pursuit distance between salamanders exposed to snake-soiled water (mean $\pm$ SE $= 6.52 \pm 1.52$ cm, $N = 8$) and their partners exposed to unsoiled water (mean $\pm$ SE $= 8.67 \pm 1.71$ cm, $N = 8$) was not different (Wilcoxon matched pairs test: $T = 12$, $N = 8$, $P = 0.401$). The power of this

TABLE 1. FORAGING ACTIONS OF ADULT *P. cinereus* EXPOSED TO UNSOILED OR SNAKE-SOILED WATER AND DEPRIVED OF FOOD[a]

	Foraging action/min (mean ± SE)				
	Days since last fed			Kruskal-Wallis test	
	4–6 days[b]	10–12 days[b]	25–27 days[b]	*H*	*P*
Treatment					
Unsoiled water	0.66 ± 0.06	0.98 ± 0.17	0.71 ± 0.23	2.538	0.281
Snake-soiled water	0.25 ± 0.14	0.51 ± 0.15	0.49 ± 0.14	2.233	0.327
Within food-deprivation level,	$Z = 1.992$	$Z = 2.201$	$Z = 0.943$		
Wilcoxon matched pairs test	$P = 0.046$	$P = 0.028$	$P = 0.345$		

[a] $N = 6$ for each mean.
[b] Days since last fed.

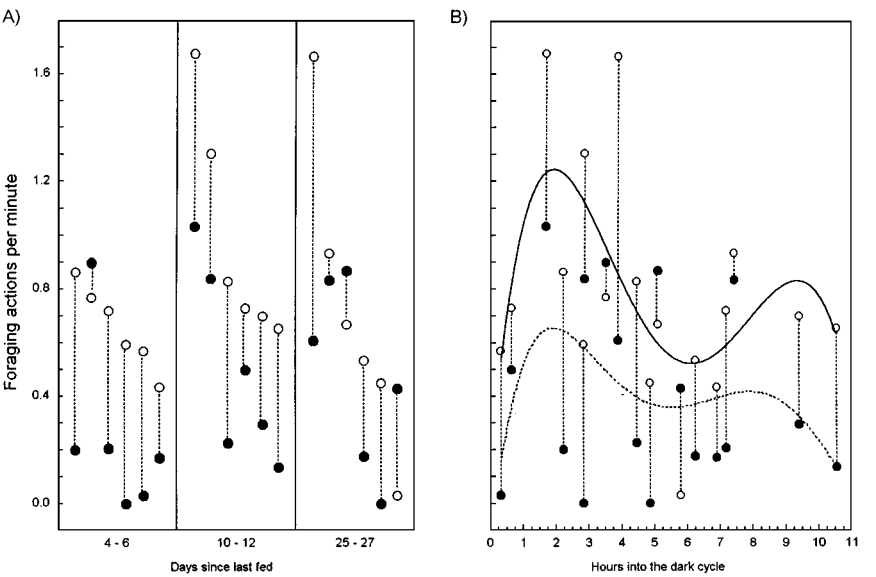


FIG. 1. Differences in the foraging activity rates of unsoiled water exposed (hollow circles) and snake-soiled water exposed (solid circles) *Plethodon cinereus*. Dashed lines connect symbols for partnered salamanders. (A) Data are arranged in descending order from most active salamander (exposed to unsoiled water) to least active within each food-deprivation group. Salamanders exposed to snake-soiled water had lower foraging activity rates than did their partners, and foraging activity rates of partnered salamanders were positively correlated. (B) Data are arranged according to the time within the dark phase when the trial was conducted. Curved lines are least-squares polynomial functions for salamanders exposed to unsoiled water (solid line) and salamanders exposed to snake-soiled water (stippled line).

comparison was weakened because a strike was a required component of pursuit; so only eight pairs were available for comparison. Overall, salamanders initiated pursuit within 18 cm of prey and only attempted a strike at prey within 2 cm.

Unexpectedly, foraging activity was positively correlated between partnered salamanders (Spearman rank correlation: $r_s = 0.663$, $N = 18$, $P = 0.003$; Figure 1A). Because salamanders were randomly partnered, this correlation suggests factors besides odor treatment influenced salamander foraging activity. Within odor treatments, male and female *P. cinereus* foraging activity was not different. Among the salamanders exposed to plain water, male foraging actions per minute (mean $\pm$ SE $= 0.82 \pm 0{:}12$, $N = 10$) were not different from female foraging activity (mean $\pm$ SE $= 0.74 \pm 0.16$, $N = 8$; Mann-Whitney U test, $U = 38.0$, $P = 0.859$). Likewise, among the salamanders exposed to snake-soiled water, male foraging actions per minute (mean $\pm$ SE $= 0.44 \pm 0.13$, $N = 10$) were not different from female foraging activity (mean $\pm$ SE $= 0.38 \pm 0.10$, $N = 8$; Mann-Whitney U test, $U = 39.5$, $P = 0.965$).

Within odor treatments, salamander foraging activity tended to increase in the high food-deprivation levels; however, some extremely high scores within those groups drove this trend and it was not significant (Table 1). Our sample sizes within food-deprivation levels were small, so the ability to detect a food-deprivation effect was limited. The positive correlation between foraging activity levels of partnered salamanders was apparent within food-deprivation levels (Figure 1A).

A least-squares polynomial curve of foraging activity as a function of the time period within the dark cycle showed bimodal foraging activity patterns in both treatment groups (Figure 1B). Salamanders in both odor treatments showed peaks in foraging activity 2 hr into the dark cycle and a smaller activity peak 10 hr into the dark cycle. Regardless of treatment, seven of the nine highest foraging activity levels were recorded 2–4 hr into the dark cycle.

DISCUSSION

Identifying potential costs associated with prey responses to alarm cues contributes to a more accurate understanding of the effects of predators on prey populations and whole communities (Lima, 1998; Persson, 1999). Even though prey responses to alarm cues might reduce predation rates, costs associated with those responses can negatively affect prey populations and alter the structure and function of whole communities. This study shows that red-backed salamander responses to chemical cues from a predator can compromise salamander foraging success. Salamanders exposed to snake-soiled water showed reduced foraging activity that resulted in most animals not attempting a strike at prey during our observation

period. Neotenic graybelly salamanders, *Eurycea multiplicata*, also reduce foraging activity and seldom attempt to capture prey when exposed to chemical cues from predatory fish (Whitham and Mathis, 2000). Reducing activity in response to alarm cues might reduce detection by visually oriented predators (Godin and Smith, 1988; Skelly, 1994), but could negatively affect salamander growth, development, and female fecundity (Bernardo, 1994; Fraser, 1980). Therefore, the potential for important nonlethal effects of predators should be incorporated into models of salamander ecology.

We do not know whether *P. cinereus* exposed to snake-soiled water failed to strike at prey because it is too conspicuous or because reduced pursuit activity results in fewer salamanders getting within strike range of prey. Regardless of treatment, salamanders generally initiated pursuit of prey from a distance of 6–8 cm and from as far as 18 cm, but needed to move within 2 cm to attempt a strike. Therefore, it is possible that reduced activity results in reduced encounters with prey at a sufficient distance to strike. Unlike the laboratory environment, the unconstrained movement of prey in the natural environment might further reduce prey encounter rates.

Our results on the effects of hunger on *P. cinereus* foraging activity are equivocal, but regardless of whether hunger affects salamander foraging activity, hunger did not appear to affect salamander responsiveness to chemical cues. It must be pointed out that all of the salamanders that we tested were deprived of food for at least four days therefore, it is likely that all were hungry to some degree. Thus, hunger did not diminish *P. cinereus* responsiveness to predator chemical cues, at least through 12 days of food deprivation. Other studies of larval anurans (Horat and Semlitsch, 1994) and neotenic adult salamanders (Whitham and Mathis, 2000) show that hunger does not diminish responsiveness to predator chemical cues even if hunger tends to increase foraging activity. In our study and the other studies of amphibians, food deprivation ranged from one to five days, and no level of food deprivation was sufficient to diminish responsiveness to predator chemical cues. Studies of other animals show interactions between the effects of hunger and exposure to predator chemical cues on foraging behavior with shorter periods of food deprivation. For example, one day of food deprivation is sufficient to increase foraging activity of three-spine sticklebacks, *Gasterosteus aculeatus* and override responsiveness to predator chemical cues (Godin and Crossman, 1994). We do not know why the effects of hunger on animal responsiveness to predator cues might differ between some amphibians and fish. Many amphibians, especially plethodontid salamanders, such as those tested in this study and by Whitham and Mathis (2000), have exceptionally high metabolic efficiencies that allow them to go for extended periods without food (Feder, 1983; Pough, 1983). High metabolic efficiency is a hallmark adaptation of amphibians and reptiles, enabling them to exploit highly variable environments where access to food can be limited frequently or for long periods.

We discovered *post hoc* that the foraging activity of partnered salamanders was positively correlated, which suggests additional influences on salamander foraging behavior independent of the effect of exposure to predator chemical cues. Because we randomly partnered salamanders, those additional factors that affected foraging activity must be conditions shared by partners. Partnered salamanders had three factors in common that might affect foraging behavior: sex, length of the food-deprivation period, and time within the dark cycle when the pair was tested. Maerz (2000) reported differences in foraging patterns between male and female *P. cinereus*, specifically that female salamanders tend to capture larger and sometimes more prey than male salamanders, and Dakin (1978) reported disproportionately high numbers of female *P. jordani* and *P. yonahlosse* during peak hours of salamander activity. However, in this study, we found no evidence of differences in foraging activity between male and female *P. cinereus*; so, sex was not a factor contributing to variation in foraging behavior among salamander pairs.

As discussed before, food deprivation did not appear to significantly affect salamander foraging activity and does not appear to account for the correlated behavior of partnered salamanders. Because of our small sample sizes within food-deprivation/odor treatment groups, we hesitate to conclude that food deprivation has no effect on *P. cinereus* foraging activity. However, Adler (1968) examined the effects of food deprivation on *P. glutinosus* activity, and although his study focused on internal activity biorhythms and not facultative adjustments to activity in the presence of prey, he reported that a month of food deprivation had no measurable affect on *P. glutinosus* activity patterns. Further, it was apparent that foraging activity was correlated between partnered salamanders even within food-deprivation groups.

We hypothesize that the positive correlation in activity level between partnered salamanders reflects internal biorhythm influences on activity. The activities of many animals are regulated by internal biorhythms that could operate independent of, or interact with, responses to chemical cues. In this study, those *P. cinereus* tested 2–4 hr into the dark cycle showed the highest foraging activity. Field studies show that when the environment is sufficiently moist, most *Plethodon* emerge from daytime retreats shortly after dusk to forage on the surface (Adler, 1968; Dakin, 1978). Salamander surface activity peaks 2–4 hr after dusk, declines late in the evening, and may peak again near dawn (Adler, 1968; Dakin, 1978). Adler (1968) suggested that increased rodent activity might account for the reduction in salamander surface activity in the middle of the night. Laboratory studies show that these daily activity patterns of *Plethodon* are regulated by an internal biorhythm tuned with extraoptic photoreceptors to the light–dark cycle (Adler, 1969, 1970). Biorhythmic regulation of activity persists at least 24 hr after the removal of photo cues (Adler, 1969).

Many studies consider the state of an animal, e.g., hunger level, when trying to understand an animal's response to stimuli, but most studies fail to factor in the

effects of biorhythms. In this study, exposure to predator chemical cues appears to reduce the amplitude of *P. cinereus* foraging activity, especially during periods of peak activity, but does not appear to affect the expression of temporal activity patterns. This suggests the need to consider biorhythms in the design of behavioral studies. Because exposure to chemical cues has a large effect on activity during activity peaks and a subtler effect on behavior during natural activity lulls, studies conducted during different times could produce different, potentially conflicting, results and lead to different conclusions about the effect of particular stimuli on an animal's behavior.

The interaction of behavioral biorhythms and chemical cues could be an important area of future research. Two key areas for research are: (1) how do behavioral biorhythms interact with facultative responses to environmental stimuli like chemical cues, and (2) are any responses to environmental cues under the control of biorhythms. We already know that *P. cinereus* responses to some chemical alarm cues show diel periodicity tied to the light–dark cycle (Madison et al., 1999a,b). Unlike *P. cinereus* responsiveness to the odors of snakes that fed on *P. cinereus*, which does not change throughout the diel cycle, *P. cinereus* responsiveness to the odors of snakes feeding on alternative prey varies depending on the time of day. *P. cinereus* respond the odors of *T. sirtalis* feeding on alternative prey during the day and for the first few hours after dusk, but stop responding late at night (Madison et al., 1999a,b; Madison, Maerz, and Sullivan, unpublished data). These temporal response patterns appear to be regulated by internal biorhythms tied to the diel cycle and are not directly affected by environmental cues such as light (Madison et al., 1999b). Circadian rhythmic control of responses to chemical cues has also been reported for *Drosophila* (Krishnan et al., 1999). Biorhythmic regulation of responses to predator cues should make prey responses more efficient. Predator activity or efficiency will vary depending on time of day and conditions, and prey can respond differently to the same cue depending on conditions.

CONCLUSIONS

This study shows that red-backed salamanders reduce foraging activity in response to exposure to chemical cues from a predator. Reduced foraging activity represents a cost associated with responses to chemical cues and suggests that predators could have nonlethal effects on terrestrial salamander populations. A positive correlation between the activity levels of randomly partnered salamanders exposed to different odor treatments indicated additional influences on salamander activity independent of odor treatment. Because activity patterns across the dark cycle were consistent with natural periodicity in foraging activity, which is under the control of internal biorhythms, we hypothesize that biorhythms and not sex or

hunger level affected salamander foraging activity. The effects of chemical cues on salamander foraging activity were strongest during natural peaks in salamander activity and subtler during natural lulls in foraging activity. Because biorhythms can affect both responses to chemical cues and other behaviors, such as foraging or mate search, and because independent rhythms could interact to produce different activity patterns under different contexts, studies of chemical cues should incorporate behavioral biorhythms into their design.

Acknowledgments—We thank Nadia Moger for her help in scoring videotapes. We thank A. Sullivan and J. Rohr for valuable comments on this manuscript. This research was partially supported by two grants from Binghamton University: a Departmental Research Award to J. Maerz and a Harpur College Undergraduate Research Award to N. Panebianco.

REFERENCES

ADLER, K. 1968. Environmental control of locomotor activity in a salamander (*Plethodon glutinosus*). PhD dissertation. University of Michigan, Ann Arbor, Michigan.

ADLER, K. 1969. Extraoptic phase shifting of circadian locomotor rhythm in salamanders. *Science* 164:1290–1291.

ADLER, K. 1970. The role of extraoptic photoreceptors in amphibian rhythms and orientation: A review. *J. Herpetol.* 4:96–112.

ARNOLD, S. J. 1982. A quantitative approach to antipredator performance: salamander defense against snake attack. *Copeia* 1982:247–253.

BERNARDO, J. 1994. Experimental analysis of allocation in two divergent, natural salamander populations. *Am. Nat*. 143:14–38.

CHIVERS, D. P., KIESECKER, J. M., WILDY, E. L., ANDERSON, M. T., and BLAUSTEIN, A. R. 1997. Chemical alarm signalling in terrestrial salamanders: Intra-and interspecific responses. *Ethology* 103:599–613.

CUPP, P. V. 1994. Salamanders avoid chemical cues from predators. *Anim. Behav*. 48:232–235.

DAKIN, S. F. 1978. The influence of interspecific competition on the microhabitat distribution of terrestrial lungless salamanders in the southern Appalachian mountains. PhD dissertation. Duke University, Durham, North Carolina.

DODD, C. K., JR. 1989. Duration of immobility in salamanders, genus *Plethodon* (Caudata: Plethodontidae). *Herpetologica* 45:467–472.

DUCEY, P. K., and BRODIE, E. D., JR. 1983. Salamanders respond selectively to contacts with snakes: Survival advantage of alternative antipredator strategies. *Copeia* 1983:1036–1041.

FEDER. M. E. 1983. Integrating the ecology and physiology of plethodontid salamanders. *Herpetologica* 39:291–310.

FRASER, D. F. 1976. Empirical evaluation of the hypothesis of food competition in salamanders of the genus *Plethodon*. *Ecology* 57:459–471.

FRASER, D. F. 1980. On the environmental control of oocyte maturation in a plethodontid salamander. *Oecologia* 46:302–307.

GODIN, J.-G. J., and CROSSMAN, S. L. 1994. Hunger-dependent predator inspection and foraging behaviours in the threespine stickleback (*Gasterosteus aculeatus*) under predation risk. *Behav. Ecol. Sociobiol*. 34:359–366.

GODIN, J.-G. J., and SMITH, S. A. 1988. A fitness cost of foraging in the guppy. *Nature* 333:69–71.

HORAT, P., and SEMLITSCH, R. D. 1994. Effects of predation risk and hunger on the behaviour of two species of tadpole. *Behav. Ecol. Sociobiol.* 34:393–401.

JAEGER, R. G. 1978. Plant climbing by salamanders: Periodic availability of plant-dwelling prey. *Copeia* 1978:686–691.

JAEGER, R. G. 1980. Fluctuations in prey availability and food limitation for a terrestrial salamander. *Oecologia* 44:335–341.

JAEGER, R. G. 1990. Territorial salamanders evaluate size and chitinous content of arthropod prey, pp. 111–126, *in* R. N. Hughes (ed.). Behavioral Mechanisms of Food Selection. Springer-Verlag, Berlin, Germany.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation by prey animals. *Ecoscience* 5:361–394.

KRISHNAN, B., DRYER, S. E., and HARDIN, P. E. 1999. Circadian rhythms in olfactory responses of *Drosophila melanogaster*. *Nature* 400:375–378.

LAURILA, A., and KUJASALO, J. 1999. Habitat duration, predation risk and phenotypic plasticity in common frog (*Rana temporaria*) tadpoles. *J. Anim. Ecol*. 68:1123–1132.

LAURILA, A., KUJASALO, J., and RANTA, E. 1998. Predator-induced changes in life history in two anuran tadpoles: Effects of predator diet. *Oikos* 83:307–317.

LIMA, S. L. 1998. Nonlethal effects in the ecology of predator–prey interactions: What are the ecological effects of anti-predator decision-making? *BioScience* 48:25–34.

LIMA, S. L., and DILL, L. M. 1990. Behavioral decisions made under the risk of predation: A review and prospectus. *Can. J. Zool*. 68:619–640.

MADISON, D. M., MAERZ, J. C., and MCDARBY, J. D. 1999a. Chemosensory avoidance of snake odors by salamanders: Freeze and flight contingencies, pp. 508–516 *in* R. E. Johnston, D. Müller-Schwarze, and P. Sorensen (eds.). Advances in Chemical Communication in Vertebrates. Kluwer Academic/Plenum Press, New York.

MADISON, D. M., MAERZ, J. C., and MCDARBY, J. D. 1999b. Optimization of predator avoidance by salamanders using chemical cues: Diet and diel effects. *Ethology* 105:1073–1086.

MAERZ, J. C. 2000. Prey availability and phenotypic differences between local terrestrial salamander populations. PhD dissertation. State University of New York at Binghamton, Binghamton, New York.

MCDARBY, J. D., Madison, D. M., and Maerz, J. C. 1999. Chemosensory avoidance of predators by the red-backed salamander, *Plethodon cinereus*, pp. 489–495, *in* R. E. Johston, D. Müller-Schwarze, and P. Sorensen (eds.). Advances in Chemical Communication in Vertebrates. Kluwer Academic/Plenum Publishers, New York.

MURRAY, D. L., and JENKINS, C. L. 1999. Perceived predation risk as a function of predator dietary cues in terrestrial salamanders. *Anim. Behav*. 57:33–39.

PEACOR, S. D., and WERNER E. E. 2000. Predator effects on an assemblage of consumers through induced changes in consumer foraging behavior. *Ecology* 81:1998–2010.

PERSSON, L. 1999. Trophic cascades: Abiding heterogeneity and trophic level concept at the end of the road. *Oikos* 85:385–397.

POUGH, F. H. 1983. Amphibians and reptiles as low-energy systems, pp. 141–188, *in* W. P. Aspey and S. I. Lustick (eds.). Behavioral Energetics: The Cost of Survival in Vertebrates. Ohio State University Press, Columbus, Ohio.

RELYEA, R. A. 2000. Trait-mediated indirect effects in larval anurans: reversing competition with the threat of predation. *Ecology* 81:2278–2289.

RELYEA, R. A., and WERNER, E. E. 1999. Quantifying the relation between predator-induced behavior and growth performance in larval amphibians. *Ecology* 80:2117–2124.

SKELLY, D. K. 1994. Activity level and the susceptibility of anuran larvae to predation. *Anim. Behav*. 47:465–468.

TURNER, A. M. 1997. Contrasting short-term and long-term effects of predation on consumer habitat use and resources. *Behav. Ecol*. 8:120–125.

TURNER, A. M., and MITTELBACH, G. G. 1990. Predator avoidance and community structure: Interactions among piscivores, planktivores, and plankton. *Ecology* 71:2241–2254.

WERNER, E. E., and ANHOLT, B. R. 1996. Predator-induced behavioral indirect effects: Consequences to competitive interactions in anuran larvae. *Ecology* 77:157–169.

WHITHAM, J., and MATHIS, A. 2000. Effects of hunger and predation risk on foraging behavior of graybelly salamanders, *Eurycea multiplicata*. *J. Chem. Ecol*. 26:1659–1665.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

SEQUESTRATION OF HOST PLANT-DERIVED COMPOUNDS BY GEOMETRID MOTH, *Milionia basalis*, TOXIC TO A PREDATORY STINK BUG, *Eocanthecona furcellata*

HIROE YASUI*

Department of Insect Physiology and Behavior
National Institute of Sericultural and Entomological Science (NISES)
Ohwashi, Tsukuba, Ibaraki 305-0851, Japan

(Received December 1, 2000; accepted March 14, 2001)

Abstract—A predatory stink bug, *Eocanthecona furcellata*, died after feeding on *Milionia basalis* larvae. The compounds toxic to *E. furcellata* were isolated from the hemolymph of *M. basalis* larvae and identified as inumakilactone A, nagilactone C, and nagilactone C glucoside. The concentrations of inumakilactone A, nagilactone C, and nagilactone C glucoside in the hemolymph of the final instar larvae were 130, 50, and 770 μg/ml, respectively. Nagilactone C showed the highest insecticidal activity against second-instar nymphs of *E. furcellata*, while nagilactone C glucoside showed the lowest, one twentieth of that of nagilactone C. When mixed compounds were given at the same concentrations as those in hemolymph of *M. basalis*, all nymphs of *E. furcellata* died with in three days. Inumakilactone A and nagilactone C were found to be in the leaves of podocarp, *Podocarpus macrophyllus*, the only host plant of *M. basalis*, at concentrations of 13 and 175 μg/g fresh weight, respectively. However, no nagilactone C glucoside was detected in the leaves of this species. These results suggested that *M. basalis* may transform nagilactone C to its glucoside.

Key Words—Toxic compounds, defense substance, stink bug, *Eocanthecona furcellata*, geometrid moth, *Milionia basalis*, podocarp, *Podocarpus macrophyllus*, nagilactone, glucosides, inumakilactone, sequestration, transformation.

INTRODUCTION

The podocarp tree, *Podocarpus macrophyllus* (Podocarpaceae), is used as building material and as a street or garden tree in southern Japan because it is rarely infested by termites and insects (Saeki et al., 1970). *P. macrophyllus* was reported to

* e-mail: yasui@affrc.go.jp

0098-0331/01/0700-1345$19.50/0

contain substances toxic to some insect species (Ito and Kodama, 1976). However, the larvae of the geometrid moth, *Milionia basalis pryeri* Druce (Geometridae) have adapted to this plant and feed exclusively on it, with frequent outbreaks that cause considerable damage. Egg batches of the predatory stink bug, *Eocanthecona furcellata* (Wolff) (Heteroptera: Pentatomidae), have been observed on the leaves of *P. macrophyllus* (Wakamura, personal communication). *E. furcellata* is considered to be a predator of *M. basalis* because the stink bug attacks larvae of many species of Lepidoptera, Coleoptera, and Hymenoptera (Chu, 1975). However, a preliminary experiment showed that second-instar nymphs of *E. furcellata* died after attacking *M. basalis* larvae (Sakakibara, personal communication), suggesting that *M. basalis* larvae contain toxic compounds to protect themselves against predators.

In the present paper, we describe the isolation and identification of the toxic compounds in the hemolymph of *M. basalis* larvae against the predatory stink bug, *E. furcellata*, and the relationship between these isolated compounds and components in *P. macrophyllus*, the host plant of *M. basalis*.

METHODS AND MATERIALS

Insects. Larvae of the geometrid moth, *M. basalis*, were obtained from podocarp trees, *P. macrophyllus*, on Okinawa Island, Japan. The larvae were reared on the podocarp leaves in the laboratory. A laboratory colony of the predatory stink bug, *E. furcellata*, was maintained on frozen preserved larvae of the armyworm, *Pseudaletia separata*, which had been reared on an artificial diet, Insecta LF (Nihon Nousan Kogyo Co., Ltd., Japan). The second-instar nymphs of the bugs were starved for one day after ecdysis before conducting bioassays. All insects were reared at 25°C and 16L:8D photoperiod.

Collection of Hemolymph from M. basalis *Larvae.* *M. basalis* larvae (sixth instars) were kept in ice water for 15 min and then their prolegs were cut. Hemolymph was collected into 1.5-ml microtubes. The hemolymph was immediately heated and then kept at 60°C for 15 min to inactivate enzymes. The turbid hemolymph was then centrifuged, and the supernatant was separated and stored at −20°C until extraction.

Isolation of Toxic Compounds from Hemolymph of M. basalis *Larvae.* The hemolymph of *M. basalis* larvae (ca. 5 ml, sixth instars) was diluted with distilled water (5 ml) and extracted with hexane, ethyl acetate, and water-saturated 1-butanol (each 10 ml × 3), successively.

Bioassay. Test material was dissolved in methanol and applied (1 larva equivalent) to a piece of absorbent cotton (3 × 3 × 1 mm) on a sheet of parafilm (1 × 1 cm) that was placed on filter paper in a 5-cm plastic Petri dish. After the solvent evaporated, 25 μl of hemolymph of the silkworm, *Bombyx mori*, which

had been heated (60°C, 15 min) and centrifuged, was added to the cotton piece. Five second-instar nymphs of *E. furcellata* were released in the dish. To keep the test materials and insects from desiccation, a moistened cotton wick was placed on the inner side of the lid of the Petri dish. Whether the nymphs fed or not was judged from the excreta traces that remained on the filter paper disk. Mortality was checked after four days and evaluated as follows: −, less than 20%; +, ∼40%; ++, ∼80%; + + +, more than 80%. Each test was replicated at least three times.

Glycoside Hydrolysis. Hydrochloric acid (1 M, 0.05 ml) was added to the methanol solution of glycoside (0.5 mg/0.2 ml). The mixture was kept at 60°C for 14 hr, and then evaporated to dryness. Water was added to the residue, and the supernatant was separated and dried. Water (3 μl) was again added for the next trimethylsilylation.

Trimethylsilyl Derivatives of Carbohydrates. Carbohydrate in water solution (0.5 mg/5 μl) was mixed with TMSI-C (*N*-trimethylsilyl imidazole in pyridine, GL Sciences Inc.; 0.5 ml) in a sealed vial and kept at 60°C for 2hr. Authentic glucose, galactose, and sorbose were purchased from Wako Pure Chem. Industries, Ltd. Mannose, allose, and gulose were from Nakarai Chem. Ltd., Aldrich Chem. Co., and Sigma Chem. Co., respectively. Reaction mixture, 1 μl diluted 100-fold with pyridine, was directly injected into GC.

Isolation and Determination of Nagilactones from P. macrophyllus *Leaves. P. macrophyllus* leaves were steamed at 60°C for 15 min to inactivate enzymes in the leaves and then frozen. Freeze-dried powdered *P. macrophyllus* leaves were extracted with methanol for 1 night. After filtration, the methanol extract was evaporated. Distilled water was added to the residue and extracted thoroughly with hexane, ethyl acetate, and water-saturated 1-butanol, successively. The 1-butanol fraction was chromatographed on silica gel (ethyl acetate–methanol 10:1) and ODS silica gel (20% methanol). The crude inumakilactone A and nagilactone C were further purified by HPLC.

Equipment. A Shimadzu LC-10Avp system was used for HPLC. Separation was achieved with a Cosmosil-5C18-AR-II, 4.6 × 250 mm (Nacalai Tesque, Inc. Japan) column. The column temperature was 40°C. The eluting solvent was water–methanol (85:15). The flow rate was 1 ml/min. The UV detector was set at 210 for detection of inumakilactone A and at 254 and 300 nm for detection of nagilactones. NMR spectra were recorded with a Bruker ARX-400 (400 MHz) spectrometer, with pyridine-d_5 as a solvent. Mass spectra (FAB) were obtained with a Jeol JMS- SX102A spectrometer. Argon was used as a bombarding gas, (5×10^{-6} torr), FAB energy was set to 10 keV, and glycerol was used as a matrix. The gas chromatograph used was a Hewlett Packard (HP) 5890 II with a splitless capillary injector and a flame ionization detector. The column employed was DB-17HT (30 m × 0.25 mm I D × 0.15-μm film thickness; J&W Scientific); injection, 270°C; splitless time, 1.0 min; column oven temperature, 50°C for 1 min, programmed to increase up to 100°C at 20°C/min, and further increased to 270°C at 5°C/min,

and held at the final temperature for 5 min; carrier gas, helium at a column head pressure of 110 kPa. Within the GC peaks of hydrocarbons (C_{12}–C_{28}), those of C_{15}–C_{22} appeared at the same time intervals, that is, this condition was suitable for calculation of Kovát's retention time index.

Spectral Data. Data of compound **1** are: ^{1}H NMR (400 MHz); δ1.41 (3H, s, CH_3-20), 1.60 (3H, s, CH_3-18), 1.61 (3H, d, J = 6.2 Hz, CH_3-16), 2.18 (1H, d, 5.1, H-5), 3.56 (1H, dd, 6.0 and 4.2, H-2), 3.68 (1H, d, 4.1, H-1), 4.39 (1H, dq, 6.2 and 2.2, H-15), 4.69 (1H, dd, 5.9 and 5.1, H-3), 4.78 (1H, d, 8.5, H-14), 5.15 (2H, overlapping H-6 and H-7), 6.84 (1H, s, H-11). UV; λ_{max} 222 nm in methanol.

Data for compound **2** are: ^{1}H NMR (400 MHz); δ 1.22 (3H, d, J = 6.7 Hz, CH_3-16), 1.31 (3H, d, 6.7, CH_3-17), 1.59 (3H, s, CH_3-20), 1.89 (3H, s, CH_3-18), 2.14 (1H, d, 8.4, H-5), 3.45 (1H, m, H-15), 3.63 (1H, m, H-2), 3.65 (1H, d, 4.0, H-1), 4.04 (1H, m, 3-glc H-5′), 4.08 (1H, t, 8.3, 3-glc H-2′), 4.18 (1H, t, 9.1, 3-glc H-4′), 4.27 (1H, t, 8.8, 3-glc H-3′), 4.38 (1H, dd, 11.6 and 5.5, 3-glc H-6′), 4.58 (1H, dd, 11.7 and 1.7, 3-glc H-6′), 4.78 (1H, d, 5.8, H-3), 5.10 (1H, t, 8.1, H-6), 5.23 (1H, d, 7.7, 3-glc H-1′), 5.65 (1H, d, 8.0, H-7), 6.65 (1H, s, H-11). ^{13}C NMR (100 MHz); δ19.69 (C-20), 20.62 (C-16), 21.28 (C-17), 27.70 (C-18), 30.19 (C-15), 38.28 (C-10), 49.96 (C-4), 51.29 (C-5), 51.41 (C-2), 58.54 (C-1), 60.43 (C-7), 63.47 (3-glc6′), 72.00 (3-glc4′), 74.66 (C-6), 76.18 (3-glc2′), 77.58 (C-3), 78.75 (3-glc5′), 79.42 (3-glc3′), 108.19 (C-11), 108.26 (3-glc1′), 112.12 (C-8), 162.51 (C-12), 164.95 (C-14), 170.94 (C-9), 178.61 (C-19).

RESULTS

Isolation of Toxic Compounds from Hemolymph of M. basalis *Larvae.* Ethyl acetate, 1-butanol, and water extracts of the hemolymph showed toxic activity (mortality of more than 80%) to the nymphs of *E. furcellata.* Each extract was further fractionated to isolate the toxic compounds.

Identification of Toxic Compounds. Each isolated compound was identified by using the NMR, MS, UV spectra, and GC retention index system (Kováts, 1965). From the ethyl acetate extract of the hemolymph, compound **1** was isolated (Figure 1). The spectral data (NMR, UV) of compound **1** were identical to those of inumakilactone A, norditerpene dilactone, that was isolated from the podocarp tree, *P. macrophyllus* (Figure 1) (Ito et al., 1968).

From the 1-butanol extract, two compounds were found to have toxic activity against *E. furcellata.* Both compounds had the same UV spectra (λ_{max} 300 nm in methanol). Polar compound **2** was present in a large amount and showed a low toxicity, while the less polar compound **3** was present in a small amount and showed a high toxicity. UV spectra of **2** and **3** were very similar to that of nagilactone C, norditerpene dilactone, that was also isolated from the podocarp tree, *P. macrophyllus* (Hayashi et al., 1968; Ito and Kodama, 1976). The NMR

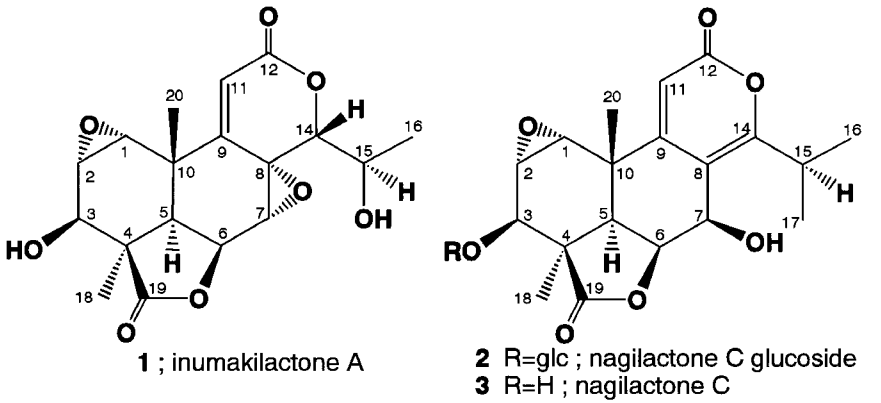


FIG. 1. Structures of toxic compounds **1**, **2**, and **3** isolated from hemolymph of *M. basalis*.

spectrum of **3** was identical with that of nagilactone C (Hayashi et al., 1968; Kubo and Ying, 1991) (Figure 1).

The mass peak (FAB) of compound **2** was observed at *m/z* 547 $(M+Na)^+$, which was higher by 162 mass units than that of nagilactone C, and indicated the presence of one additional mole of the glycosyl group in **2**. The ^1H and ^{13}C NMR spectra of **2** were very similar to those of nagilactone C (Hayashi et al., 1968; Kubo and Ying, 1991), except for the appearance of glycopyranosyl group signals. In addition, the signal at Me-18 in **2** (δ 1.89) shifted ca. 0.22 ppm upfield (nagilactone C; δ 2.11) and the carbon signal at C-3 in **2** (δ 77.58) shifted ca. 9.57 ppm downfield (nagilactone C; δ 68.01). Thus, the additional glycopyranosyl moiety appeared to link to the C-3 hydroxyl group.

In order to identify the unknown carbohydrate moiety in **2**, the GC retention index system (Kováts, 1965) was used. The data of FAB of 162 mass units difference between **2** and nagilactone C indicated that the molecular weight of the unknown carbohydrate moiety in **2** was 180. Therefore, some naturally occurring carbohydrates with the chemical formula of $C_6H_{12}O_6$ were used as standards. Each of the standard trimethylsilylated carbohydrate solutions (glucose, galactose, sorbose, mannose, allose, and gulose, 1 μl) as well as a solution of the hydrolyzed and trimethylsilylated compound **2** (1 μl) was injected into the GC together with the standard hydrocarbons in hexane solution 1 μl (C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, and C_{28}, each 10 ng/μl). Retention indices were calculated. Since the retention index of the trimethylsilyl derivative of hydrolyzed compound **2** (peak 1: 1857, and peak 2: 1893; peak 1 and 2 were derived from α- and β-anomers during the trimethylsilylation of carbohydrates) agreed with that of glucose 5TMS (peak 1: 1858, and peak 2: 1893), the carbohydrate in **2** proved to be glucose. A signal at δ 5.23 (d, $J = 7.7$ Hz) was assigned to a glucosyl anomeric proton and the J value

suggested that the glycosidic bond had a β linkage. From the above evidence, **2** was identified as nagilactone C glucoside, 3-*O*-β-glucopyranoside of nagilactone C (Figure 1). Signals of ^{1}H and ^{13}C NMR spectra of **2** were all assigned by H-H cosy and HSQC spectra.

From the water extract of the hemolymph, the toxic fraction included nagilactone C glucoside (**2**) and nagilactone C (**3**), the same compounds as those of the 1-butanol extract.

*Quantification of Inumakilactone A (**1**), Nagilactone C glucoside (**2**), and Nagilactone C (**3**).* The concentrations of the three compounds in the hemolymph of *M. basalis* were calculated based on HPLC analysis, and found to be as high as 130, 770, and 50 μg/ml, respectively. The concentrations of **1** and **3** in the leaves of *P. macrophyllus* were 13 and 175 μg/g fresh weight, respectively.

*Relationship Among Concentrations of Compounds **1**, **2**, and **3** and* E. furcellata *Mortality.* When the test compound was applied to the absorbent cotton at concentrations of 1000, 200, 40, and 10 μg/ml each in silkworm hemolymph, the concentrations of inumakilactone A, nagilactone C glucoside, and nagilactone C, that showed 50% mortality of *E. furcellata*, were 260, 650, and 30 μg/ml, respectively (Figure 2A). Nagilactone C showed the highest insecticidal activity

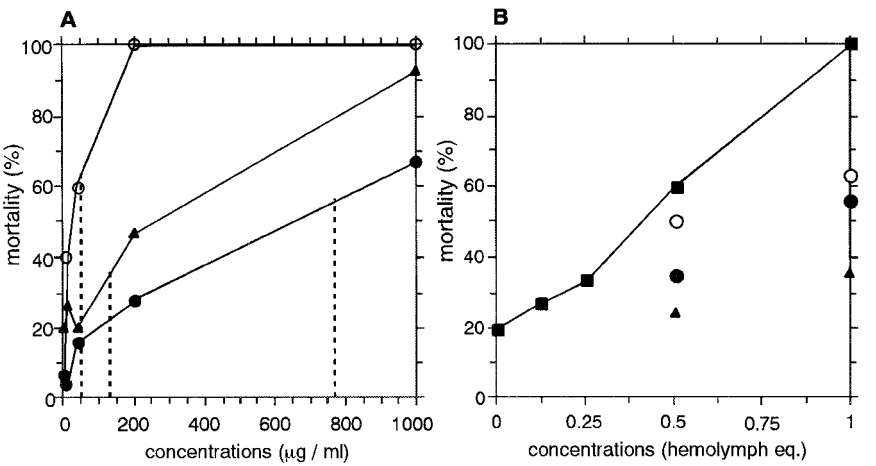


FIG. 2. Relationship between concentration of inumakilactone A, nagilactone C and nagilactone C glucoside and mortality of *E. furcellata*. (A) Test compound was applied at concentrations of 1000, 200, 40, and 10 μg/ml. Triangles: inumakilactone A, open circles: nagilactone C, closed circles: nagilactone C glucoside. Dotted lines indicate the contents of each compound in the hemolymph of *M. basalis* larvae. (B) Test compounds were applied at the same concentrations as hemolymph of *M. basalis*. Squares: three compounds mixed at the hemolymph of *M. basalis* equivalent, i.e., 1 eq mixture of inumakilactone A (130 μg/ml), nagilactone C (50 μg/ml), and nagilactone C glucoside (770 μg/ml); triangles: inumakilactone A, open circles: nagilactone C, closed circles: nagilactone C glucoside.

against *E. furcellata*, while nagilactone C glucoside showed the lowest, one twentieth of that of nagilactone C. When the three compounds mixed at the same concentrations as those in hemolymph of *M. basalis* larvae (**1**, 130; **2**, 770; and **3**, 50 μg/ml) were given to *E. furcellata*, none of the nymphs survived after three days (Figure 2B).

DISCUSSION

In this study, we identified that *M. basalis* larvae possess high concentrations of inumakilactone A (**1**), nagilactone C (**3**), and its glucoside (**2**) in the hemolymph as compounds toxic to *E. furcellata* (Figure 1). It has been reported that inumakilactone A and nagilactone C are contained in *P. macrophyllus*, the only host plant of *M. basalis* (Hayashi et al., 1968; Ito et al., 1968). Inumakilactone A shows antitermitic activity (Saeki et al., 1970), and nagilactone C is toxic to housefly larvae (Russell et al., 1972) and larvae of codling moth and light-brown apple moth (Singh et al., 1978, 1979). In the present study, inumakilactone A and nagilactone C were confirmed to be contained in the leaves of *P. macrophyllus* at concentrations of 13 and 175 μg/g fresh leaves, respectively. In *M. basalis* hemolymph, inumakilactone A, nagilactone C, and its glucoside were found to be at concentrations of 130, 50, and 770 μg/ml, respectively. Taking into account these results and the fact that no nagilactone C glucoside was detected in the leaves of *P. macrophyllus*, *M. basalis* is considered to sequester inumakilactone A and nagilactone C from the host plant and transform nagilactone C to its glucoside and store it. The conflict that the concentration of nagilactone C in the hemolymph is lower than that of the fresh leaves of *P. macrophyllus* can be explained by the likelihood of transformation of nagilactone C to its glucoside in *M. basalis* larvae. It is reported that larvae of the lycaenid butterfly, *Polyommatus icarus*, reared on inflorescences of *Coronilla varia* and *Medicago sativa* sequester host-plant derived flavonoids and glycosylated flavonoids (Wiesen et al., 1994). Wiesen et al. (1994) indicated that these flavonoids might be biotransformed to their glycosides by the insects or their gut flora. A similar transformation might occur in the case of *M. basalis*. Although it is uncertain, one of the reasons for transformation to glucoside form in *M. basalis* larvae may be to reduce the toxicity of nagilactone C for *M. basalis* itself (Figure 2A).

Inumakilactone A (**1**), nagilactone C (**3**), and its glucoside (**2**) in *M. basalis* hemolymph were contained at 130, 50, and 770 μg/ml, respectively. Each of these compounds showed insecticidal activity against *E. furcellata* at concentrations of 260, 30, and 650 μg/ml, respectively (50% mortality; Figure 2A). Furthermore, when the three compounds were given as a mixture at the same concentrations as in the hemolymph of *M. basalis* larvae, none of the *E. furcellata* nymphs survived. Thus, the concentrations of those compounds in the *M. basalis* hemolymph were

toxic enough to kill the *E. furcellata* nymphs. It is unclear whether the observed toxicity of *E. furcellata* larvae is due to postingestive effects, direct contact, or a combination of both. However, when *E. furcellata* larvae bloodsuck *M. basalis* larvae, they contact only at proboses, and other parts of the body and legs seldom contact *M. basalis* larvae. In addition, from HPLC analysis of the skin extract, only trace amounts of nagilactones were detected. Taking into account the above results, the observed toxicity of the *E. furcellata* larvae is regarded to be due to only postingestive effects.

In this report, we focused on the toxicity of host-derived compounds sequestered in *M. basalis* larvae. Since *M. basalis* larvae have an aposematic coloration, orange head and white–latticed black skin, the coloration may also play a part in the prey–predator interaction. Furthermore, other aspects, such as deterrence of the compounds, may contribute to the protection of *M. basalis* from predators. It has been reported that some of the defense substances directly toxic to predators have an unpalatable or deterrent nature (Nishida and Fukami, 1989; Nishida, 1994). For example, physiologically toxic grayanoid diterpenes that are sequestered from leaves of *Pieris japonica* by larvae of a geometrid moth, *Arichanna gaschkevitchii*, have potent deterrent activity (Nishida, 1994). The generalist predatory stink bugs, *E. furcellata*, put their proboses into lepidopteran larvae in response to (*E*)-phytol, which is produced by larvae from chlorophyll in food plants (Yasuda, 1997, 1998). *E. furcellata* put their proboses into *M. basalis* larvae, but soon pulled back, although they repeated short-time sucking. They then died after three days under confined conditions. This suggests a deterrent nature for *E. furcellata*. The deterrent nature may also play a part in the protection of *M. basalis* from *E. furcellata* in fields. Further investigation is needed to determine whether toxic compounds identified in this study have deterrent activity to predators.

Acknowledgments—I am grateful to Dr. J. Arnason of the University of Ottawa, Dr. M. Hattori and Dr. S. Wakamura for critical reading of the manuscript and valuable comments, and Dr. S. Wakamura for GC operation. I also thank Dr. M. Sakakibara for giving me the idea for this study, and Drs. N. Arakaki, K. Yasuda, M. Sakakibara, and K. Kinjo of the Okinawa Prefectural Agricultural Experiment Station for kindly collecting the *Milionia basalis* larvae, Dr. T. Yasuda for providing *Eocanthecona furcellata*, and R. Murakami for measurement of NMR spectra.

REFERENCES

CHU, Y. 1975. Rearing density of *Eocanthecona furcellata*, with special consideration to its mass production (Asopinae: pentatomidae). *Rostria* 24:135–140 (in Japanese with English summary).

HAYASHI, Y., TAKAHASHI, S., ONA, H., and SAKAN, T. 1968. Structures of nagilactone A, B, C and D, novel nor-and bisnorditerpenoids. *Tetrahedron Lett.* 17:2071–2076.

ITO, S. and KODAMA, M. 1976. Norditerpene dilactones from Podocarpus species. *Heterocycles* 4:595–624.

ITO, S., KODAMA, M., SUNAGAWA, M., TAKAHASHI, T., IMAMURA, H., and HONDA, O. 1968. Structure of inumakilactone A, bisnorditerpenoid. *Tetrahedron Lett.* 17:2065–2070.

KOVÁTS, E. 1965. Gas chromatographic characterization of organic substances in the retention index system. *Adv. Chromatog.* 1:229–247.

KUBO, I. and YING, B.-P. 1991. A bisnorditerpene dilactone from *Podocarpus nagi*. *Phytochemistry* 30:3476–3477.

NISHIDA, R. 1994. Sequestration of plant secondary compounds by butterflies and moths. *Chemoecology* 5/6:127–138.

NISHIDA, R. and FUKAMI, H. 1989. Ecological adaptation of an Aristolochiaceae-feeding swallowtail butterfly, *Atrophaneura alcinous*, to aristolochic acids. *J. Chem. Ecol.* 15:2549–2563.

RUSSELL, G. B., FENEMORE, P. G., and SINGH, P. 1972. Insect-control chemicals from plants. Nagilactone C, a toxic substance from the leaves of *Podocarpus nivalis* and *P. hallii*. *Aust. J. Biol. Sci.* 25:1025–1029.

SAEKI, I., SUMIMOTO, M., and KONDO, T. 1970. The termiticidal substances from the wood of *Podocarpus macrophyllus* D. Don. *Holzforschung* 24:83–86.

SINGH, P., FENEMORE, P. G., and DUGDALE, J. S. 1978. The insecticidal activity of foliage from New Zealand conifers. *Biochem. Syst. Ecol.* 6:103–106.

SINGH, P., RUSSELL, G. B., HAYASHI, Y., GALLAGHER, R. T., and FREDERICKSEN, S. 1979. The insecticidal activity of some norditerpene dilactones. *Entomol. Exp. Appl.* 25:121–127.

WIESEN, B., KRUG, E., FIEDLER, K., WRAY, V., and PROKSCH, P. 1994. Sequestration of host-plant-derived flavonoids by lycaenid butterfly *Polyommatus icarus*. *J. Chem. Ecol.* 20:2523–2538.

YASUDA, T. 1997. Chemical cues from *Spodoptera litura* larvae elicit prey-locating behavior by the predatory stink bug, *Eocanthecona furcellata*. *Entomol. Exp. Appl.* 82:349–354.

YASUDA, T. 1998. Effects of (*E*)-phytol of several lepidopteran species in prey-locating behavior of generalist predatory stink bug, *Eocanthecana furcellata* (Heteroptera: Pentatomidae). *Entomol. Sci.* 1:159–164.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

COMPARISON OF CULTIVARS OF ORNAMENTAL CROP *Gerbera jamesonii* ON PRODUCTION OF SPIDER MITE-INDUCED VOLATILES, AND THEIR ATTRACTIVENESS TO THE PREDATOR *Phytoseiulus persimilis*

O. E. KRIPS,[1] P. E. L. WILLEMS,[1,4] R. GOLS,[1] M. A. POSTHUMUS,[2] G. GORT,[3] and M. DICKE[1,*]

[1]*Laboratory of Entomology*
[2]*Laboratory of Organic Chemistry*
[3]*Subdepartment of Mathematics*
Wageningen University
Wageningen, The Netherlands

(Received November 16, 1999; accepted March 8, 2001)

Abstract—We investigated whether volatiles produced by spider mite-damaged plants of four gerbera cultivars differ in attractiveness to *Phytoseiulus persimilis*, a specialist predator of spider mites, and how the mite-induced odor blends differ in chemical composition. The gerbera cultivars differed in resistance, as expressed in terms of spider mite intrinsic rate of population increase (r_m). In order of increasing resistance these were Sirtaki, Rondena, Fame, and Bianca. To correct for differences in damage inflicted on the cultivars, we developed a method to compare the attractiveness of the blends, based on the assumption that a larger amount of spider mite damage leads to higher attraction of *P. persimilis*. Spider mite-induced volatiles of cultivars Rondena and Bianca were preferred over those of cultivar Sirtaki. Spider mite-induced volatiles of cultivars Sirtaki and Fame did not differ in attractiveness to *P. persimilis*. Sirtaki plants had a lower relative production of terpenes than the other three cultivars. This was attributed to a low production of *cis*-α-bergamotene, *trans*-α-bergamotene, *trans*-β-bergamotene, and (*E*)-β-farnesene. The emission of (*E*)-β-ocimene and linalool was lower in Sirtaki and Fame leaves than in Bianca and Rondena. The importance of these chemical differences in the differential attraction of predatory mites is discussed.

* To whom correspondence should be addressed. e-mail: Marcel.Dicke@users.ento.wau.nl
[4] Present address: Nunhems Zaden bv, Voort 6, 6083 AC Nunhem, The Netherlands.

0098-0331/01/0700-1355$19.50/0

Key Words—Acarina, Phytoseiidae, Tetranychidae, cultivars, herbivore-induced plant volatiles, attraction, chemical analysis, terpenes.

INTRODUCTION

The predatory mite *Phytoseiulus persimilis* Athias-Henriot is a specialist predator of the spider mite *Tetranychus urticae* Koch, an important pest in greenhouses. Since the predators feed almost exclusively on spider mite species from the genus *Tetranychus* and are unable to survive on alternative food sources such as pollen or nectar (Sabelis, 1985), they rely on their ability to locate areas where prey is present.

Several species of host plants respond to damage by spider mites with the production of volatiles that attract *P. persimilis* (Sabelis and van de Baan, 1983; Sabelis et al., 1984; Dicke and Sabelis, 1988; Dicke et al., 1990a,b, 1998; Sabelis and van der Weel, 1994; Sabelis and Afman, 1994). *Phytoseiulus persimilis* uses these odors to find leaves with colonies of its prey. The predators do not leave a prey patch until all prey is exterminated. This is most likely the result of arrestment in response to volatiles from damaged plants (Sabelis and van der Meer, 1986; Sabelis and Afman, 1994). Arrestment of the predators in a prey patch has a major influence on the population growth of the predators and their prey (van Baalen and Sabelis, 1995). Therefore, the response of the predators to spider mite-induced volatiles is likely to be important for the success of spider mite control with *P. persimilis*.

Different species of plants show qualitative and quantitative differences in the production of spider mite-induced volatiles, and these blends differ in attractiveness to *P. persimilis*. Even cultivars of the same species may differ in production of volatiles and attractiveness (see Takabayashi and Dicke, 1996, and Dicke et al., 1998, for reviews). Cultivar differences also have been found in the composition or the attractiveness of blends of volatiles that are produced in response to damage by other herbivores (Loughrin et al., 1995, 1996; Souissi, 1999). The success of *P. persimilis* in localizing spider mite-infested leaves and the speed at which prey populations are exterminated may, therefore, depend on the plant species or cultivar.

Gerbera jamesonii Bolus is an ornamental crop of which a large number of cultivars are grown commercially. During the breeding process, cultivars are mainly selected for color and shape of the flowers. However, quite a number of other differences exist, such as leaf shape and texture, density of trichomes, and resistance to the spider mite *Tetranychus urticae* Koch (Süterlin and van Lenteren, 1997; Krips et al., 1998, 1999a). Possibly, gerbera cultivars with spider mite damage differ as well in the production of volatiles, and these volatile blends may differ in attractiveness. This may result in cultivar effects on the ability of *P. persimilis* to localize its prey and the speed at which a spider mite population is exterminated.

The aim of this study was to compare the composition of the blends of volatiles produced by spider mite damaged leaves of four gerbera cultivars and to determine

the attractiveness of these blends to *P. persimilis*. We tried to relate differences in blends to differences in attractiveness to *P. persimilis*. However, gerbera cultivars differ largely in resistance to *T. urticae* (Krips et al., 1998), which results in unequal consumption rates by the spider mites. Thus, placing a standardized number of spider mites on gerbera leaves for a standardized amount of time does not result in equal damage on all cultivars. Because of these differences in host plant resistance, we estimated the amount of spider mite damage on the leaves by counting the number of eggs produced by the infesting spider mites on each set of leaves used in the experiments. In this way, we had an indication of whether or not cultivar differences in volatile production or attractiveness could be explained by differences in damage by spider mites.

METHODS AND MATERIALS

Plant Material

Gerbera (*Gerbera jamesonii*) plants were obtained from the gerbera breeding companies Prego Rijsenhout bv and Terra Nigra bv. They were grown at 20–30°C, 50–70% relative humidity, and at least 16 hr of light per day in a greenhouse at Wageningen University, The Netherlands. High-pressure mercury lamps were switched on when the light intensity outside dropped below 150 W/m^2 and switched off when it increased above 250 W/m^2. Plants used for the experiments were 6–12 months old. The youngest fully unfolded leaves of the cultivars Bianca, Fame, Rondena, and Sirtaki were used. Sirtaki is most susceptible to spider mites, closely followed by Rondena, while Fame is more resistant, and Bianca is most resistant. The intrinsic rate of population increase (r_m) of *T. urticae* is 0.24/day on Sirtaki, 0.21/day on Rondena, 0.16/day on Fame, and 0.09/day on Bianca (Krips et al., 1998). The cultivars Fame, Sirtaki, and Rondena are related to each other (see pedigree in Figure 1). Cultivar Bianca is unrelated to the other three cultivars.

Spider Mites

Spider mites (*Tetranychus urticae*) were collected from a commercial gerbera greenhouse at Mijdrecht, The Netherlands, in the spring of 1994 and were subsequently reared on the gerbera cultivar Sirtaki. Plants with mites were kept at 20–30°C, 50–70% relative humidity, and at least 16 hr of light per day in a greenhouse at Wageningen University.

Predatory Mites

Predatory mites (*Phytoseiulus persimilis*) were originally obtained from Entocare CV, a commercial mass rearing company for biological control. In our

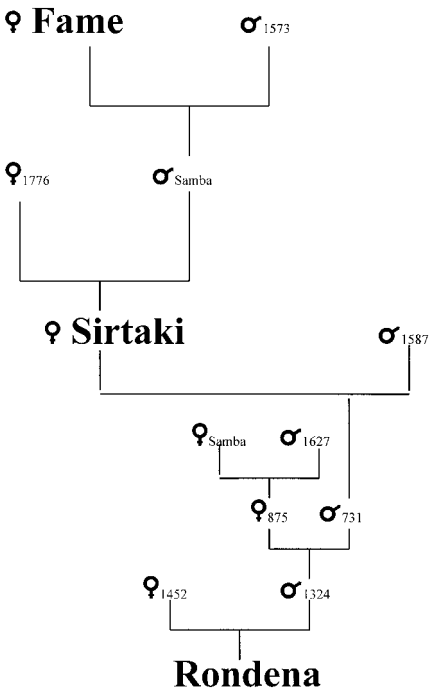


FIG. 1. Pedigree showing the relatedness of the gerbera cultivars Rondena, Fame and Sirtaki.

laboratory, they were reared on spider mites (*T. urticae*) on gerbera leaves of the cultivar Sirtaki. The predators were kept in Petri dishes of 9 cm diameter with an opening in the lid of 2.7 cm diameter, covered with fine-mesh gauze, and were offered pieces of leaves with spider mites three times a week. Once a week, five gravid female *P. persimilis* were transferred from each Petri dish to new ones. The Petri dishes were kept in a climate room at $23 \pm 2^{\circ}$C, $60 \pm 5\%$ relative humidity with a 16L:8D photoperiod.

Experiment 1: Preference of P. persimilis

Incubation of Leaves. We compared the attractiveness of the spider mite-induced volatiles of cultivar Sirtaki with those of cultivars Bianca, Fame, and Rondena. For each replicate, detached leaves of Sirtaki and one of the other cultivars were placed with their petioles in vials with tap water. The weight of the leaf samples was 30–70 g per cultivar, but was equal for both cultivars per replicate. Depending on the cultivar, 50–200 adult female spider mites were placed on each leaf, after which the leaves were incubated for seven days at $23 \pm 1^{\circ}$C, $60 \pm 5\%$

relative humidity, and a 16L:8D photoperiod. After seven days, we counted the number of eggs on the leaves as a measure of the spider mite damage inflicted. Approximately 20% of the leaf material ingested by spider mites is converted into eggs (Sabelis, 1981, p. 31), and at constant temperature and relative humidity, the number of eggs produced is directly related to the amount of ingested leaf material. Since we placed only adult female mites on the leaves, the leaves were not damaged by males. Eggs that were produced on the first day of incubation had, on some cultivars, developed into larvae during incubation. In these cases, the larvae were counted together with the eggs. Damage inflicted by these larvae was negligible compared to the damage inflicted by adult females (Sabelis, 1981).

Olfactometer Set-Up. Leaves of both cultivars were placed in separate jars that contained a small amount of water. The jars were connected to a Y-tube olfactometer, described in more detail by Takabayashi and Dicke (1992). Air with volatiles from spider mite-damaged leaves of one cultivar was led through one arm at 4 liters/min, and air from the other cultivar through the other arm at 4 liters/min. Air was extracted at the base of the olfactometer at 8 liters/min. In this way, two well-separated laminar airflows were generated in the olfactometer.

Bioassay. Adult female *P. persimilis*, from the culture on gerbera with spider mites, were starved for 3 hr and introduced individually into the olfactometer onto an iron wire running through the center of the olfactometer glass tube and parallel to the tube walls. Predators were placed at the base of the Y-tube and were allowed to choose between two olfactometer arms. The observation of a predator ended when it reached the far end of one of the olfactometer arms. Observations lasted for a maximum of 5 min per predator. Predators that had not reached the end of one of the olfactometer arms within this time were excluded from the statistical analysis. This happened in 44 cases out of a total number of 600 predators. For each replicate set of leaves, 20 predators were tested, and after every five predators, the connections of the odor sources to the two arms of the olfactometer were interchanged.

Statistical Analysis. For the statistical analysis, each olfactometer test with 20 predators and a separate set of leaves was used as a statistical unit. We used a generalized linear model (Crawley, 1993) for the number of predators choosing Sirtaki per replicate experiment with 20 predators. This model contained a binomial distribution with p for the proportion of predators choosing Sirtaki and a linear relationship between the logit of p and the amount of damage (=number of spider mite eggs) on each of two cultivars. This amount of damage is quantified by l_x, which is the logarithm of $x/(1-x)$, with x the fraction of eggs found on Sirtaki [eggs on Sirtaki/(eggs on Sirtaki + eggs on other cultivar)]. If $1_x > 0$, more eggs are found on Sirtaki than on the other cultivar, so we expect to find a higher rate of predators choosing for Sirtaki. If $1_x < 0$, more eggs are found on the other cultivar, and we expect a higher rate of predators to choose for the other cultivar.

We allowed the residual variance to be larger than prescribed by the binomial distribution through a multiplicative scale parameter. The main interest focused

on the probability p_0 of choosing Sirtaki at $1_x = 0$, i.e., with equal amount of damage on both gerbera cultivars. We tested the null hypothesis: $p_0 = 0.5$ versus the alternative $p_0 <> 0.5$. In other words, we tested whether the chance the predators chose for Sirtaki was 0.5 at an equal number of spider mite eggs on both cultivars. We further tested whether there is an effect of amount of damage on predator choice as follows: H_0: regression coefficient = 0 vs. H_1: regression coefficient >0. The model was fitted by using procedure GENMOD from the statistical program SAS version 6.12 (SAS Institute Inc., 1997).

We present results from analyses for the comparison of Sirtaki with each of the three other cultivars separately and from an overall analysis with all results combined, allowing for different success rates for different experimental setups, but with a common effect of amount of damage 1_x and a common overdispersion factor.

Experiment 2: Chemical Composition of Blends of Volatiles

We incubated detached leaves of the four gerbera cultivars with adult female spider mites following the procedure described for experiment 1. The fresh weight of the leaf samples was 34.6 ± 1.96 ($N = 5$), 29.7 ± 3.30 ($N = 6$), 32.8 ± 1.12 ($N = 6$) and 33.3 ± 3.73 ($N = 4$) g (mean $\pm$ SD) for Bianca, Fame, Rondena, and Sirtaki, respectively. The number of leaves varied between 3 and 10 per sample. Two additional samples were taken from Sirtaki with a smaller total weight (15.7 and 23.3 g) because some of the leaves had wilted during incubation and were discarded. After seven days, we counted the eggs that were present on the leaves to determine the amount of damage.

The leaves were placed with their petioles in a 200-ml glass vial containing tap water. The vial was subsequently placed in a 5-liter glass jar. Air entered the jar through a glass tube that nearly reached the bottom of the jar. Before entering the jar, air was cleaned through silica gel, molecular sieves, and activated charcoal, following the procedure described by Mattiacci et al. (1994).

An airstream of 450 ml/min was generated, and air was purged through the system for 1 hr prior to collection of volatiles in order to remove all contaminants from the jar. Subsequently, a Pyrex glass tube (161×6.4 mm OD, 3 mm ID) containing 90 mg Tenax-TA was connected to the outlet of the system for 15 min (6.75 liter). The volatiles from the Tenax tubes were analyzed by GC-MS by using the Thermodesorption Cold Trap Unit as an inlet on the gas chromatograph (Mattiacci et al., 1994).

Headspace samples were taken in four batches between April 1997 and November 1998. For all samples of Bianca, Fame, and Sirtaki and one sample of Rondena, we used a Supelcowax 10 fused silica capillary column, 60 m $\times$ 0.25 mm ID, 0.25-μm film thickness, with helium as carrier gas at an initial linear velocity of 22 cm/sec. The oven temperature was raised from 40°C to 270°C

at 4°C/min. For the other Rondena samples, a Restek Rtx200 column was used, 60 m × 0.25 mm ID, 0.25-μm film thickness. The oven temperature was raised from 40°C to 250°C at 4°C/min. Compounds were identified by comparison of the mass spectra (Finnigan MAT 95 mass spectrometer, 70 eV EI ionization mode, scanning from mass 24 to 400 at 0.7 sec/decade) with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index. One additional sample of Sirtaki was analyzed on a BP5 column and was used only to verify the identification of some sesquiterpenoids by comparison with the retention indices as described in Adams (1989) and Joulain and Koenig (1998). An approximate measure of the sensitivity of the GC-MS was obtained by regular quantification of the peak areas of a mixture of standard compounds. The sensitivity was variable among batches of samples and was approximately 0.26, 0.10, and 0.05 ng/peak area unit for the three batches.

RESULTS

Experiment 1: Preference of P. persimilis *for Volatiles of Gerbera Cultivars with Spider Mite Damage*

In Figure 2 three pairwise comparisons of the attractiveness of spider mite-induced volatiles from different cultivars are shown. Each point in Figure 2 represents a separate olfactometer test with 20 predators. For each set of leaves, we have estimated the amount of mite damage by counting the number of eggs present on the leaves. The fraction of eggs on Sirtaki [eggs on Sirtaki/(eggs on Sirtaki + eggs on other cultivar)] is plotted on the *x* axis and the fraction of predators that chose for the volatiles of Sirtaki [predators to Sirtaki/(predators to Sirtaki + predators to other cultivar)] is plotted on the *y* axis.

Our null hypothesis was that the choice of the predators for the volatiles of one of the cultivars is determined by the amount of damage on that cultivar, in comparison with the amount of damage on the other cultivar. If this hypothesis is correct, one would expect the predators to choose for the volatiles of the cultivar with the most damage. Hence, in absence of preference, we expect points in the left-lower quarter and right-upper quarter of the graphs. With a generalized linear model and a subsequent contrast, we tested whether the predators had equal preference for the volatiles from both cultivars when spider mite damage on both cultivars was equal.

In the comparison between cultivars Sirtaki and Rondena (Figure 2A), most dots fall within the lower-right quarter of the graph, which means that, although the leaves of Sirtaki were more severely damaged by spider mites, the predators in the Y-tube olfactometer chose the volatiles of the less-damaged Rondena leaves. This preference for the volatiles of Rondena leaves was significant ($P = 0.004$). In total, 63% of the 179 predators chose Rondena. A similar result was obtained

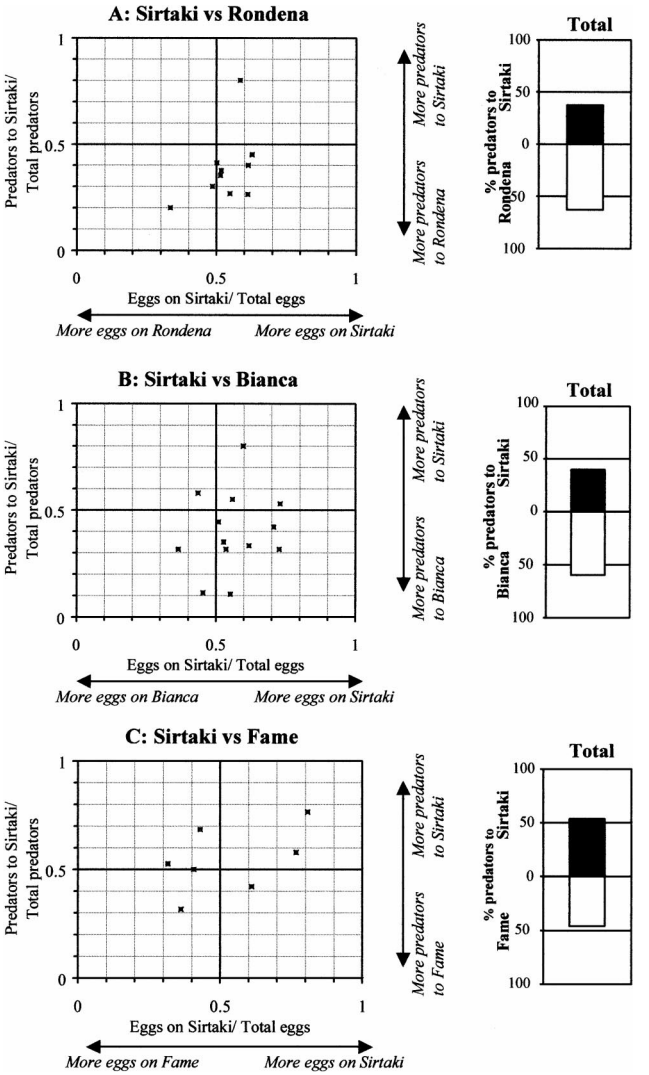


FIG. 2. Response of adult female *Phytoseiulus persimilis* to volatiles from leaves of gerbera cultivars infested by *Tetranychus urticae*. Each data point represents a Y-tube olfactometer test in which 20 adult female *P. persimilis* chose between volatiles from damaged leaves of two cultivars. The fraction of predators that chose for the spider mite-induced volatiles of either cultivar is plotted against the fraction of spider mite eggs (as a relative measure of damage) on the leaves of both odor sources. On the right of each graph, the pooled data for all replicates together is shown as the percentage of all predators that chose for each of the two cultivars.

in the comparison of Sirtaki and Bianca (Figure 2B). Although Sirtaki leaves had more damage than Bianca, there was no significant preference ($P = 0.08$). Of the 245 predators, 60% chose Bianca. When the predators were given a choice between spider mite-induced volatiles from Sirtaki and Fame, they did not show any preference ($P = 0.63$; Figure 2C). In total, 54% of the 132 predators chose Sirtaki.

If we perform a generalized linear model analysis on all data, we find the following results: a significant preference of the predators for Rondena volatiles over Sirtaki volatiles ($P = 0.01$), a significant preference for Bianca volatiles over Sirtaki volatiles ($P = 0.01$), and no significant preference for Sirtaki volatiles over Fame volatiles ($P = 0.70$). The amount of damage to Sirtaki, 1_x, showed a significant positive relationship with the rate of predators choosing Sirtaki ($P = 0.05$).

Experiment 2: Chemical Composition of Volatile Blends

The volatiles that are emitted by spider mite-damaged leaves of cultivars Bianca, Sirtaki, Fame, and Rondena are shown in Table 1. Since the total production of volatiles was variable, we calculated the relative production for each compound, based on the total amount of identified compounds. An approximation of the total production of volatiles by each cultivar is given in Table 1.

A large number of volatiles produced through different biosynthetic pathways is emitted by spider-mite-infested gerbera plants (Figures 3A and 3B). The major portions of the volatile blends consist of terpenoids (Figure 3A). Sirtaki produces the lowest relative amount of terpenoids, which is reflected in the highest relative production of aldehydes, ketones, acetates, nitrogenous compounds, and aromatic compounds (Figure 3B). Fame has a higher relative production of ketones and compounds that contain nitrogen than Bianca and Rondena, while Rondena produces more esters than the other two cultivars.

The compounds that represent more than 5% of the average total volatile blend of at least one cultivar are presented in Figure 4. The major compound is the homoterpene, 4,8-dimethyl-1,3(*E*),7-nonatriene, with a relative production that varies from 18% on Bianca to 26% on Sirtaki. Another homoterpene, 4,8, 12-trimethyl-1,3(*E*),7(*E*),11-tridecatetraene, has a relative production that varies from 6.7% on Bianca to 14% on Fame.

The most striking cultivar differences among the blends are found in the production of *cis*-α-bergamotene, *trans*-α-bergamotene, and *trans*-β-bergamotene. These three compounds each make up about 1% of the blend of Sirtaki, whereas they represent around 5%, 9%, and 4% of the blends of the other cultivars, respectively (Figure 4). The near absence of these compounds in the headspace of Sirtaki accounts to a large extent for the low total production of terpenoids by this cultivar. The production of the terpenoid (*E*)-β-ocimene is around 4% in Sirtaki and Fame, while it represents more than 8% in the blends of the other

TABLE 1. HEADSPACE CHEMICALS OF DETACHED LEAVES OF FOUR GERBERA CULTIVARS INFESTED FOR 7 DAYS BY SPIDER MITES (*T. urticae*)[a]

	Gerbera cultivar			
	Bianca ($N = 5$)	Fame ($N = 6$)	Rondena ($N = 6$)	Sirtaki ($N = 6$)
Aldehydes				
2-Methyl-2-propenal	0.05 ± 0.048	0.6 ± 0.36	n.d.	1.5 ± 0.79
Hexanal	0.007 ± 0.0068	0.06 ± 0.060	0.13 ± 0.045	0.014 ± 0.0136
(*E*)-2-Hexenal	n.d.	0.17 ± 0.175	0.021 ± 0.0151	n.d.
Nonanal	0.28 ± 0.093	0.15 ± 0.099	0.23 ± 0.094	0.41 ± 0.219
Decanal	0.64 ± 0.274	0.45 ± 0.207	0.22 ± 0.119	0.89 ± 0.299
Alcohols				
Ethanol	0.10 ± 0.062	n.d.	n.d.	n.d.
2-Butanol	0.30 ± 0.040	0.36 ± 0.086	0.30 ± 0.050	1.14 ± 0.194
2-Methyl-1-propanol	n.d.	0.15 ± 0.095	0.013 ± 0.0092	0.20 ± 0.098
1-Butanol	0.09 ± 0.045	0.19 ± 0.129	0.017 ± 0.0166	n.d.
1-Hexanol	0.14 ± 0.113	0.6 ± 0.34	0.10 ± 0.044	n.d.
(*Z*)-3-Hexen-1-ol	0.56 ± 0.226	1.8 ± 1.10	0.72 ± 0.236	0.08 ± 0.056
(*E*)-2-Hexen-1-ol	n.d.	0.11 ± 0.113	n.d.	n.d.
1-Dodecanol	0.07 ± 0.046	n.d.	n.d.	0.12 ± 0.080
Esters				
Ethyl acetate	0.06 ± 0.038	0.7 ± 0.44	0.025 ± 0.0249	0.21 ± 0.124
Methyl 2-methylbutanoate	0.017 ± 0.0167	n.d.	n.d.	0.13 ± 0.042
Methyl 3-methyl-2-butenoate	1.1 ± 0.50	0.15 ± 0.104	n.d.	1.1 ± 0.36
(*Z*)-3-Hexen-1-ol, acetate	1.20 ± 0.246	1.8 ± 0.73	4.7 ± 0.75	7.9 ± 1.24
3-Methylbutyl 3-methylbutanoate	n.d.	n.d.	n.d.	0.15 ± 0.118
(*Z*)-3-Hexen-1-ol, 3-methylbutanoate	0.09 ± 0.039	0.05 ± 0.047	0.47 ± 0.146	0.32 ± 0.227
(*Z*)-3-Hexen-1-ol, tiglate	n.d.	n.d.	0.15 ± 0.038	n.d.
Ketones				
2-Butanone	3.9 ± 0.57	7.3 ± 1.73	4.3 ± 0.58	11.6 ± 2.23
3-Buten-2-one	0.11 ± 0.070	0.13 ± 0.065	n.d.	0.26 ± 0.180
2- or 3-Pentanone	0.008 ± 0.0081	0.14 ± 0.072	0.042 ± 0.0205	0.13 ± 0.072
3-Penten-2-one	0.30 ± 0.202	0.59 ± 0.234	0.10 ± 0.066	0.21 ± 0.105
6-Methyl-5-hepten-2-one	0.114 ± 0.0286	0.08 ± 0.051	0.011 ± 0.0110	0.10 ± 0.066
4-Hydroxy-2-pentanone	0.8 ± 0.51	0.6 ± 0.39	0.39 ± 0.141	n.d.
Unidentified ketone[b]	0.021 ± 0.0212	0.012 ± 0.0118	n.d.	0.17 ± 0.098

TABLE 1. CONTINUED

	Gerbera cultivar			
	Bianca ($N = 5$)	Fame ($N = 6$)	Rondena ($N = 6$)	Sirtaki ($N = 6$)
Nitrogenous compounds				
2-Methyl-2-propenenitrile	0.77 ± 0.130	0.39 ± 0.193	0.27 ± 0.079	1.15 ± 0.253
2-Methylpropanenitrile	1.36 ± 0.119	1.94 ± 0.195	0.43 ± 0.078	4.0 ± 1.16
2-Methylbutanenitrile	0.34 ± 0.077	0.54 ± 0.134	0.14 ± 0.037	1.0 ± 0.42
Unidentified nitriles[b]	0.30 ± 0.065	0.32 ± 0.119	0.16 ± 0.057	0.52 ± 0.195
2-Methyl-1-nitropropane	0.44 ± 0.086	0.60 ± 0.108	0.113 ± 0.0249	0.51 ± 0.178
Isomer of previous compound	0.10 ± 0.084	0.18 ± 0.094	0.004 ± 0.0044	0.37 ± 0.187
2-Methylpropanal-*O*-methyloxime	0.7 ± 0.40	1.3 ± 0.33	0.55 ± 0.180	1.7 ± 0.37
2-Methylbutanal-*O*-methyloxime	0.028 ± 0.0281	0.15 ± 0.072	n.d.	0.31 ± 0.150
Isomer of previous compound	0.9 ± 0.41	2.2 ± 0.72	0.93 ± 0.240	5.5 ± 1.75
2-Methylpropanal, oxime	0.04 ± 0.036	0.6 ± 0.37	0.11 ± 0.049	0.027 ± 0.0271
2-Methylbutanal, oxime	n.d.	0.4 ± 0.39	0.016 ± 0.0155	n.d.
Aromatic compounds				
Benzaldehyde	0.9 ± 0.31	1.0 ± 0.47	0.81 ± 0.272	3.8 ± 0.97
Benzonitrile	n.d.	0.16 ± 0.104	n.d.	0.11 ± 0.070
Methyl benzoate	0.16 ± 0.125	0.11 ± 0.086	0.077 ± 0.0154	0.03 ± 0.035
Methyl salicylate	0.44 ± 0.118	n.d.	0.015 ± 0.0037	0.005 ± 0.0054
2-Methylpropyl-benzoate	n.d.	0.33 ± 0.209	n.d.	n.d.
(*Z*)-3-Hexen-1-ol, benzoate	n.d.	n.d.	0.13 ± 0.075	n.d.
Terpenes				
Sabinene	0.15 ± 0.041	n.d.	n.d.	n.d.
Myrcene	0.136 ± 0.0256	n.d.	0.040 ± 0.0130	n.d.
Limonene	0.256 ± 0.0252	0.21 ± 0.069	0.046 ± 0.0215	0.05 ± 0.034
(*Z*)-β-ocimene	0.31 ± 0.056	0.11 ± 0.054	0.137 ± 0.0186	0.09 ± 0.044
(*E*)-β-ocimene	8.5 ± 1.71	4.2 ± 1.27	9.3 ± 1.12	3.5 ± 1.54
4,8-Dimethyl-1,3(*Z*), 7-nonatriene	0.72 ± 0.165	0.53 ± 0.127	0.24 ± 0.109	1.03 ± 0.215
4,8-Dimethyl-1,3(*E*), 7-nonatriene	18 ± 4.2	19.0 ± 1.29	25.1 ± 2.88	26.4 ± 1.61
Linalool	0.9 ± 0.40	0.12 ± 0.119	1.46 ± 0.277	0.6 ± 0.32
(*E*)-Nerolidol,	1.4 ± 0.42	0.08 ± 0.079	0.8 ± 0.35	0.19 ± 0.093
α-Copaene	0.03 ± 0.030	n.d.	0.084 ± 0.0184	1.03 ± 0.244
β-Cubebene	n.d.	0.04 ± 0.037	n.d.	0.9 ± 0.42
cis-α-Bergamotene	6.3 ± 1.77	4.2 ± 0.78	4.7 ± 0.57	1.2 ± 0.43
trans-α-Bergamotene	9.9 ± 1.54	8.3 ± 0.84	9.0 ± 0.73	1.5 ± 0.54

TABLE 1. CONTINUED

	Gerbera cultivar			
	Bianca ($N = 5$)	Fame ($N = 6$)	Rondena ($N = 6$)	Sirtaki ($N = 6$)
β-Elemene	2.6 ± 1.14	1.7 ± 0.39	2.0 ± 0.45	2.3 ± 0.30
β-Caryophyllene	2.9 ± 0.46	3.9 ± 0.94	3.8 ± 0.44	2.4 ± 0.41
β-Santalene	0.10 ± 0.067	n.d.	0.010 ± 0.0098	n.d.
(E)-β-Farnesene	3.3 ± 0.71	1.4 ± 0.31	2.2 ± 0.34	0.05 ± 0.039
α-Humulene	0.11 ± 0.084	0.32 ± 0.229	0.39 ± 0.073	0.16 ± 0.083
trans-β-Bergamotene	5.3 ± 1.16	4.4 ± 0.58	3.0 ± 0.58	0.72 ± 0.082
γ-Curcumene	n.d.	n.d.	0.12 ± 0.43	n.d.
α-Zingiberene	2.7 ± 0.58	0.26 ± 0.260	2.7 ± 0.51	n.d.
Germacrene D	1.0 ± 0.35	1.4 ± 0.72	1.40 ± 0.254	1.3 ± 0.60
β-Bisabolene	1.3 ± 0.51	0.18 ± 0.114	1.5 ± 0.46	n.d
(*Z*,*E*)-α-Farnesene	0.06 ± 0.057	n.d.	n.d.	0.20 ± 0.099
(*E*,*E*)-α-Farnesene	3.03 ± 0.133	3.1 ± 0.50	2.3 ± 0.39	2.3 ± 0.88
β-Sesquiphellandrene	1.82 ± 0.296	1.1 ± 0.45	0.99 ± 0.147	n.d.
ar-Curcumene	1.6 ± 0.48	1.7 ± 0.51	0.91 ± 0.087	n.d.
Isomer of next compound	0.14 ± 0.141	0.08 ± 0.083	0.25 ± 0.075	0.39 ± 0.175
4,8,12-Trimethyl-1,3(E) 7(E),11-tridecatetraene	6.7 ± 1.02	14 ± 3.7	9.5 ± 0.89	7.0 ± 2.51
(*Z*,*E*,*E*)-Allofarnesene	0.32 ± 0.116	n.d.	0.008 ± 0.0079	n.d.
Unidentified terpenes[b]	3.0 ± 1.37	2.6 ± 1.06	1.8 ± 0.37	0.41 ± 0.200
Indole	0.09 ± 0.033	0.062 ± 0.0133	0.39 ± 0.170	0.23 ± 0.082
Total	99.6%	99.5%	99.6%	99.5%
Unidentified compounds (% of total peak area)	4.1 ± 1.47	7.4 ± 2.78	1.53 ± 0.208	9.6 ± 2.98
Approximate total volatile production (ng)[c]	4456 ± 1137	5645 ± 3339	5057 ± 2282	1672 ± 151[d]
Average number of spider mite eggs per sample	4379 ± 814	7349 ± 2450	8024 ± 904	3706 ± 410

[a] For each compound the percentage (mean ± SE) of the total amount of identified volatiles is given. Only compounds that represent at least 0.1% of the blend of one cultivar are presented. n.d. = not present in detectable levels in any of the samples of this cultivar.
[b] These unidentifiable compounds were present in many samples and are therefore not excluded from calculation of the total amount of volatiles.
[c] Based on the approximate sensitivity of the GCMS analysis per batch.
[d] Two samples with low total leaf weight of Sirtaki excluded.

two cultivars. Furthermore, (E)-β-farnesene is almost absent in the Sirtaki blend, whereas it represents 1.4–3.3% of the blends of the other cultivars (Table 1).

The relative production of (Z)-3-hexen-1-yl acetate, 2-butanone, and 2-methylbutanal-*O*-methyloxime is higher in Sirtaki than in the other three cultivars (Figure 4). Furthermore, Sirtaki has a higher production of 2-methylpropanenitrile and benzaldehyde (Table 1).

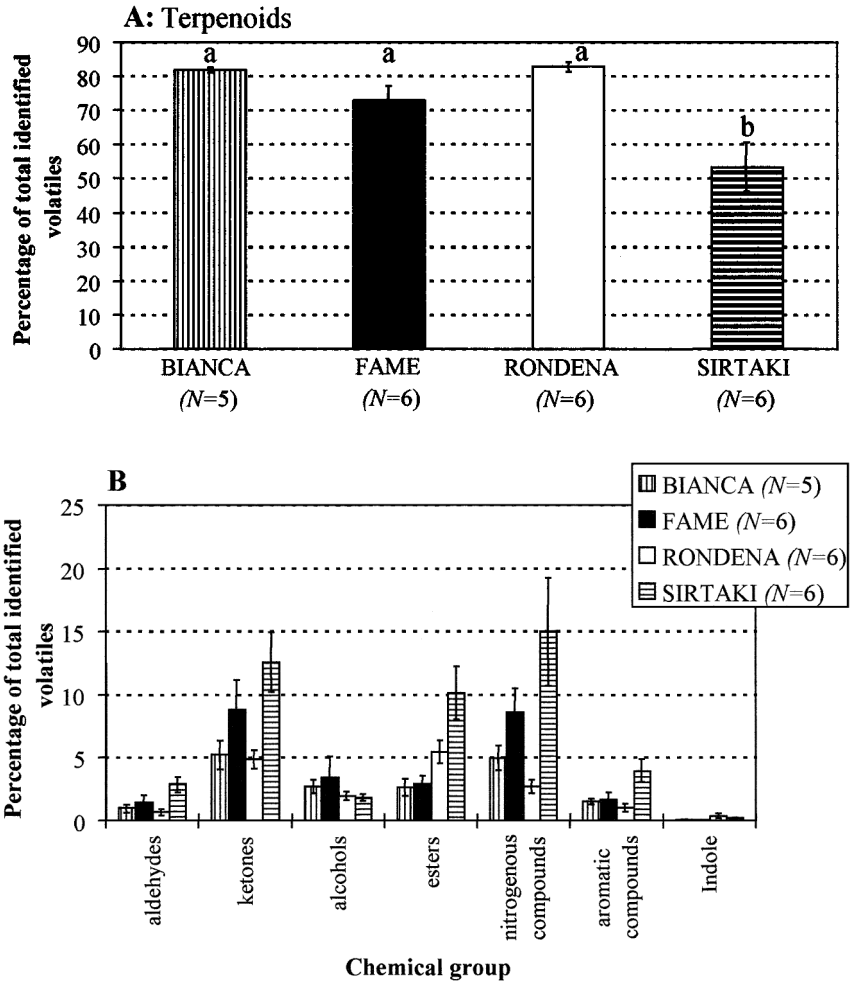


FIG. 3. Volatiles of four gerbera cultivars with *Tetranychus urticae* damage, divided into chemical groups: (A) percentage of terpenoids (different letters above bars indicate significant differences; ANOVA followed by LSD test, $\alpha = 0.05$). (B) percentages of the remaining groups. Vertical lines represent standard errors.

The total volatile production by Sirtaki was less than half the production rate of the other three cultivars (Table 1). The number of spider mite eggs found on this cultivar was also about half what was found on Fame and Rondena. Hence, the difference in total volatile production between Sirtaki as compared to Fame and Rondena can be explained by a difference in spider mite damage. However, the

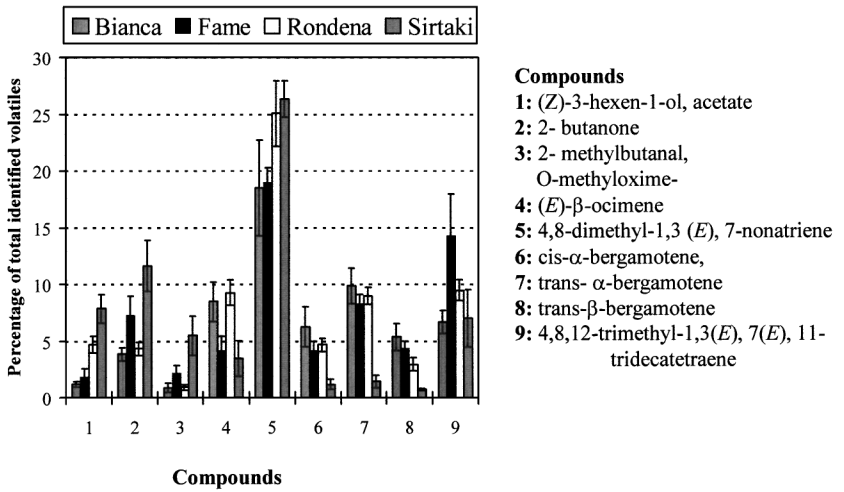


FIG. 4. Volatiles produced by leaves of four gerbera cultivars with damage of *Tetranychus urticae*. For each compound the average peak area divided by the total peak area of identified compounds is shown. Only compounds that represent at least 5% of the total volatile blend of a cultivar are presented. Vertical lines represent standard errors.

number of eggs on Sirtaki and Bianca is on the same order of magnitude, while the total production of volatiles of Sirtaki is less than half the production by Bianca. This difference in total production of volatiles, therefore, can not be explained by a difference in spider mite damage.

DISCUSSION

We have presented a method to compare the attractiveness of volatiles produced by spider mite-damaged leaves of four gerbera cultivars without ignoring differences in damage on the leaves as a result of differences in host plant resistance. We based our method on the assumption that attraction of *P. persimilis* is positively related to the amount of spider mite damage on gerbera leaves. We found a correlation between the amount of spider mite damage on the leaves and the attraction of *P. persimilis* towards the volatiles produced by these leaves. Previous studies showed that the response of *P. persimilis* to volatiles from spider mite-damaged bean leaves increases with the number of damaged leaves offered (Sabelis and van de Baan, 1983). Hence, the total amount of damaged leaf area is important for attraction of the predators. As the data in our paper demonstrate, however, the attractiveness of volatiles from spider mite-damaged leaves is also determined by the amount of damage per leaf.

The cultivars we investigated differ in resistance, as expressed in terms of spider mite intrinsic rate of population increase (r_m). In order of increasing resistance these were Sirtaki, Rondena, Fame, and Bianca, on which spider mite r_m is 0.24, 0.21, 0.16, and 0.09/day, respectively (Krips et al., 1998). *P. persimilis* has a preference for spider mite-induced Rondena and Bianca volatiles over those of Sirtaki, while no difference in attractiveness of Sirtaki and Fame volatiles was found. The predators that were used had been reared on spider mites on Sirtaki. The response of *P. persimilis* to spider mite-induced volatiles of a new host plant can be enhanced by experience (Takabayashi et al., 1994; Krips et al., 1999b). However, *P. persimilis* does not prefer the volatiles of Sirtaki over those of other cultivars. Hence, the differences in attractiveness can not be explained by rearing history or experience of the predators.

A low attractiveness of Sirtaki volatiles as compared with the volatiles of Rondena and Bianca may be the result of a lower production rate of volatiles by Sirtaki leaves, a different composition of the volatile blend, or a combination of the two. The results of the chemical analyses indicate that at equal damage, the total production of volatiles by Sirtaki leaves is lower than by Bianca leaves. However, this does not seem to be the case in the comparison between volatiles of Sirtaki leaves and Rondena leaves. Hence, the composition of the blend of volatiles appears to be important for the attractiveness to *P. persimilis*.

The blend of spider mite-damaged Sirtaki leaves consists of a lower relative amount of terpenes than the blends of the other three cultivars. Similarly, Loughrin et al. (1995) found that differences in volatile production of cotton cultivars were mainly based on differences in the production of terpenes. Several terpenes are known to attract *P. persimilis* (Dicke et al., 1990a). The low production of terpenes by gerbera cultivar Sirtaki in our studies is likely responsible for the low attractiveness of the blend for *P. persimilis*. The low production of terpenes by Sirtaki leaves was mainly due to the low production of *cis*-α-bergamotene, *trans*-α-bergamotene, *trans*-β-bergamotene, and (E)-β-farnesene. However, the low attractiveness of Sirtaki volatiles compared to those of Rondena and Bianca, can not be explained by the low production of these four terpenes because volatiles from Fame are equally attractive as volatiles from Sirtaki, although Fame has a much higher production of these compounds. On the other hand, the relative production of (E)-β-ocimene is lower in the blends of both Sirtaki and Fame than in those of Bianca and Rondena leaves. Interestingly, this compound is known to be attractive to *P. persimilis* when offered as an individual compound in a Y-tube olfactometer (Dicke et al., 1990a) and may, therefore, be partly responsible for the low attractiveness of Sirtaki volatiles in comparison to those of Rondena and Bianca.

Loughrin et al. (1996) found that cultivar differences of volatiles from undamaged apple leaves in attractiveness for the Japanese beetle *Popillia japonica* were correlated with the production of linalool. They also found indications that the

homoterpene, 4,8-dimethyl-1,3(*E*),7-nonatriene, played a role. Both compounds are attractive to *P. persimilis* (Dicke et al., 1990a). Our data indicate that the cultivar differences in attractiveness of gerbera volatiles may be related to differences in the production of linalool. The relatively unattractive Fame has the lowest production of linalool. The attractive Rondena leaves have the highest production rate. Sirtaki and Bianca have similar relative production rates of linalool, but Bianca had a total volatile production that was twice as high as the production by Sirtaki and, therefore, also produced twice as much linalool as Sirtaki.

The relative and absolute production of 4,8-dimethyl-1,3(*E*),7-nonatriene was equal in Sirtaki and Rondena, although the attractiveness of the blends differed. Furthermore, the attractive cultivar Bianca had a lower relative production of this compound than the unattractive cultivar Sirtaki. Therefore, the cultivar differences in attractiveness can not be explained by differences in the production of this homoterpene.

The relative production of 2-butanone, (*Z*)-3-hexen-1-yl-acetate, 2-methylpropane nitrile, and 2-methylpropanal-*O*-methyloxime is higher in the leaves of Sirtaki than in the leaves of the other three cultivars. (*Z*)-3-Hexen-1-yl-acetate is neither attractive nor repellent for *P. persimilis* (Dicke et al., 1990a), and the effect of the other compounds is not known. However, Takabayashi et al. (1994) found indications that two other oximes might be repellent to *P. persimilis*. Possibly, oximes play a role in the low attractiveness of the blend of Sirtaki leaves.

It has been hypothesized that plants that spend much of their resources on direct defense to herbivores may spend less on indirect defense by attracting natural enemies of the herbivores (Dicke, 1994, 1999). This raises the question whether there is a negative correlation between resistance to *T. urticae* and attraction of *P. persimilis*. The cultivars Sirtaki and Rondena are both highly susceptible to *T. urticae* (Krips et al., 1998). The volatiles of Rondena were more attractive to *P. persimilis* than the volatiles of Sirtaki. The cultivar Bianca is resistant to *T. urticae* (Krips et al., 1998), but the volatiles of this cultivar were more attractive to *P. persimilis* than the volatiles of the susceptible Sirtaki. Furthermore, the cultivar Fame is much more resistant than Sirtaki, while no difference in attractiveness of the volatiles of these two cultivars was found. Thus, no relation between direct and indirect defense of gerbera plants to *T. urticae* was found.

In conclusion, differences exist in the preference of *P. persimilis* for the blends of spider mite-damaged gerbera cultivars and the composition of these blends. The behavioral response of *P. persimilis* results in arrestment in a spider mite patch until all prey are exterminated. If only a slight fraction of the predators leaves the prey patch before all prey is exterminated, the prey population would reach much higher numbers and the moment at which all prey is eliminated by the predators would be postponed dramatically (van Baalen and Sabelis, 1995; Pels and Sabelis, 1999). If the less preferred volatile blends of Sirtaki and Fame result in a reduced arrestment of the predators on these cultivars, the success of biological control on

these cultivars will be much lower. However, it is possible that the production of volatiles by all four cultivars is above a threshold at which *P. persimilis* is arrested until prey extermination. Therefore, it will be necessary to investigate in future greenhouse studies whether the arrestment response of the predators is related to the preference of the predators for the volatile blends of certain cultivars and to the composition of these blends.

Acknowledgments—Prego Rijsenhout bv is thanked for the free supply of plants and Entocare CV for the free supply of predatory mites. M. W. Sabelis, C. Schütte, and J. C. van Lenteren are acknowledged for useful comments on the manuscript. This project was funded by the Technology Foundation (STW), grant WBI 22.2859.

REFERENCES

ADAMS, R. P. 1989. Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy. Aacemic Press, San Diego, 302 pp.

CRAWLEY, M. J. 1993. GLIM for Ecologists. Blackwell, Oxford., 379 pp.

DICKE, M., 1994. Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant–carnivore mutualism. *J. Plant Physiol*. 143:465–472.

DICKE, M. 1999. Specificity of herbivore-induced plant defences, pp. 43–59, *in* D. J. Chadwick and J. Goode (eds.). Insect–Plant Interactions and Induced Plant Defence. Wiley, Chicester, UK (Novartis Foundation Symposium 223).

DICKE, M., and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool*. 38:148–165.

DICKE, M., BEEK, T. A. VAN, POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, Æ. 1990a. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions. Involvement of host plant in its production. *J. Chem. Ecol*. 16:381–396.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990b. Plant strategies for manipulating predator–prey interactions through allelochemicals: Prospects for application in pest control. *J. Chem. Ecol*. 16:3091–3118.

DICKE, M., TAKABAYASHI, J., POSTHUMUS, M. A., SCHÜTTE, C., and KRIPS, O. E. 1998. Plant–phytoseiid interactions mediated by herbivore-induced plant volatiles: Variation in production of cues and in responses of predatory mites. *Exp. Appl. Acarol*. 22:311–333.

JOULAIN, D., and KOENIG W. A. 1998. The Atlas of Spectral Data of Sesquiterpene Hydrocarbons. EBV, Hamburg, 358 pp.

KRIPS, O. E., WITUL, A., WILLEMS, P. E. L., and DICKE, M. 1998. Intrinsic rate of population increase of the spider mite *Tetranychus urticae* on the ornamental crop gerbera: Intraspecific variation in host plant and herbivore. *Entomol. Exp. Appl*. 89:159–168.

KRIPS, O. E., KLEIJN, P. W., WILLEMS, P. E. L. GOLS, R., and DICKE, M. 1999a. Leaf hairs influence the searching efficiency and predation rate of the predatory mite *Phytoseiulus persimilis. Exp. Appl. Acarol*. 23:119–131.

KRIPS, O. E., WILLEMS, P. E. L., Gols, R., POSTHUMUS, M. A., and DICKE, M. 1999b. The response of *Phytoseiulus persimilis* to spider mite-induced volatiles from gerbera: influence of starvation and experience. *J. Chem. Ecol*. 25:2623–2641.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1995. Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol*. 21:1217–1227.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON-KEMP, T. R., and BYERS, M. E. 1996. Volatile compounds from crabapple (*Malus* spp.) cultivars differing in susceptibility to the Japanese beetle (*Popillia japonica* Newman). *J. Chem. Ecol*. 22:1295–1305.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1994. Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of *Pieris brassicae* larvae: Role of mechanical damage and herbivore elicitor. *J. Chem. Ecol*. 20:2229–2247.

PELS, B., and SABELIS, M. W. 1999. Local dynamics, overexploitation and predator dispersal in an acarine predator–prey system. *Oikos* 86:573–583.

SABELIS, M. W. 1981. Biological control of two-spotted spider mites using phytoseiid predators. Part 1: Modelling the predator-prey interaction at the individual level. PhD thesis. Pudoc, Wageningen, 242 pp.

SABELIS, M. W. 1985. Life history: Capacity for population increase, pp. 35–41, *in* W. Helle and M. W. Sabelis (eds.). Spider Mites. Their Biology, Natural Enemies and Control, World Crop Pests, Vol 1B. Elsevier, Amsterdam.

SABELIS, M. W., and AFMAN, B. P. 1994. Synomone-induced suppression of take-off in the phytoseiid *Phytoseiulus persimilis* Athias-Henriot. *Exp. Appl. Acarol*. 18:711–721.

SABELIS, M. W., and VAN DE BAAN, H. E. 1983. Location of distant spider mite colonies by phytoseiid predators: Demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl*. 33:303–314.

SABELIS, M. W., and VAN DER MEER, J. 1986. Local dynamics of the interaction between predatory mites and two-spotted spider mites, pp. 322–343, *in* J. A. J. Metz and O. Diekman (eds.). Dynamics of Physiologically Structured Populations. Lecture Notes in Biomathematics, Springer, Berlin.

SABELIS, M. W., and VAN DER WEEL, J. J. 1994. Anemotactic responses of the predatory mite, *Phytoseiulus persimilis* Athias-Henriot, and their role in prey finding. *Exp. Appl. Acarol*. 17:521–529.

SABELIS, M. W., VERMAAT, J. E., and GROENEVELD, A. 1984. Arrestment responses of the predatory mite, *Phytoseiulus persimilis*, to steep odour gradients of a kairomone. *Physiol. Entomol*. 9:437–446.

SAS Institute Inc. 1997. SAS/STAT Software: Changes and Enhancements through Release 6.12, SAS Institute Inc., Cary, North Carolina, 1167 pp.

SOUISSI, R. 1999. The influence of the host plant of the cassava mealybug *Phenacoccus manihoti* on the plant and host preferences of its parasitoid *Apoanagyrus lopezi. Biol. Control* 15:64–70.

SÜTTERLIN, S., and VAN LENTEREN, J. C. 1997. Influence of hairiness of *Gerbera jamesonii* leaves on the searching efficiency of the parasitoid *Encarsia formosa. Biol. Control* 9:157–165.

TAKABAYASHI, J., and DICKE, M. 1992. Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomol. Exp. Appl*. 64:187–193.

TAKABAYASHI, J., and DICKE, M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci*. 1:109–113.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1994. Volatile herbivore-induced terpenoids in plant-mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol*. 20:1329–1354.

VAN BAALEN, M., and SABELIS, M. W. 1995. The milker-killer dilemma in spatially structured predator-prey interactions. *Oikos* 74:391–400.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

SINGLE AND BLENDED MAIZE VOLATILES AS ATTRACTANTS FOR DIABROTICITE CORN ROOTWORM BEETLES[1]

LESLIE HAMMACK*

USDA, ARS, Northern Grain Insects Research Laboratory
2923 Medary Avenue
Brookings, South Dakota 57006

(Received September 20, 2000; accepted March 13, 2001)

Abstract—Synthetic maize volatiles and analogs dispensed singly and blended were tested for attractiveness to western (WCR, *Diabrotica virgifera virgifera*) and northern corn rootworm beetles (NCR, *D. barberi*) in maize fields. Newly identified attractants included *syn*-benzaldoxime, especially for NCR, and β-caryophyllene for WCR females. (±)-Linalool was more effective than was (−)-linalool. Myrcene, (+)-β-pinene, and (−)-β-pinene were unattractive. Adding methyl salicylate to (±)-linalool, (+)-α-terpineol, or β-ionone appeared to synergistically increase capture of WCR females, but dispensing the terpenes in binary blends did not. Dose–response data for methyl salicylate, (±)-linalool, and a blend of both compounds confirmed the synergy. β-Caryophyllene, but not (−)-α-pinene, added to the latter blend produced a further synergistic increase in WCR female capture that did not vary with sesquiterpene dose from 1.0 to 100 mg. Indole addition to the same blend caused an increase in WCR female captures indicative of synergy, assuming that each did not individually lure different segments of the WCR female population. The green leaf volatiles *(Z)*-3-hexenyl acetate and (*Z*)-3-hexen-1-ol were unattractive alone and had no influence on efficacy of traps baited with 3.3 mg each of (±)-linalool, methyl salicylate, and β-caryophyllene. The latter mixture captured about half as many WCR females as did 10 mg of 4-methoxycinnamaldehyde, a potent WCR attractant standard. Substituting β-ionone for (±)-linalool yielded a ternary blend that captured more beetles than did the aldehyde and was unaffected by aldehyde addition. Olive oil, which has been used to sustain attractant volatilization, did not affect captures. The results show that the blending of maize volatiles has the

* To whom correspondence should be addressed. e-mail: LHammack@ngirl.ars.usda.gov

[1] Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

0098-0331/01/0700-1373$19.50/0

potential to greatly improve efficacy of lures having promising applications in corn rootworm population management.

Key Words—Coleoptera, Chrysomelidae, *Diabrotica virgifera virgifera*, *Diabrotica barberi*, *Zea mays*, maize volatiles, host plant attractant, kairomone, synergy.

INTRODUCTION

Diabroticite rootworms (Coleoptera: Chrysomelidae) are major pests of maize, *Zea mays* L., in the United States, where most damage results from western (WCR) and northern (NCR) corn rootworms, *Diabrotica virgifera virgifera* LeConte and *D. barberi* Smith and Lawrence, respectively. The WCR was also recently introduced into Europe, where it is spreading and attaining pest status (Enserink, 1999). Principal rootworm management options are crop rotation and chemical insecticides. The latter account for nearly a fifth of the insecticides applied to U.S. field crops (Delvo, 1993). New management tools are needed to address pesticide-related environmental concerns and the emergence of corn rootworm populations adapted to resist insecticides (Meinke et al., 1998) or survive in maize rotated with another crop (Krysan, 1993; Stewart et al., 1995; Onstad et al., 1999). One promising approach is the exploitation of insect behavior-modifying chemicals. These include cucurbitacin feeding stimulants, currently under evaluation in area wide corn rootworm trials, and host plant kairomones or their analogs with odors attractive to adult diabroticite beetles (Levine and Oloumi-Sadeghi, 1991; Sutter and Lance, 1991; Metcalf and Lampman, 1997).

Because most crop damage from corn rootworms results from larval feeding, kairomones attracting adults could be most effectively used to monitor the potential for damaging larval populations in subsequent growing seasons or to interfere with reproduction by mass trapping or annihilation techniques. Unlike diabroticite sex pheromones, the kairomonal attractants, which apparently influence host seeking and selection, usually trap at least as many females as males, enhancing their potential as rootworm management tools (Quiring and Timmins, 1990).

Attraction of diabroticite beetles to host plant odors has been studied in considerable detail. Phytochemicals from Cucurbitaceae, the hypothesized ancestral hosts of diabroticite corn rootworms (Metcalf and Metcalf, 1992), have been most extensively studied (Metcalf and Lampman, 1997; Cossé and Baker, 1999); however, synthetic maize volatiles were examined in recent field trials (Hammack, 1996, 1997). Existing research stresses phenylpropanoids from cucurbits and terpenoids from maize, but diabroticite attractants show considerable chemical heterogeneity, and some, such as β-ionone, indole, 2-phenyl-1-ethanol, and phenylacetaldehyde, occur in both maize and squash blossoms (Flath et al., 1978; Buttery et al., 1980; Andersen, 1987; Andersen and Metcalf, 1987; Turlings et al., 1993). The green leaf

volatiles (GLVs), aliphatic six-carbon primary alcohols, aldehydes, and acetates that are ubiquitously distributed in the plant kingdom, also occur in both host groups, but have received little attention as corn rootworm attractants.

The chemical diversity of diabroticite attractants identified to date and their widespread distribution among plant families increase the likelihood that odorant blends are important for specificity and strength of adult host-finding responses. Although existing studies emphasize individual compounds, several blends of cucurbit blossom volatiles are known to produce a synergistic increase in capture of WCR, NCR, or southern corn rootworm, *D. undecimpunctata howardi* Barber (SCR). These blends include equal-weight mixtures of veratrol, indole, and phenylacetaldehyde (VIP) for SCR and 1,2,4-trimethoxybenzene, indole, and (*E*)-cinnamaldehyde (TIC) for all three species (Lampman and Metcalf, 1987). Indole was recently identified as a key synergist in both VIP and TIC mixtures and in combination with 4-methoxycinnamaldehyde, the most potent single-component attractant available for WCR and a possible contributor to cucurbit blossom aroma (Metcalf et al., 1995; Metcalf and Lampman, 1997). More limited evidence that lure efficacy can be increased by blending attractants has also been reported (Hammack, 1996; Metcalf and Lampman, 1997; Petroski and Hammack, 1998), including evidence for synergy between maize terpenoids.

Improved efficacy of corn rootworm kairomonal lures is desirable, at least for mass trapping and annihilation applications. This is because the kairomonal lures generally function least well, if at all, during maize silking, and intervention with these techniques would work best during or shortly after silking when females have emerged but not yet oviposited to any extent (Lance, 1993; Hesler et al., 1994; Hammack and Hesler, 1995).

The present study tested primarily maize terpenoids, GLVs, and blends of these chemicals for attractiveness to WCR and NCR beetles. The main goal was to assess the blending of individual attractants as a means of improving lure efficacy, emphasizing inclusion of maize headspace volatiles in test blends. Attention to blends also stimulated evaluation of the need for a previously used olive oil extender (Hammack, 1997) because of the possible existence of behaviorally active oil volatiles and the greater potential for interaction of any such volatiles with blended test components.

METHODS AND MATERIALS

Odorants. The structure and purity of the test compounds are shown in Figure 1. They were purchased from Fluka Chemical Co., Ronkonkoma, New York {(−)-linalool, β-caryophyllene [(-)-*trans*-caryophyllene], (+)-α-terpineol, (+)- and (−)-β-pinene}; Sigma, St. Louis, Missouri [(*Z*)-3-hexenyl acetate];

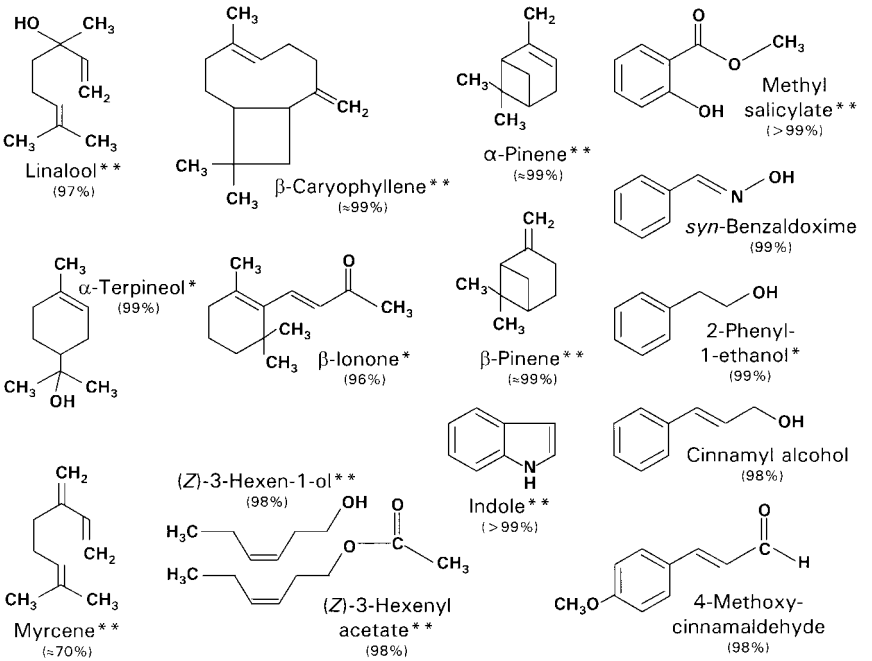


FIG. 1. Structure and purity (in parentheses) of test compounds. Single asterisks indicate maize volatiles (Flath et al., 1978). Double asterisks indicate those maize volatiles found in headspace analyses (see text for references).

Schweizerhall Inc., South Plainfield, New Jersey (4-methoxycinnamaldehyde); or Aldrich, Milwaukee, Wisconsin (all others).

Experiments. Ten field experiments were conducted between 1996 and 1999. One 1996 test examined the effect of omitting the olive oil extender on WCR and NCR responses to 2-phenyl-1-ethanol and to unbaited controls. This alcohol was used because it lures beetles of both species (Petroski and Hammack, 1998).

A second 1996 test screened single compounds for attractiveness. Four terpenoids (β-caryophyllene, linalool, myrcene, and β-pinene) were assayed because of their presence in maize headspace (Buttery and Ling, 1984; Light et al., 1993; Turlings et al., 1993; Takabayashi et al., 1995). *syn*-Benzaldoxime was tested because of its structural similarity with 2-phenyl-1-ethanol, a lure shared by maize and cucurbits, and with 2-phenyl-1-ethylamine, an effective NCR attractant not reported from host plants (Metcalf and Lampman, 1991; Petroski and Hammack, 1998). Cinnamyl alcohol was included as a reference standard for NCR, for lack of a maize headspace volatile highly attractive to this species. (±)-Linalool, previously

shown to lure primarily WCR (Hammack, 1997), served as a standard for WCR and was compared with (−)-linalool.

The remaining 1996 test screened for synergy among four maize volatiles known to individually attract primarily WCR: (±)-linalool, (+)-α-terpineol, β-ionone, and methyl salicylate (Lampman and Metcalf, 1988; Hammack, 1996, 1997). Screening was done by comparing responses to one-component, binary, and quaternary blends. The compounds were mixed in equal weights for convenience.

One of two 1997 tests yielded dose–response data for (±)-linalool, methyl salicylate, and an equal weight blend of both compounds to confirm synergy between them. Linalool was used for confirmation, rather than α-terpineol or β-ionone, because it is the only one of the three terpenes so far reported in maize headspace analyses (Buttery and Ling, 1984; Light et al., 1993; Takabayashi et al., 1995; Turlings et al., 1993).

The other 1997 test appraised synergy upon β-caryophyllene addition to the blend of (±)-linalool and methyl salicylate. This test was repeated twice, except that β-caryophyllene was replaced with one of two maize headspace volatiles: the attractant indole in 1998 and (−)-α-pinene in 1999. (−)-α-Pinene earlier showed evidence of slight attractiveness to WCR in one of two tests when dispensed by itself (Hammack, 1997).

The effect on beetle captures of altering the ratio of blend components was examined in 1998 by varying from 1 to 100 mg the amount of β-caryophyllene dispensed with 10 mg each of (±)-linalool and methyl salicylate. Another 1998 test evaluated attractiveness of two GLVs released by maize, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate (Buttery and Ling, 1984; Light et al., 1993; Takabayashi et al., 1995; Turlings et al., 1993). The GLVs were dispensed individually and blended with 3.3 mg each of (±)-linalool, methyl salicylate, and β-caryophyllene. A final 1998 test aimed to enhance captures by replacing (±)-linalool with β-ionone in the ternary blend and to compare efficacy of the maize blends with that of 4-methoxycinnamaldehyde. The latter aldehyde was also dispensed in combination with the maize volatiles.

Bioassays. Cotton dental rolls (1 cm diam. 3.8 cm long) were used in all of the experiments to dispense the test odorants. Each roll was also treated in 1996 with 2.0 ml of olive oil in hexane (1:4 by volume) (Hammack, 1997), except for the test which compared attractiveness of 2-phenyl-1-ethanol dispensed with and without the extender. No extender was used after 1996. Odorant-treated dental rolls were attached for bioassay to yellow Pherocon AM (Trécé, Inc., Salinas, California) sticky traps as the traps were placed in grower maize fields in Brookings County, South Dakota. When multiple test compounds were to be dispensed from the same trap, each was applied to a half dental roll (1.9 cm long) that was separately affixed to the trap. The number of dental rolls and solvent volumes per trap (oil and hexane in 1996, acetone used to dissolve indole and 4-methoxycinnamaldehyde in 1998) were held constant within experiments. Control traps were identical to the treated

ones, except that no test odorant(s) was applied to the dental roll(s) affixed to the controls. Odorants were considered attractive if they captured significantly more beetles than did the control.

The experiments were all laid out in a randomized complete block design. Each consisted of eight blocks, except for six blocks in two 1996 tests: the test that examined effects of extender and the one that screened individual compounds for attractiveness. Traps were positioned at least 30 m from one another at ear height on maize plants, and crop phenology was determined during testing, as previously detailed (Hammack, 1996, 1997). Traps were left in the field for 48 hr, except for 96 hr when 2-phenyl-1-ethanol was dispensed with and without extender and 24 hr in the (−)-α-pinene test (for convenience). The species and sex of captured beetles were recorded once the traps were returned to the laboratory.

Odorant Interactions. Interactions were evaluated using dose–response data and ratios of interaction (ROI), where $ROI = [(A + B) + \text{Control}]/[(A) + (B)]$, A is the capture on a trap baited with odorant A, B is the capture on a trap baited with odorant B or blend B, and $A + B$ is the capture on a trap baited with both A and B (Hammack, 1996). Odorants were deemed to interact synergistically when the response to a blend exceeded that predicted from their individual dose–response curves (Barenbaum, 1989) and, in addition, the ROI value was greater than one (Hammack, 1996). If the first criterion was met but the $\text{ROI} = 1$, then synergy could still be occurring if the individual odorants were similarly affecting the same segment of the test population. Although such similar action was likely, it was not unequivocally proven in these field experiments.

Statistical Analyses. The SAS Institute (1989) statistical package was used for data analysis. To determine whether ROI values differed from 1, Student's *t* test for paired samples or Wilcoxon's signed rank test (*S* statistic) was used to test the null hypothesis of no difference between ROI numerator and denominator (PROC Univariate). Wilcoxon's test was used instead of the *t* test if the Shapiro-Wilk test for normality approached significance at $P \leq 0.1$ (PROC Univariate). Otherwise, data from each experiment were transformed $[\ln(x + 1)]$ to ensure homogeneity of variances and then examined by analysis of variance (PROC ANOVA). The Student-Newman-Keuls option was used to separate means after a significant ANOVA. The tables and figures show untransformed data. $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extender. Dose significantly influenced capture of both WCR and NCR on traps baited with 0–30 mg of 2-phenyl-1-ethanol, although WCR males were unaffected (Table 1). In contrast, treatment with the oil extender had no effect on captures of either sex or species nor did it interact with dose to alter captures (Table 1).

TABLE 1. CAPTURE OF CORN ROOTWORM ADULTS ON TRAPS BAITED WITH 2-PHENYL-1-ETHANOL ON DISPENSERS WITH AND WITHOUT OLIVE OIL[a]

2-Phenyl-1-ethanol dose (mg/trap)	Oil	Capture (mean ± SE)			
		Western corn rootworm		Northern corn rootworm	
		Female	Male	Female	Male
30	yes	87.8 ± 17.6	36.3 ± 3.9	97.0 ± 17.6	61.0 ± 9.7
30	no	91.7 ± 17.1	46.7 ± 7.2	85.3 ± 11.4	57.2 ± 6.1
3	yes	36.5 ± 5.2	43.8 ± 4.9	28.7 ± 5.9	46.2 ± 9.6
3	no	45.5 ± 7.6	51.7 ± 7.4	37.5 ± 5.1	55.5 ± 12.1
0	yes	12.8 ± 2.1	51.0 ± 10.3	4.0 ± 1.7	34.7 ± 6.5
0	no	14.8 ± 3.1	48.8 ± 8.8	5.8 ± 0.8	39.8 ± 2.8
F statistics[b]					
Oil ($df = 1, 25$)		0.36 NS	0.94 NS	2.79 NS	0.73 NS
2-Phenyl-1-ethanol dose ($df = 2, 25$)		48.90**	0.82 NS	114.4**	3.74*
Oil × dose ($df = 2, 25$)		0.14 NS	0.41 NS	1.45 NS	0.28 NS

[a] Test conducted August 30–September 3, 1996 ($N = 6$). Mean beetle count per plant ± SE on September 3 was 1.7 ± 0.3 WCR and 1.0 ± 0.2 NCR. Corn was in the dough stage (R4).
[b] Asterisks denote statistical significance at $^{*}P < 0.05$ or $^{**}P < 0.0001$. NS denotes $P > 0.05$.

Many extenders including olive and mineral oils have been used to sustain volatilization of candidate attractants from lure dispensers in field trials (Lampman and Metcalf, 1987; Petroski and Hammack, 1998; Cossé and Baker, 1999), although some, like olive oil, could release behaviorally active volatiles. The present study detected no effect of olive oil, either by itself or in combination with 2-phenyl-1-ethanol, on WCR or NCR captures. However, omission of the oil was still deemed desirable, at least in these short-term tests, because it precluded interaction with test compounds other than the alcohol and will facilitate future gravimetric measurement of attractant release rates.

Screening of Individual Terpenoids/Benzaldoxime. The screening of individual compounds (100 mg/trap) showed *syn*-benzaldoxime attractive to WCR females and to NCR of both sexes, while β-caryophyllene attracted only WCR females (Table 2). *syn*-Benzaldoxime captured more NCR than did the cinnamyl alcohol standard, considered a strong NCR attractant (Metcalf and Lampman, 1991), and as many WCR females as did (±)-linalool. β-Caryophyllene, in contrast, lured fewer WCR females than did the (±)-linalool standard. (−)-Linalool also proved less attractive than the racemic standard, especially to WCR but also to NCR females. Neither β-pinene stereoisomer nor myrcene was attractive (Table 2).

This is the first demonstration that syn-benzaldoxime and β-caryophyllene attract corn rootworm adults. Activity of the oxime may derive from a structural similarity with 2-phenyl-1-ethanol or 2-phenyl-1-ethylamine lures, or perhaps

TABLE 2. CAPTURE OF CORN ROOTWORM ADULTS ON TRAPS BAITED WITH CANDIDATE ATTRACTANTS[a]

Candidate/standard attractant (100 mg/compound)	Capture (mean ± SE)			
	Western corn rootworm		Northern corn rootworm	
	Female	Male	Female	Male
syn-Benzaldoxime	277.3 ± 17.4a	17.5 ± 2.6	410.7 ± 33.5a	144.5 ± 11.1a
β-Caryophyllene	65.7 ± 9.7b	13.0 ± 2.3	3.2 ± 1.6ef	11.7 ± 2.0d
(−)-Linalool	70.5 ± 8.0b	13.5 ± 2.0	10.3 ± 2.7d	20.3 ± 3.6c
(+)-β-Pinene	13.8 ± 1.9c	9.0 ± 1.4	1.0 ± 0.5f	9.7 ± 0.6d
(−)-β-Pinene	12.7 ± 2.0c	10.3 ± 0.7	2.2 ± 0.5ef	8.5 ± 2.0d
Myrcene	9.0 ± 2.0c	11.7 ± 3.1	1.7 ± 0.2ef	8.7 ± 0.9d
None (control)	15.2 ± 2.7c	13.0 ± 1.9	2.7 ± 0.3e	12.0 ± 2.0d
(±)-Linalool (WCR standard)	270.5 ± 39.0a	20.2 ± 3.2	20.3 ± 3.2c	22.3 ± 4.5c
Cinnamyl alcohol (NCR standard)	88.5 ± 19.2b	17.3 ± 1.9	180.3 ± 25.9b	93.3 ± 10.1b
F statistic[b]	63.56**	2.11 NS	109.58**	61.24**

[a] Test conducted September 9–11, 1996 ($N = 6$). Mean beetle count per corn plant ± SE on September 11 was 1.2 ± 0.2 WCR and 1.1 ± 0.2 NCR. Corn was in the dent stage (R5).
[b] Asterisks denote statistical significance at $P < 0.0001$, NS denotes $P > 0.05$ ($df = 8, 40$). Means within a column followed by the same letter do not differ by Student-Neuman-Keuls test, $P > 0.05$.

with phenylacetaldoxime, a common floral volatile (Kaiser, 1991). Oximes have not previously been reported as corn rootworm attractants and merit further study given the magnitude of WCR and especially NCR captures reported here.

β-Caryophyllene is released by a wide assortment of flowering plants (Knudsen et al., 1993; Borg-Karlson et al., 1994) and attracts insects other than WCR, including boll weevils (Minyard et al., 1969) and green lacewings (Flint et al., 1979). Like several other corn rootworm attractants, such as linalool and indole, β-caryophyllene is among volatiles released by maize in response to insect feeding (Turlings et al., 1993; Takabayashi et al., 1995). Its hydrocarbon structure renders β-caryophyllene an atypical attractant for diabroticite beetles. Several other maize terpenoid hydrocarbons, namely myrcene, β-pinene, and limonene (Light et al., 1993; Takabayashi et al., 1995), were tested here or earlier (Hammack, 1996), but failed to attract either WCR or NCR. (−)-α-Pinene, but not (+)-α-pinene, did attract WCR females in one of two earlier tests, although that response was only just detectable (Hammack, 1997) and not reproducible in the current study.

Unlike benzaldoxime, β-caryophyllene was a more effective WCR than NCR lure. Despite much overlap in the array of host compounds and analogs attractive to NCR and WCR beetles, response patterns of the two species are generally distinct (Hammack, 1996, 1997; Metcalf and Lampman, 1997; Petroski and Hammack, 1998). Distinct response patterns might be expected, as WCR and NCR likely

evolved their association with maize independently of one another and differ as adults in their reliance on maize for food (Branson and Krysan, 1981). Metcalf et al. (1998) considered the greater reliance of WCR on maize atypical of diabroticite adults, which as a group tend to feed on pollen from a variety of grasses and forbs, perhaps in response by some *Diabrotica* species to widely distributed floral volatiles shared with ancestral Cucurbitaceae.

The weaker response of both species to (−)- than to (±)-linalool implies that(+)-linalool, or a blend containing this enantiomer, is the more effective lure. The (+)-isomer was not, however, available for testing. Chirality is known to be important to WCR reacting to α-terpineol (Hammack, 1996), as well as to female cabbage looper moths and solitary bees, which both preferentially respond to (*S*)-(+)-linalool acting as a pheromone (Heath et al., 1992; Borg-Karlson et al., 1996).

Traps baited with effective lures usually attracted more females than males within each species, and the disparity between the sexes tended to be greater for WCR than NCR. These results agree with those of previous studies, where trap catch was examined by sex (see Hammack, 1997 for references and further discussion), except that NCR females do not always outnumber NCR males. Assuming attractant involvement in host seeking/selection, higher female captures likely relate to nutritional demands of oogenesis, which could be more stringent in the more fecund WCR than NCR (Naranjo and Sawyer, 1987). Captures would also reflect sex ratios in maize fields at testing. Female to male ratios could tend to rise in late season with earlier mortality of males due to protandry (Quiring and Timmins, 1990) or, in the case of NCR, which are more apt than WCR to move out of maize to feed on a variety of flowering forbs, with the migration of females back into maize to oviposit (Branson and Krysan, 1981).

Terpenoid and Methyl Salicylate Blends. When methyl salicylate and the three terpenes were assayed at 30 mg per component per trap, each binary blend captured significantly more WCR females than did its individual constituents only when the blend contained methyl salicylate (Figure 2). A significant increase occurred with mixing when methyl salicylate was added to (±)-linalool, (+)-α-terpineol, or β-ionone, but not when blends contained any two of the three terpenes. Mixing all four chemicals failed to increase the capture of WCR females beyond that achieved by mixing only methyl salicylate and β-ionone. The latter two were the least and most active, respectively, of the four single compounds (Figure 2). The other two, (±)-linalool and (+)-α-terpineol, showed an intermediate attractiveness. WCR males demonstrated significant, albeit weak, olfactory attraction only when treatments included β-ionone; however, none of the β-ionone blends was significantly better than β-ionone by itself (Figure 2). Only treatments containing (+)-α-terpineol or (±)-linalool attracted NCR in numbers significantly higher than control, although mean captures per trap failed to exceed 25 and showed no tendency to increase when chemicals were blended (data not shown).

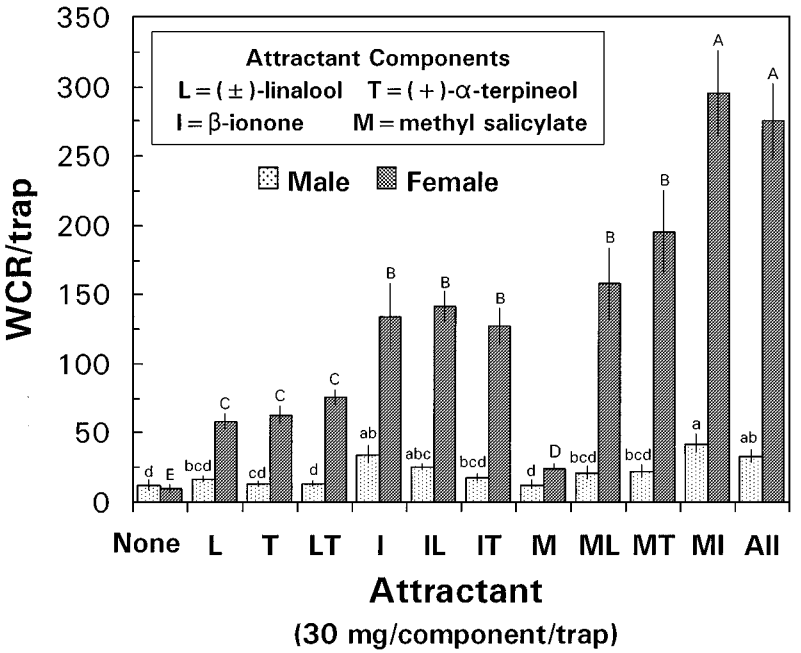


FIG. 2. Effect of blending (±)-linalool, (+)-α-terpineol, β-ionone, and methyl salicylate on WCR capture (mean ± SE, $N = 8$). Means within sexes topped by different letters differ at $P \leq 0.05$ by Student-Newman-Keuls test after ANOVA ($F = 66.62$ and 8.09 for females and males, respectively; $df = 11, 77$; $P < 0.0001$). On September 6, 1996 when the test ended, corn was dough to dent stage (R4–R5) and mean beetle count per plant ± SE was 0.9 ± 0.2 WCR and 1.2 ± 0.2 NCR.

Doses as low as 30 mg/trap (15 mg/chemical) of a 1:1 blend of (±)-linalool and methyl salicylate produced captures higher than those seen with as much as 120 mg of either compound by itself (Figure 3). ROI values calculated for WCR females reacting to the two chemicals, each chemical at doses of 7.5, 15, 30, or 60 mg dispensed from the same and separate traps, were very similar across doses, at 1.9 ± 0.2, 1.8 ± 0.3, 2.1 ± 0.5, or 1.9 ± 0.3 (mean ± SE), respectively. All four doses were, therefore, used to calculate a joint ROI of 1.9 ± 0.2, which differed from 1 ($S = 244$, $N = 32$, $P < 0.001$). Only the higher doses of the blend (60 and 120 mg) attracted NCR females, but the best mean capture (6.4 per trap) did not differ from that to any single component (data not shown). No odorant treatment attracted males of either species (data not shown).

The present tests clearly demonstrated a synergistic increase in capture of WCR females when methyl salicylate and linalool were dispensed from the same as compared with different traps, with observed captures at least 1.9 times those

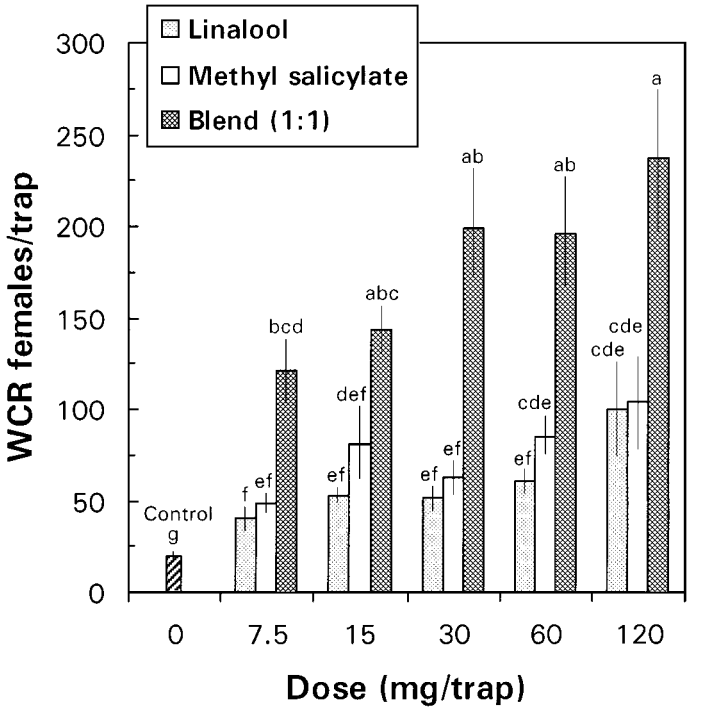


FIG. 3. Relationship between attractant dose and capture (mean ± SE, $N = 8$) of WCR females for (±)-linalool, methyl salicylate, and a 1:1 by weight blend of both. Means topped by different letters differ at $P \leq 0.05$ by Student-Newman-Keuls test after ANOVA ($F = 18.50$; $df = 15, 105$; $P < 0.0001$). On August 29, 1997, when the test ended, corn was dough stage (R4) and mean beetle count per plant ± SE was 1.4 ± 0.2 WCR and 0.9 ± 0.2 NCR.

expected had there been no synergy. The dose–response data showed that a rise of this magnitude would only be expected after about a 10-fold increase in dose of the individual compounds, assuming similar action of both odorants on the same segments of the field population.

Adding β-caryophyllene or indole, but not (−)-α-pinene, to a blend of (±)-linalool and methyl salicylate increased capture of WCR females above levels expected from simply increasing the dose of either the single compound or the binary blend (Figure 4). ROI values calculated in each series from captures on traps baited with 20 mg per component per trap were 2.5 ± 0.7, 1.1 ± 0.1, and 1.2 ± 0.3 for β-caryophyllene, indole, and (−)-α-pinene, respectively. Only the β-caryophyllene value differed significantly from 1 ($t = 2.36, N = 8, P = 0.05$; for (−)-$\alpha$-pinene, $t = 0.48, N = 8, P = 0.65$; for indole, $S = 5, N = 8, P = 0.55$).

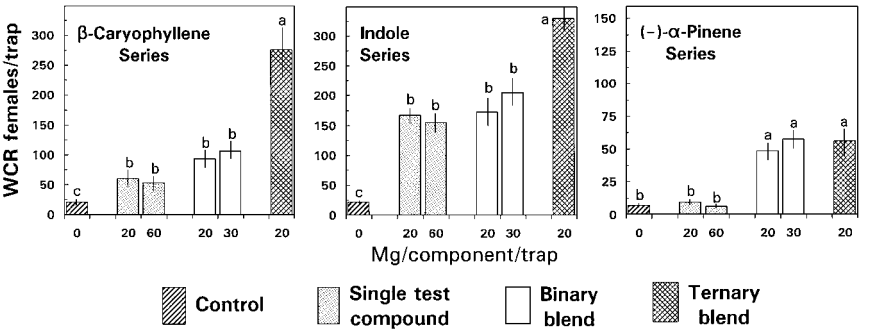


FIG. 4. Mean capture ± SE ($N = 8$) of WCR females with and without addition of β-caryophyllene, indole, or (−)-α-pinene to a 1:1 by weight blend of (±)-linalool and methyl salicylate (20 mg/component/trap for each ternary blend). Means in a series topped by different letters differ at $P \leq 0.05$ by Student-Newman-Keuls test after ANOVA ($F = 17.12$, 98.83, and 36.91, respectively; $df = 5, 35$; $P < 0.0001$). On August 28, 1997, and August 19, 1998, when the β-caryophyllene and indole series ended, respectively, corn was dough stage (R4) and mean beetle count per plant ± SE was 2.3 ± 0.3 WCR and 1.8 ± 0.2 NCR (1997) and 2.8 ± 0.4 WCR and 1.8 ± 0.3 NCR (1998). At the start of the (−)-α-pinene series on August 26, 1999, corn was dent stage (R5) and beetle counts were 1.3 ± 0.2 WCR and 1.0 ± 0.2 NCR.

Odorants lured WCR males just in the indole series, but responses were barely detectable and did not vary among olfactory treatments (data not shown). NCR reacted only to scents containing (±)-linalool, albeit in low numbers, and did not distinguish between binary and ternary blends (data not shown).

Thus, β-caryophyllene, which was only weakly attractive by itself, but not (−)-α-pinene, synergistically elevated female WCR captures when added to the linalool-methyl salicylate blend. Captures were again at least twice as high as expected without synergy. Indole addition to the binary blend produced a WCR response increase that was smaller than the three- to fivefold increase reported for its mixture with 4-methoxycinnamaldehyde or cucurbit volatiles (Metcalf et al., 1995). Indeed, the smaller increase could be explained without invoking synergy if indole and the binary blend were to attract different segments of the WCR female population. For example, one lure could attract females with immature ovaries seeking food and the other affect gravid females seeking oviposition sites. This hypothetical situation is perhaps less likely for WCR, which tend to oviposit where they feed, than for NCR, which feed on a variety of flowering forbs once maize silks dry and then return to maize to oviposit (Branson and Kryson, 1981).

Even 1.0 mg of β-caryophyllene added to 10 mg each of (±)-linalool and methyl salicylate increased captures of WCR females and yielded responses that were not lower than those obtained with additions up to 100 mg (Figure 5). WCR

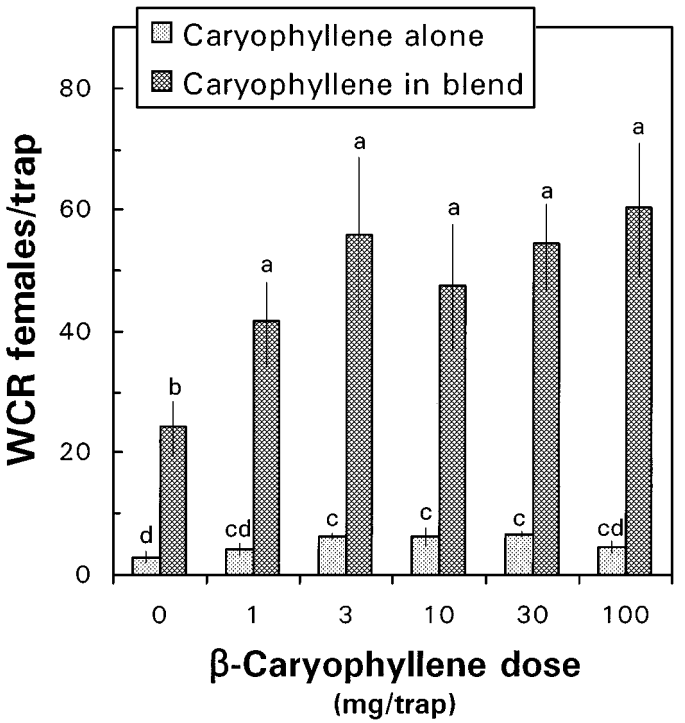


FIG. 5. Relationship between β-caryophyllene dose and capture (mean $\pm$ SE, $N = 8$) of WCR females upon caryophyllene addition to an attractant blend consisting of 10 mg each of (±)-linalool and methyl salicylate. Means topped by different letters differ at $P \leq 0.05$ by Student-Newman-Keuls test after a significant ANOVA ($F = 41.31$; $df = 11, 77$; $P < 0.0001$). On August 20, 1998, when the test ended, corn was dough to dent stage (R4–R5) and mean beetle count per plant $\pm$ SE was 1.8 ± 0.2 WCR and 3.1 ± 0.4 NCR.

males and NCR of both sexes failed to respond to any odorant treatment in numbers that were different from control (data not shown). Metcalf et al. (1995) also reported only a very gradual linear increase in efficacy as the dose of one member of a binary blend of floral volatiles attractive to WCR was increased over three orders of magnitude (varying dose of indole dispensed with 4-methoxycinnamaldehyde). Stronger dependence on attractant ratios might have been predicted (Loughrin et al., 1996) given the relatively specialized feeding habits of WCR adults (Krysan, 1993; Metcalf and Lampman, 1998) and the ubiquity of many WCR lures. A wider range of component test ratios and quantitative data on component release rates will ultimately be needed to critically evaluate component ratio effects on blend attractiveness.

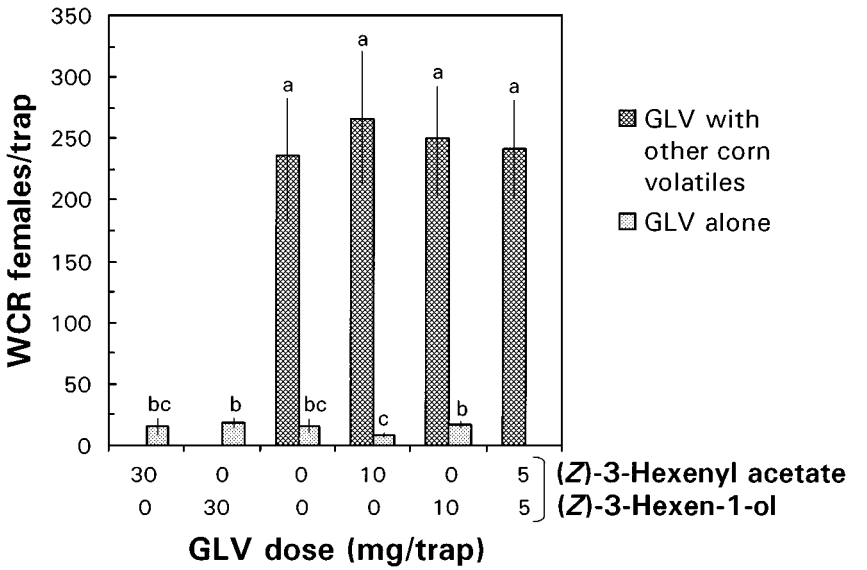


FIG. 6. Effect of the corn green leaf volatiles (GLVs), (*Z*)-3-hexenyl acetate and (*Z*)-3-hexen-1-ol, on WCR female captures (mean ± SE, $N = 8$) when the volatiles were dispensed singly and blended with other corn volatiles [3.3 mg/trap each of (±)-linalool, methyl salicylate, and β-caryophyllene]. Means topped by different letters differ at $P \leq 0.05$ by Student-Newman-Keuls test after a significant ANOVA ($F = 82.77$; $df = 8, 71$; $P < 0.0001$). On August 20, 1998, when the test ended, corn was dough to dent stage (R4–R5) and mean beetle count per plant ± SE was 3.6 ± 0.4 WCR and 1.8 ± 0.2 NCR.

Green Leaf Volatiles. Neither (*Z*)-3-hexenyl acetate nor (*Z*)-3-hexen-1-ol was attractive to WCR females when tested at 10 or 30 mg/trap (Figure 6), despite abundance of these GLVs in the headspace of maize leaves during plant tasseling and silking, when feeding conditions are optimal for corn rootworm adults (Krysan, 1993; Light et al., 1993). Moreover 10 mg of either volatile or a 1:1 (w/w) blend of both also did not affect captures when added to 3.3 mg each of (±)-linalool, methyl salicylate, and β-caryophyllene (Figure 6). Although GLVs attracting some insects can become inhibitory at higher doses, addition of the acetate and alcohol to the attractive blend of maize volatiles without effect argues against any such inhibition in the present tests. No olfactory treatment in this test significantly affected capture of WCR males or either NCR sex (data not shown).

Blend Efficacy/Enhancement. A blend of (±)-linalool, methyl salicylate, and β-caryophyllene (each at 3.3 mg/trap) captured fewer WCR of both sexes than did 10 mg of the 4-methoxycinnamaldehyde reference; however, substituting β-ionone for (±)-linalool yielded a ternary blend that was more attractive than was the

TABLE 3. CAPTURE OF CORN ROOTWORM ADULTS ON TRAPS BAITED WITH (±)-LINALOOL OR β-IONONE BLENDED WITH METHYL SALICYLATE AND β-CARYOPHYLLENE, COMPARED WITH 4-METHOXYCINNAMALDEHYDE STANDARDS[a]

		Capture (mean ± SE)			
		Western corn rootworm		Northern corn rootworm	
Attractant	Dose (mg/trap)	Female	Male	Female	Male
(±)-Linalool blend[b]	10	159.0 ± 27.7c	52.1 ± 10.0c	3.0 ± 1.1	12.0 ± 1.7
4-Methoxycinnamaldehyde	10	306.1 ± 46.9b	102.8 ± 25.3b	1.1 ± 0.4	10.1 ± 1.3
β-Ionone blend[b]	10	542.3 ± 50.1a	138.9 ± 24.7ab	0.6 ± 0.4	14.3 ± 1.5
β-Ionone blend[b]	10				
+ 4-methoxycinnamaldehyde	10	664.9 ± 48.2a	188.6 ± 42.0a	1.5 ± 0.5	7.9 ± 1.7
None (control)	0	18.8 ± 4.5d	26.1 ± 6.5d	2.6 ± 0.8	12.8 ± 2.3
F statistic[c]		203.26**	32.79**	2.40 NS	2.30 NS

[a] Test conducted August 12–14, 1998 ($N = 8$). Mean beetle count per corn plant ± SE on August 14 was 3.7 ± 0.5 WCR and 1.4 ± 0.2 NCR. Corn was in the milk to dough stage (R3–R4).

[b] (±)-Linalool or β-ionone (3.3 mg) blended with 3.3 mg each of methyl salicylate and β-caryophyllene for a total of 10 mg/trap.

[c] Asterisks denote statistical significance at $P < 0.0001$, NS denotes $P > 0.05$ ($df = 4, 28$). Means within a column followed by the same letter do not differ by Student-Neuman-Keuls test, $P > 0.05$.

aldehyde (Table 3). Addition of the aldehyde to the ternary blend containing β-ionone did not affect captures of either WCR sex. No odorant influenced NCR (Table 3).

SUMMARY AND CONCLUSIONS

This study identified two new attractants for corn rootworm adults: *syn*-benzaldoxime and β-caryophyllene. The oxime was notable for its strong attractiveness to NCR, whereas β-caryophyllene synergistically elevated captures of WCR females when blended with other maize headspace volatiles. Methyl salicylate showed a similar synergy when dispensed with one of several maize terpenoids. Indole also increased WCR captures in a manner suggesting synergy but to a lesser extent than reported for its interaction with cucurbit volatiles and their analogs. The results support the conclusion that olfactory synergism plays a key role in host finding by diabroticite corn rootworm adults, in agreement with studies of cucurbit-diabroticite interactions (Metcalf and Lampman, 1997), and demonstrate that the blending of maize volatiles has the potential to dramatically improve efficacy of corn rootworm lures with applications in pest management. Attaining full potential will likely entail adjustment of compound release rates to match natural conditions optimal for corn rootworm attraction, conditions not yet fully elucidated. Most chemical studies of maize volatiles have examined younger crop developmental stages than those preferred by corn rootworm adults or else used cut or macerated tissues, which may release qualitatively and quantitatively different blends than do intact or herbivore-damaged tissues (Buttery and Ling, 1984; Turlings et al., 1993; Takabayashi et al., 1995).

Acknowledgments—I thank Kent Howell and Vanderwal Farms, Inc., for generously permitting experimentation in their maize fields. Janet Fergen provided excellent technical assistance, aided by Sherri Brende, Jennifer Denkinger, Julie Marler, Mary Roduner, Holly Schultz, and Cara Wulf. Robert Bartelt (ARS, Peoria, Illinois), Astrid Eben (Instituto de Ecologia, A.C., Xalapa, Veracruz, Mexico), Wade French (ARS, Brookings), Julio Rojas (El Colegio de la Frontera Sur, Tapachula, Chiapas, Mexico), and two anonymous reviewers read an earlier version of the manuscript and provided many useful suggestions.

REFERENCES

ANDERSEN, J. F. 1987. Composition of the floral odor of *Cucurbita maxima* Duchesne (Cucurbitaceae). *J. Agric. Food Chem*. 35:60–62.

ANDERSEN, J. F., and METCALF, R. L. 1987. Factors influencing distribution of *Diabrotica* spp. in blossoms of cultivated *Cucurbita* spp. *J. Chem. Ecol*. 13:681–699.

BARENBAUM, M. C. 1989. What is synergy? *Pharmacol. Rev*. 1989:93–141.

BORG-KARLSON, A., VALTEROVA, I., and NILSSON, L. A. 1994. Volatile compounds from flowers of six species in the family Apiaceae: Bouquets for different pollinators? *Phytochemistry* 35:111–119.

BORG-KARLSON, A., UNELIUS, C. R., VALTEROVA, I., and NILSSON, L. A. 1996. Floral fragrance chemistry in the early flowering shrub, *Daphne mezereum*. *Phytochemistry* 41:1477–1483.

BRANSON, T. F., and KRYSAN, J. L. 1981. Feeding and oviposition behavior and life cycle strategies of *Diabrotica*: An evolutionary view with implications for pest management. *Environ. Entomol*. 10:826–831.

BUTTERY, R. G., and LING, L. C. 1984. Corn leaf volatiles: Identification using Tenax trapping for possible insect attractants. *J. Agric. Food Chem*. 32:1104–1106.

BUTTERY, R. G., LING, L. C., and TERANISHI, R. 1980. Volatiles of corn tassels: Possible corn ear worm attractants. *J. Agric. Food Chem*. 28:771–774.

COSSÉ, A. A., and BAKER, T. C. 1999. Electrophysiologically and behaviorally active volatiles of buffalo gourd root powder for corn rootworm beetles. *J. Chem. Ecol*. 25:51–66.

DELVO, H. W. 1993. Pesticide use down 3 percent in 1993, pp. 13–20, *in* Agricultural Resources: Inputs Situation and Outlook. USDA Report AR-29, Washington, D.C.

ENSERINK, M. 1999. Biological invaders sweep in. *Science* 285:1834–1836.

FLATH, R. A., FORREY, R. R., JOHN, J. O., and CHAN, B. G. 1978. Volatile components of corn silk (*Zea mays* L.): Possible *Heliothis zea* (Boddie) attractants. *J. Agric. Food Chem*. 26:1290–1293.

FLINT, H. M., SALTER, S. S., and WALTERS, S. 1979. Caryophyllene: An attractant for the green lacewing. *Environ. Entomol*. 8:1123–1125.

HAMMACK, L. 1996. Corn volatiles as attractants for northern and western corn rootworm beetles (Coleoptera: Chrysomelidae: *Diabrotica* spp.). *J. Chem. Ecol*. 22:1237–1253.

HAMMACK, L. 1997. Attractiveness of synthetic corn volatiles to feral northern and western corn rootworm beetles (Coleoptera: Chrysomelidae). *Environ. Entomol*. 26:311–317.

HAMMACK, L., and HESLER, L. S. 1995. Seasonal response to phenylpropanoid attractants by northern corn rootworm beetles (Coleoptera: Chrysomelidae). *J. Kans. Entomol. Soc*. 68:169–177.

HEATH, R. R., LANDOLT, P. J., DUEBEN, B. D., MURPHY, R. E., and SCHNEIDER, R. E. 1992. Identification of male cabbage looper sex pheromone attractive to females. *J. Chem. Ecol*. 18:441–453.

HESLER, L. S., LANCE, D. R., and SUTTER, G. R. 1994. Attractancy of volatile nonpheromonal semiochemicals to northern corn rootworm beetles (Coleoptera: Chrysomelidae) in eastern South Dakota. *J. Kans. Entomol. Soc*. 67:186–192.

KAISER, R. 1991. Trapping, investigation and reconstitution of flower scents, pp. 213–250, *in* P. M. Müller and D. Lamparsky (eds.). Perfumes: Art, Science and Technology. Elsevier, London.

KNUDSEN, J. T., TOLLSTEN, L., and BERGSTRÖM, L. G. 1993. Floral scents—a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry* 33:253–280.

KRYSAN, J. L. 1993. Adaptations of *Diabrotica* to habitat manipulations, pp. 361–373, *in* K. C. Kim and B. A. McPheron (eds.). Evolution of Insect Pests/Patterns of Variation. John Wiley & Sons, New York.

LAMPMAN, R. L., and METCALF, R. L. 1987. Multicomponent kairomonal lures for southern and western corn rootworms (Coleoptera: Chrysomelidae: *Diabrotica* spp.). *J. Econ. Entomol*. 80:1137–1142.

LAMPMAN, R. L., and METCALF, R. L. 1988. The comparative response of *Diabrotica* species (Coleoptera: Chrysomelidae) to volatile attractants. *Environ. Entomol*. 17:644–648.

LANCE, D. R. 1993. Effects of a nonpheromonal attractant on movement and distribution of adult *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). *Environ. Entomol*. 22:654–662.

LEVINE, E., and OLOUMI-SADEGHI, H. 1991. Management of diabroticite rootworms in corn. *Annu. Rev. Entomol*. 36:229–255.

LIGHT, D. M., FLATH, R. A., BUTTERY, R. G., ZALOM, F. G., RICE, R. E., DICKENS, J. C., and JANG, E. B. 1993. Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera).*Chemoecology* 4:145–152.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON-KEMP, T. R., and BYERS, M. E. 1996. Role of feeding-induced plant volatiles in aggregative behavior of the Japanese beetle (Coleoptera: Scarabaeidae). *Environ. Entomol*. 25:1188–1191.

MEINKE, L. J., SIEGFRIED, B. D., WRIGHT, R. J., and CHANDLER, L. D. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. *J. Econ. Entomol*. 91:594–600.

METCALF, R. L., and LAMPMAN, R. L. 1991. Evolution of diabroticite rootworm beetle (Chrysomelidae) receptors for *Cucurbita* blossom volatiles. *Proc. Natl. Acad. Sci. U.S.A.* 88:1869–1872.

METCALF, R. L., and LAMPMAN, R. L. 1997. Plant kairomones and kairomone mimetics in basic and applied research with diabroticite rootworms. *Trends Entomol*. 1:49–62.

METCALF, R. L., and METCALF, E. R. 1992. Plant Kairomones in Insect Ecology and Control. Chapman & Hall, New York, 168 pp.

METCALF, R. L., LAMPMAN, R. L., and DEEM-DICKSON, L. 1995. Indole as an olfactory synergist for volatile kairomones for diabroticite beetles. *J. Chem. Ecol.* 21:1149–1162.

METCALF, R. L., LAMPMAN, R. L., and LEWIS, P. A. 1998. Comparative kairomonal chemical ecology of diabroticite beetles (Coleoptera: Chrysomelidae: Galerucinae: Luperini: Diabroticina) in a reconstituted tallgrass prairie ecosystem. *J. Econ. Entomol*. 91:881–890.

MINYARD, J. P., HARDEE, D. D., GUELDNER, R. C., THOMPSON, A. C., WIYGUL, G., and HEDIN, P. A. 1969. Constituents of the cotton bud: Compounds attractive to the boll weevil. *J. Agric. Food Chem*. 17:1093–1097.

NARANJO, S. E., and SAWYER, A. J. 1987. Reproductive biology and survival of *Diabrotica barberi* (Coleoptera: Chrysomelidae): Effect of temperature, food, and seasonal time of emergence. *Ann. Entomol. Soc. Am.* 80:841–848.

ONSTAD, D. W., JOSELYN, M. G., ISARD, S. A., LEVINE, E., SPENCER, J. L., BLEDSOE, L. W., EDWARDS, C. R., DI FONZO, C. D., and WILLSON, H. 1999. Modeling the spread of western corn rootworm (Coleoptera: Chrysomelidae) populations adapting to soybean-corn rotation. *Environ. Entomol*. 28:188–194.

PETROSKI, R. J., and HAMMACK, L. 1998. Structure activity relationships of phenyl alkyl alcohols, phenyl alkyl amines, and cinnamyl alcohol derivatives as attractants for adult corn rootworm (Coleoptera: Chrysomelidae: *Diabrotica* spp.). *Environ. Entomol*. 27:688-694.

QUIRING, D. T., and TIMMINS, P. R. 1990. Influence of reproductive ecology on feasibility of mass trapping *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). *J. Appl. Ecol*. 27:965–982.

SAS INSTITUTE. 1989. SAS/STAT User's Guide, Version 6, 4th edn., Vols. 1 and 2, SAS Institute Inc., Cary, North Carolina.

STEWART, J. W., COCKE, J., JR., TAYLOR, J., and WILLIAMS, R. 1995. Mexican corn rootworm emergence from corn fields where sorghum was grown the previous season. *Southwest. Entomol*. 20:229–230.

SUTTER, G. R., and LANCE, D. R. 1991. New strategies for reducing insecticide use in the corn belt, pp. 231–249, *in* B. J. Rice (ed.). Sustainable Agriculture Research and Education in the Field. National Academy Press, Washington, D.C.

TAKABAYASHI, J., TAKAHASHI, S., DICKE, M., and POSTHUMUS, M. A. 1995. Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J. Chem. Ecol*. 21:273–287.

TURLINGS, T. C. J., MCCALL, P. J., ALBORN, H. T., and TUMLINSON, J. H. 1993. An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps. *J. Chem. Ecol*. 19:411–425.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

A CHIRAL SEX PHEROMONE SYSTEM IN THE PEA MIDGE, *Contarinia pisi*

YLVA HILLBUR,[1,*] MARIE BENGTSSON,[1] JAN LÖFQVIST,[1] ANTHONY BIDDLE,[2] OLIVIER PILLON,[3] ERNST PLASS,[4] WITTKO FRANCKE,[4] and ERIC HALLBERG[5]

[1]*Department of Crop Science*
Swedish University of Agricultural Sciences
S-230 53 Alnarp, Sweden

[2]*Processors and Growers Research Organisation*
Thornhaugh, Peterborough, PE8 6HJ, UK

[3]*Service Régional de la Protection des Végétaux*
Antenne Régionale Aube–Haute Marne
10120 Saint Pouange, France

[4]*Institute of Organic Chemistry*
University of Hamburg
D-20146 Hamburg, Germany

[5]*Department of Zoology*
Lund University
S-223 62 Lund, Sweden

(Received November 14, 2000; accepted March 6, 2001)

Abstract—The sex pheromone of the pea midge consists of 2-acetoxytridecane, (2*S*,11*S*)-diacetoxytridecane and (2*S*,12*S*)-diacetoxytridecane. The responses of male pea midges to the corresponding stereoisomers of (2*S*,11*S*)-diacetoxytridecane and (2*S*,12*S*)-diacetoxytridecane were tested in field trapping experiments and by electroantennographic recordings. When added at 20% of the pheromone component to the sex pheromone blend, the (2*S*,11*R*)- and (2*R*,11*S*)-stereoisomers of (2*S*,11*S*)-diacetoxytridecane, were shown to have a strong inhibitory effect on male attraction in the field. At the same dose, (2*R*,11*R*)-diacetoxytridecane, (2*R*,12*R*)-diacetoxytridecane, and *meso*-2,12-diacetoxytridecane, did not have a significant effect on male behavior. It was also shown that substitution of either (2*S*,11*S*)-diacetoxytridecane or (2*S*,12*S*)-diacetoxytridecane with the related stereoisomers reduced trap catches to the level of blank traps. The electroantennographic recordings showed similar dose–response curves for the pheromone components and the stereoisomers shown to have an inhibitory effect. It seems likely that male antennae have receptors for both

* To whom correspondence should be addressed. e-mail: ylva.hillbur@vv.slu.se

0098-0331/01/0700-1391$19.50/0

pheromone components and for inhibitory stereoisomers. Scanning electron microscopy and transmission electron microscopy of the antennae revealed three types of sensilla involved in chemoreception: sensilla circumfila, sensilla trichodea, and sensilla coeloconica. The sensilla circumfila and trichodea are both innervated by two sensory cells, whereas the sensilla coeloconica are innervated by four to five cells.

Key Words—*Contarinia pisi*, Cecidomyiidae, Diptera, sex pheromone, (2*S*,11*S*)-diacetoxytridecane, (2*S*,12*S*)-diacetoxytridecane, stereoisomers, inhibition, EAG recordings, antennal morphology.

INTRODUCTION

The pea midge, *Contarinia pisi* Winn. (Diptera: Cecidomyiidae), a serious pest of commercial peas in northern Europe (Wall et al., 1985; Pillon and Thieuleux, 1995), was the first species of the family Cecidomyiidae, gall midges, for which the full sex pheromone blend was identified (Hillbur et al., 1999, 2000). It consists of 2-acetoxytridecane, (2*S*,11*S*)-diacetoxytridecane, and (2*S*,12*S*)-diacetoxytridecane. All three compounds elicited a response from antennae of males when pheromone gland extract from females was analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Hillbur et al., 1999). The low concentration of 2-acetoxytridecane in gland extract prevented determination of its absolute configuration (Hillbur et al., 1999). However, in a wind tunnel bioassay a blend of racemic 2-acetoxytridecane, (2*S*,11*S*)-diacetoxytridecane, and (2*S*,12*S*)-diacetoxytridecane was as attractive to males as gland extract, indicating that if females produce only one enantiomer of 2-acetoxytridecane (*R* or *S*), the other one is not strongly inhibitory (Hillbur et al., 2000). A related compound, (2*S*)-(*E*)-10-tridecen-2-yl acetate, has been identified as a sex pheromone component in another gall midge, the Hessian fly, *Mayetiola destructor* (Foster et al., 1991). However, results from behavioral tests suggested that the complete Hessian fly sex pheromone contains one or more additional components (Harris and Foster, 1991, 1999). Recently, (2*S*,7*S*)-2,7-nonadienyl dibutyrate has been identified as sex pheromone of the orange wheat blossom midge, *Sitodiplosis mosellana* (Gries et al., 2000).

A general problem in gall midge pest management is to detect the often inconspicuous adults (Harris and Foster, 1999). Results from field trapping experiments with synthetic pea midge sex pheromone (Hillbur et al., 2000) showed that pheromone traps, as outlined by Wall et al. (1985), could be useful for monitoring of the emergence of adult pea midges. For this purpose, it would have been economically advantageous if the racemic mixtures of the diacetates instead of pure (2*S*,11*S*)- and (2*S*,12*S*)-diacetoxytridecane could be used as lures, since enantiopure compounds are far more costly to produce. However, traps baited with a blend of the racemates of all three compounds were not attractive to pea midge males

in the field, indicating that one or several of the stereoisomers not produced by the females are inhibitory (Hillbur et al., 2000). For the design of an economic chemical synthesis, the continued development of a monitoring system for the pea midge would benefit from a more detailed knowledge about requirements for stereochemical purity of the compounds, and specifically, which stereoisomers are inhibitory.

Thus, the aim of this study was to investigate the response of male pea midges to all stereoisomers of the female-produced sex pheromone components, (2*S*,11*S*)-diacetoxytridecane and (2*S*,12*S*)-diacetoxytridecane. Both behavioral responses in the field and electroantennographic (EAG) recordings were studied. Male responses to various doses of the synthetic sex pheromone blend in the field were also tested. In addition, the structures of the antennal sensilla were investigated by scanning electron microscopy and transmission electron microscopy.

METHODS AND MATERIALS

Insects. Soil containing cocoons with diapausing *C. pisi* larvae was collected near Chalons-sur-Marne in the Champagne district in France in August 1999. The soil was kept in cold storage at 6°C for approximately 5 months for completion of larval diapause. The cocoons were then separated from the soil with water using a sieving method (Doane et al., 1987), and the adults were allowed to emerge at 18L:6D photoregime, 25°C, and 70% relative humidity. Immediately upon emergence and before mating, males and females were separated and transferred to glass cages (28 × 28 × 33 cm) kept at 18L:6D photoregime, 15°C, and 75% relative humidity. Males used for electrophysiological recordings were one day old.

Chemicals. 2-Acetoxytridecane, racemic (2,11)-diacetoxytridecane, racemic (2,12)-diacetoxytridecane, and the stereoisomers of the diacetates were synthesized as described by Hillbur et al. (1999). The enantiomeric excess, ee, of the stereoisomers was at least 98%, as determined by chiral gas chromatography (Hillbur et al., 1999).

Electrophysiology. For electrophysiological recordings a Plexiglas holder with two wells connected to an amplifier by gold wire electrodes was used (JoAC, Lund, Sweden) (Zhang et al., 1997). Males were suspended between the wells by pressing the thorax, until fixed, into a slit in the well wall facing the 2-mm gap separating the wells. The head and antennae protruded into the gap. The tips of the antennae were then positioned in a small glass capillary, kept in place by dental wax in an equivalent slit in the opposite well. Finally, both wells and, by capillary force, the glass capillary were filled with Beadle-Ephrussi Ringer solution, thus providing contact. Antennal preparations were continuously exposed to a charcoal-filtered and humidified airstream (0.5 m/sec) through a glass tube (8 mm diam.). Stimuli

were delivered in 0.2-sec puffs at a flow rate of 10 ml/sec (stimulus controller, Syntech, Hilversum, The Netherlands), injected into the airstream 20 cm from the antenna. During dose–response recordings, all doses of one compound, starting with the lowest dose, were presented to the antenna before the next compound was tested. The order in which compounds were presented was varied between antennae. The test compounds (0.1, 1, 10, and 100 μg) in 10 μl of redistilled hexane (Labscan) were applied directly onto the glass in the tip of Pasteur pipets. The pipets were not used until 1 hr after compound application and were renewed after approximately 10 stimulations. Between tests, pipets were kept at -18°C. After every second test stimulus the response to a reference stimulus (10 μg of (2*S*,12*S*)-diacetoxytridecane on filter paper) was recorded. In order to correct EAG values for antennal fatigue, each test response was normalized by dividing it with the average response to the two adjacent reference stimuli. Antennal signals were amplified (JoAC) before they were recorded and analyzed with ElectroAntenno-Graphy software (Syntech).

Field Tests. Field tests were done in France between May 19 and 27, 2000, at three sites, Saint Pouange, Sompuis, and Songy, in the Champagne district. A parallel to one of the experiments was done at two sites in the UK (Market Weighton and Pocklington, Yorkshire) between June 11 and July 4, 2000. Sticky delta traps were placed in *C. pisi* emergence fields, i.e., in wheat fields after peas, 10–20 cm above ground. At each site, five replicates (only four in the UK) were placed along five well-separated transects. Within replicates traps were separated by 10 m. Trap positions were maintained unchanged during the experiment to avoid possible effects of contaminated vegetation. Dental cotton rolls (Celluron No. 2, Paul Hartmann S.A., France) cut in thirds were used as dispensers (Hillbur et al., 2000). Chemicals were applied to the dispensers as a hexane solution. Dispensers were not changed during the experimental period. In France, trap catches were counted and sticky inserts were changed daily. In the UK, catches were counted and inserts changed every three to four days.

In all experiments, a standard treatment, 10 μg (2*S*,11*S*)-diacetoxytridecane + 10 μg (2*S*,12*S*)-diacetoxytridecane + 10 μg 2-acetoxytridecane, and a blank, where the dispensers were not loaded with any chemicals, were used as reference traps. In the first experiment, two two-component blends, 10 μg (2*S*,11*S*)-diacetoxytridecane + 10 μg 2-acetoxytridecane and 10 μg (2*S*,12*S*)-diacetoxytridecane + 10 μg 2-acetoxytridecane, were tested (see also Figure 1, lower part). In the second experiment, three-component blends were tested where the two diacetates, (2*S*,11*S*)-diacetoxytridecane and (2*S*,12*S*)-diacetoxytridecane, had been substituted, one by one, by the other stereoisomers (Figure 2, lower part). A possible inhibitory effect of the stereoisomers was tested in a third experiment by addition of 2 μg of them, separately, to the standard blend (Figure 3a, lower part). In a continuation of this experiment, the dose of the stereoisomers proven to be

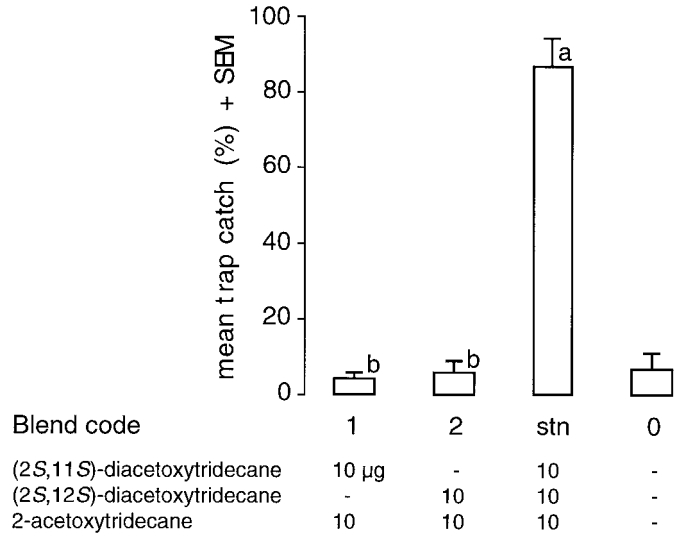
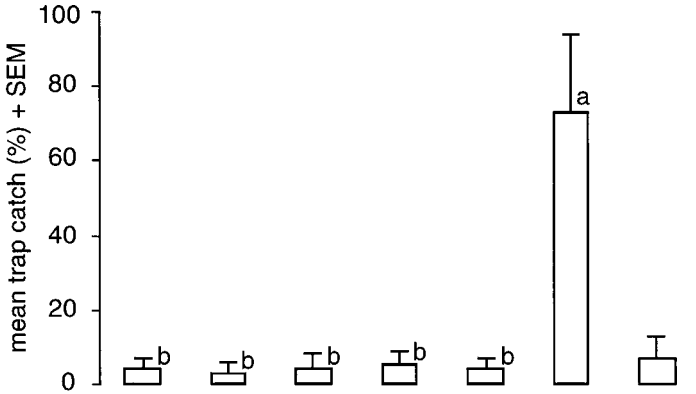


FIG. 1. Field captures of pea midge males in traps baited with the standard three-component blend and with two-component blends where one of the diacetates has been omitted. Treatments labeled with the same letter are not significantly different according to nonparametric Kruskal-Wallis analyses of variance, followed by paired Mann-Whitney *U* test with Bonferroni correction ($0.01 > P > 0.001$) (Systat, 1992).

inhibitory at 2 μg was lowered to 0.5 μg, whereas the dose of the stereoisomers that did not have any effect at 2 μg was raised to 10 μg (Figure 3b, lower part). Finally, a dose–response experiment compared traps baited with 1, 10, and 100 μg, respectively, of the standard three-component blend. This experiment was done both in France and in the UK.

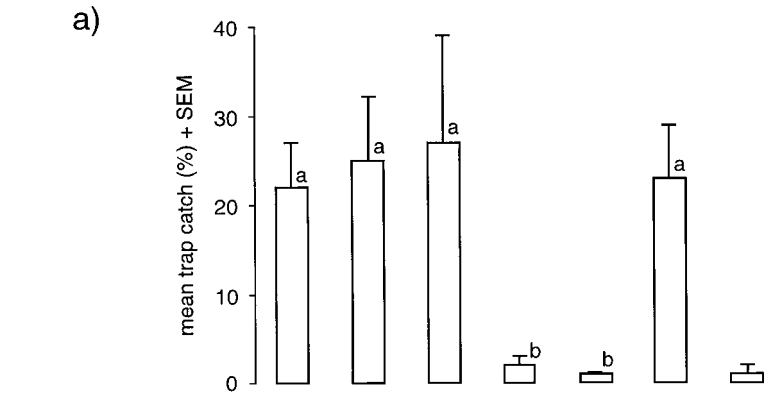
Electron Microscopy. For transmission electron microscopy (TEM), antennae from both sexes were dipped for a few seconds in 70% ethanol to reduce surface tension: after this treatment they were rapidly transferred to 3% glutaraldehyde in 0.15 M cacodylate buffer and kept overnight. After dehydration in ethanol, the preparations were brought to Epon via propylene oxide. Polymerization was carried out at 60°C for 48 hr. The blocks were trimmed and the ultrathin sections on the grids were stained in uranyl acetate and lead citrate in an LKB Ultrostainer. The sections were examined in a Jeol 1200 EX transmission electron microscope operated at 60 kV. For scanning electron microscopy (SEM), preparations fixed and dehydrated as above were critical point dried, mounted on holders, and sputter-coated with gold–palladium 40:60. The preparations were studied in a Jeol T330 scanning electron microscope.



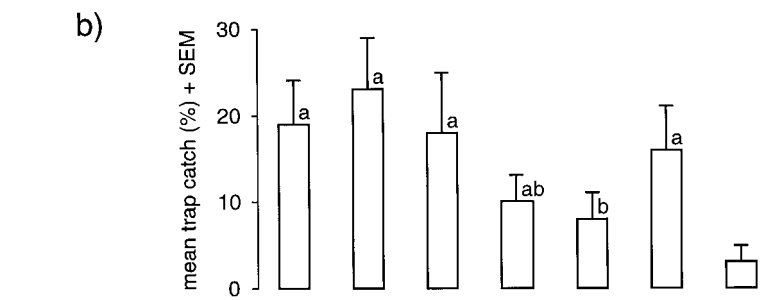
Blend code	3	4	5	6	7	stn	0
(2*S*,11*S*)-diacetoxytridecane	10 µg	10	-	-	-	10	-
(2*R*,11*R*)-	-	-	10	-	-	-	-
(2*R*,11*S*)-	-	-	-	10	-	-	-
(2*S*,11*R*)-	-	-	-	-	10	-	-
(2*S*,12*S*)-diacetoxytridecane	-	-	10	10	10	10	-
(2*R*,12*R*)-	10	-	-	-	-	-	-
meso-2,12-	-	10	-	-	-	-	-
2-acetoxytridecane	10	10	10	10	10	10	-

FIG. 2. Field captures of pea midge males in traps baited with the standard three-component blend, 2-acetoxytridecane, (2*S*,11*S*)-diacetoxytridecane, and (2*S*,12*S*)-diacetoxytridecane (stn), and with three-component blends where one of the diacetates has been substituted by stereoisomers. Treatments labeled with the same letter are not significantly different.

Statistics. The proportion of males caught by different treatments did not differ between sites, even if there was a large difference in population density. Therefore, to make sites comparable, the proportions within replicates, i.e. the number of males caught by one treatment in a replicate divided by the total number of males caught in that replicate, were used for statistical analyses. Differences were analyzed by nonparametric Kruskal-Wallis analyses of variance, followed by pairwise Mann-Whitney *U* test with Bonferroni correction (Systat, 1992). The number of replicates of the blank traps was lower than for the other treatments and, consequently, they were not included in the analysis. The dose–response curves obtained by EAG recordings were analyzed by two-way analysis of variance (ANOVA) followed by a Duncan's multiple-range test (Statistica, 1993).



Blend code	8	9	10	11	12	stn	0
(2*S*,11*S*)-diacetoxytridecane	10 µg	10	10	10	10	10	-
(2*R*,11*R*)-	-	-	2	-	-	-	-
(2*R*,11*S*)-	-	-	-	2	-	-	-
(2*S*,11*R*)-	-	-	-	-	2	-	-
(2*S*,12*S*)-diacetoxytridecane	10	10	10	10	10	10	-
(2*R*,12*R*)-	2	-	-	-	-	-	-
meso-2,12-	-	2	-	-	-	-	-
2-acetoxytridecane	10	10	10	10	10	10	-



Blend code	13	14	15	16	17	stn	0
(2*S*,11*S*)-diacetoxytridecane	10 µg	10	10	10	10	10	-
(2*R*,11*R*)-	-	-	10	-	-	-	-
(2*R*,11*S*)-	-	-	-	0.5	-	-	-
(2*S*,11*R*)-	-	-	-	-	0.5	-	-
(2*S*,12*S*)-diacetoxytridecane	10	10	10	10	10	10	-
(2*R*,12*R*)-	10	-	-	-	-	-	-
meso-2,12-	-	10	-	-	-	-	-
2-acetoxytridecane	10	10	10	10	10	10	-

FIG. 3. Field captures of pea midge males in traps baited with the standard three-component blend, 2-acetoxytridecane, (2*S*,11*S*)-diacetoxytridecane, and (2*S*,12*S*)-diacetoxytridecane (stn), in combination with: (a) 20% of each of the stereoisomers separately, and (b) 5% of the stereoisomers proven to be inhibitory at the 20% level and 100% of the stereoisomers that had no effect at the 20% level. Treatments labeled with the same letter are not significantly different.

RESULTS

The results from the field experiment with the two-component blends (1 and 2) showed that all three components are needed to attract male pea midges (Figure 1). Of the total number of males caught in this experiment, 87% were caught in traps baited with the three-component blend (stn) (Figure 1). Substitution of (2*S*,11*S*)-diacetoxytridecane and (2*S*,12*S*)-diacetoxytridecane with the corresponding stereoisomers (blends 3–7) reduced trap catches to the level of the blank trap (0) (Figure 2). Only the standard three-component blend (stn) caught significant numbers of midges (73%). Addition of 20% (2 μg) of stereoisomers to the standard blend (stn) showed that at this dose (2*R*,11*S*)-diacetoxytridecane (11) and (2*S*,11*R*)-diacetoxytridecane (12) have a strongly inhibitory effect on male attraction (Figure 3a). Both treatments reduced trap catches to the same level as the blank traps (0). Addition of (2*R*,12*R*)-diacetoxytridecane, *meso*-2,12-diacetoxytridecane, and (2*R*,11*R*)-diacetoxytridecane, to the standard three-component blend (blends 8, 9, and 10) did not affect male attraction. Catches in those traps did not differ significantly from the number of males caught with the standard blend (stn) (Figure 3a). A decrease in the dose of the inhibitory stereoisomers to 5% (blends 16 and 17) and an increase of the dose of the other stereoisomers to 100% (blends 13, 14, and 15) resulted in an overall decrease in differences in trap catches between treatments (Figure 3b). Only in traps where 5% (2*S*,11*R*)-diacetoxytridecane had been added to the standard blend (17) did catches differ significantly from those baited with the standard blend (stn) (Figure 3b).

In France, the dose–response experiment showed that 1 μg of the standard blend was the most attractive dose to males (Figure 4a). It attracted 60% of the males, whereas traps baited with 10 and 100 μg caught 24 and 16%, respectively (Figure 4a). In the UK, however, there were no significant differences in trap catches between the doses (Figure 4b). Population densities were higher in France than in the UK. The total number of males caught in traps baited with 1 μg of the standard blend was 13,200 in France (experimental period 8 days) and 5,100 in the UK (experimental period 23 days).

The male antennal response to the female-produced stereoisomers, (2*S*,11*S*)-diacetoxytridecane and (2*S*,12*S*)-diacetoxytridecane increased with an increasing dose of the stimuli (Figure 5). Similar dose–response curves were obtained for the inhibitory stereoisomers (2*R*,11*S*)-diacetoxytridecane and (2*S*,11*R*)-diacetoxytridecane, but the responses were slightly lower than to the pheromone compounds (Figure 5). Even lower responses were elicited by the behaviorally inactive stereoisomers, (2*R*,11*R*)-diacetoxytridecane, (2*R*,12*R*)-diacetoxytridecane, and *meso*-2,12-diacetoxytridecane (Figure 5). An analysis of variance showed that the factors compound and dose, as well as their interaction, had a significant effect (two-way ANOVA; factor 1: compound, $df = 160$, $F = 10.426$, $P < 0.001$, factor 2: dose, $df = 160$, $F = 16.296$, $P < 0.001$, compound $\times$ dose, $df = 160$, $F = 1.692$,

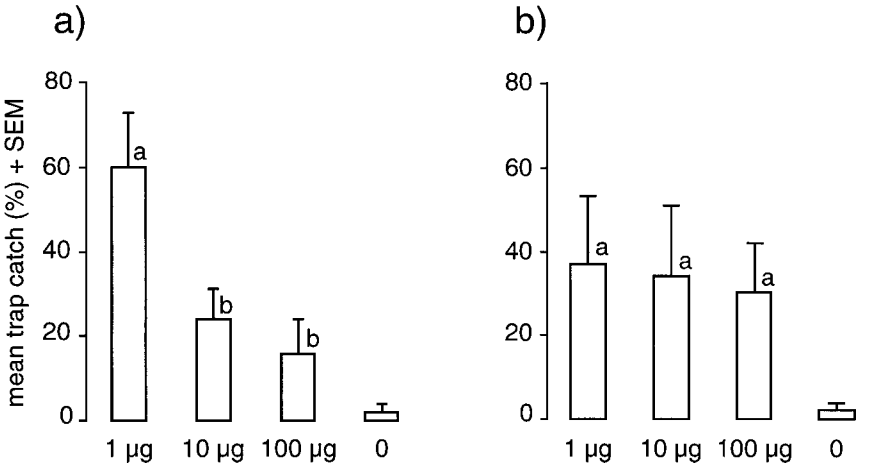


FIG. 4. Field captures of male pea midges in traps baited with different doses of the standard 3-component blend, 2-acetoxytridecane, (2*S*,11*S*)-diacetoxytridecane, and (2*S*,12*S*)-diacetoxytridecane (stn): (a) in France, and (b) in the UK. Treatments labeled with the same letter are not significantly different.

$P < 0.05$). The interaction term shows that there is a significant difference between the slopes of the dose–response curves.

The analyses of the structure of the antennae showed that antennae of males consist of 12 antennal segments (flagellomeres), in addition to the two basal segments, scapus and pedicellus. Each flagellomere is about 100 μm long and consists of two swollen nodes connected with a cylindrical portion (Figure 6). The sensilla are confined to the nodes, which are found in the proximal and middle portions of the flagellomeres. In the females, the 12 flagellomeres are cylindrical (40×25 μm) (Figure 7). In both sexes, small protrusions (microtricha) cover the surface of the flagellomeres (Figures 6 and 7). Three types of chemosensory sensilla are found on the antenna: compound sensilla with wall pores (s. circumfila), hairlike single sensilla with wall pores (s. trichodea), and double-walled sensilla (s. coeloconica). The circumfila have a peculiar shape and exhibit different structures in males and females. Long uninnervated bristles are also present among the antennal sensilla (Figures 6 and 7). These companion bristles have a shaft provided with small teeth.

In the males, there is one wreath of circumfila on each node. In each of these, there are about 10 forked sensilla that are joined apically. The circumfila are found close to a transverse row of companion hairs (Figure 6). Inside the antenna, two dendritic segments from sensory cells are enclosed within an enveloping cell, corresponding to the thecogen cell of other sensilla. At the distal part of the innermost enveloping cell, there is no dendritic sheath as in other

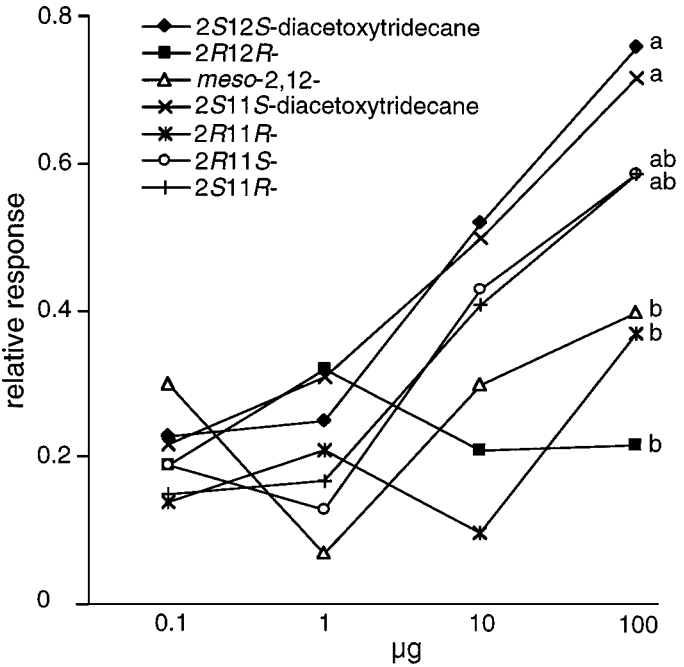


FIG. 5. Electroantennogram (EAG) responses from male pea midge antennae to different doses of the stereoisomers of (2,11)-diacetoxytridecane and (2,12)-diacetoxytridecane ($N = 6$). EAG values were corrected by relating the measured values to a standard measurement before and after the test compound. Dose–response curves followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$) (Statistica, 1993).

insect sensilla (Figure 8). The transition zone between the inner and outer dendritic segments is situated about 5 μm below the base of the hair. Lymph cavities are lacking in this region. The basal part of the hair is invaded by the sensory cells and the enveloping cells that send protrusions into the hair (Figure 8). The basal part of the hair is about 2 μm long and has a smooth outer surface. Above this zone the two branches of the sensillum turn transversely until they are close to the companion hairs, which they accompany in a distal direction (Figure 9). The proximal curved parts of the branches have a transverse cuticular pattern, whereas the distal part has longitudinal furrows (Figure 9). The sensillar hair branches follow the companion hairs to their tips, and here the branches from two adjoining sensilla fuse (Figure 6). In the distal part of the circumfila there are one or two branches of the outer dendritic segments. The density of the pores is quite low in the males.

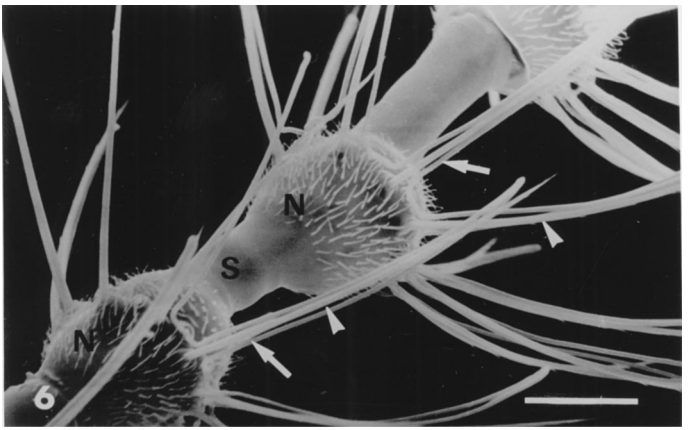


FIG. 6. Flagellomere from male consisting of two nodes (N), that are connected with portions of smaller diameter (S). On the nodes are sensilla circumfila (arrows) and companion hairs (arrowheads). Scale bar = 20 μm.

Sensilla circumfila in the females form two transverse loops connected with two axial connections (Figure 7). A large number, approximately 25–30, of sensilla form this continuous structure. The circumfila of the females also possess two sensory cells, and the other cellular arrangements below the antennal surface are similar to those of the male (Figure 8). The surface of the cirumfila in the females

FIG. 7. Flagellomere from female showing sensilla circumfila (asterisks), sensilla trichodea (arrows), and companion hairs (arrowheads). Scale bar = 20 μm.

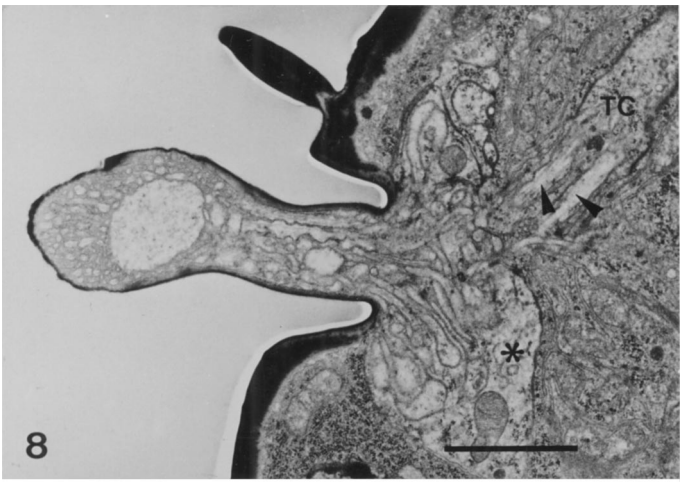


FIG. 8. Section through the base of a sensillum circumfilum in a male. The outer dendritic segments (arrowheads) are enclosed within the innermost enveloping cell (TC). Another enveloping cell (asterisk) has a more superficial position, sending processes into the base of the cuticular hair. Scale bar = 0.5 μm.

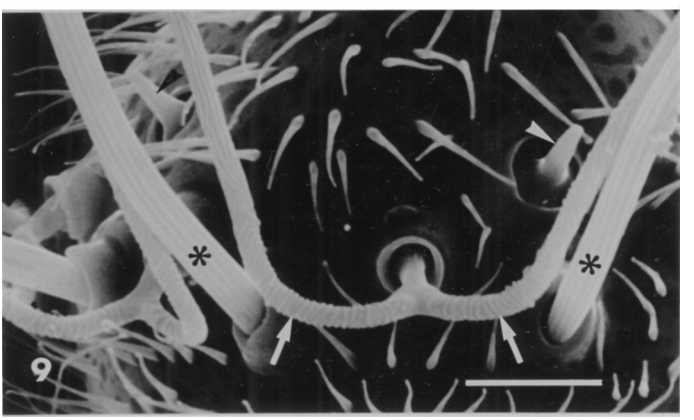


FIG. 9. The sensilla circumfila (arrows) are forked and the branches follow the companion hairs (asterisks). Sensilla coeloconica are indicated with arrowheads. Scale bar = 5 μm.

is smooth (Figure 7). The cuticle possesses a large number of small pores, which lack pore tubules. The outer dendritic segments branch profusely in the hairs.

The sensilla trichodea are found in relatively low numbers, about five on each flagellomere. They are innervated with two sensory cells, and the arrangement of the enveloping cells is similar to that of the circumfila, but the single wall-pore sensilla possess a dendritic sheath, which originates from the thecogen cell. Basally, the surface structure of the hair consists of longitudinal folds. Distally, there are transverse ridges. The dendritic outer segments branch moderately in the outer part of the hair. The pore density is lower than in the circumfila, and the pores lack tubules.

There are a few sensilla coeloconica on each flagellomere. They are innervated with four to five sensory cells, and they possess a dendritic sheath as well as inner and outer lymph cavities. The cuticular hair is short, 2 μm, and has apical slitlike pores.

DISCUSSION

In several species that use only one stereoisomer of a chiral compound as a sex pheromone component, the inhibitory effect of other stereoisomers on sex attraction forms a basis for reproductive isolation. This has been shown in species as diverse as scarab beetles (Leal, 1996), corn rootworms (Guss et al., 1985; Krysan et al., 1986), winter-flying geometrid moths (Szöcs et al., 1993), and bark beetles (e.g., Birch, 1984; Seybold, 1993). The ratio at which stereoisomers have an inhibitory effect varies between species. Leal (1996) suggested that in species where the chiral compound is just one constituent of a multicomponent pheromone blend the antagonistic effect of the antipode might be better tolerated, because species separation is then additionally supported by other compounds in the specific blend. Separation in time or space may also contribute to reproductive isolation between closely related species (e.g., Löfstedt et al., 1991; Szöcs et al., 1993).

It is not possible to say whether species separation is the reason for the inhibitory effect of (2*S*,11*R*)- and (2*R*,11*S*)-diacetoxytridecane observed in this study, because the inhibitory stereoisomers are not known to be pheromones of any related species. However, there are indications that the same or similar compounds are used as sex pheromone components within the Cecidomyiidae. The pea midge compounds and the compound identified for the Hessian fly are similar in structure, and, when tested in the field, the latter did not attract Hessian fly males but large numbers of males of another gall midge species (Harris and Foster, 1999). Moreover, all presently identified compounds within the family are chiral (Foster et al., 1991; Hillbur et al., 1999, Gries et al., 2000). Therefore, it seems reasonable to assume that the inhibitory effect of the stereoisomers (2*S*,11*R*)- and (2*R*,11*S*)-diacetoxytridecane observed in this study may be a mating barrier

between the pea midge and one or several related sympatric species. Furthermore, species specificity in the pheromone signal seems also to be maintained by the blend composition. Removal of either of the diacetates from the blend yielded trap catches comparable to those in the blank traps (Figure 1), as did removal of the third component, 2-acetoxytridecane (Hillbur et al., 2000).

The response at the antennal level can be discussed in view of both the behavioral and the electrophysiological data. The field experiments show that in order to attract pea midge males, neither of the diacetates can be substituted by the corresponding stereoisomers (Figure 2). The males must thus be able to distinguish (2*S*,11*S*)- and (2*S*,12*S*)-diacetoxytridecane from the other stereoisomers, which suggests that they have antennal receptors specifically tuned to these compounds. Evidence for enantiospecific pheromone receptor neurons has been reported in e.g. scarab beetles (Wojtasek et al., 1998). The strong inhibitory effect of (2*S*,11*R*)- and (2*R*,11*S*)-diacetoxytridecane on male attraction (Figure 3a) indicates that the antennae also have receptors specific for these antagonists. This is also supported by the EAG results, where similar dose-response curves were obtained from the pheromone components and the antagonists (Figure 5). The other stereoisomers do not seem to be of importance to male pea midges (Figures 3a,b and 5).

Earlier fine structural investigations of gall midge antennae have revealed five sensillum types involved in chemoreception: sensilla circumfila, sensilla trichodea, sensilla basiconica, sensilla coeloconica, and uniporous peg sensilla (Slifer and Sekhon, 1971; Solinas and Nuzzaci, 1987; Crook and Mordue (Luntz), 1999). Three of these, sensilla circumfila, sensilla trichodea, and sensilla coeloconica, were found on pea midge antennae. The circumfila are a unique type of sensilla found only in gall midges (Hallberg and Hansson, 1999). In the pea midge, the pronounced sexual dimorphism in the circumfila and their looped, surface-enlarging structure in the males indicate that they are important in the detection of female sex pheromones. This has also been suggested for the sorghum midge, *Contarinia sorghicola* (Slifer and Sekhon, 1971) and another gall midge, *Mycodiplosis erysiphes* (Solinas and Nuzzaci, 1987). In the blackcurrant leaf midge, *Dasineura tetensi*, however, sensilla circumfila are more elaborate in females than in males (Crook and Mordue (Luntz), 1999). Instead, sensilla trichodea, shown to be more common in males than in females in this species, were assumed to be more important in sex pheromone detection (Crook and Mordue (Luntz), 1999).

Transmission electron microscopy showed that in the pea midge both sensilla circumfila and sensilla trichodea possess two sensory cells. In view of the results of the present study, one would expect to find at least four receptor neurons associated with sex pheromone reception on male pea midge antennae, one for each of the pheromone components and at least one for the antagonists. Which of the sensillum types house these neurons and how they are organized remains to be investigated by single sensillum recordings. As pointed out by Baker

et al. (1998), cocompartmentalization within the same sensillum of receptors tuned to the pheromone components and receptors tuned to the antagonist should optimize both the spatial and temporal resolution ability of the male. Cocompartmentalization of pheromone and antagonist neurons is commonly found in Lepidoptera (e.g., Löfstedt et al., 1990; Cossé et al., 1998; Todd and Baker, 1999) and has also been shown in a diprionid sawfly (Hansson et al., 1991) and in scarab beetles (Wojtasek et al., 1998).

This study provides valuable information for the continued development of a pea midge monitoring system, both with regard to purity and amount of chemicals required. The results indicate that only (2*S*,11*S*)-diacetoxytridecane has to be of high stereoisomeric purity. The configuration of (2,12)-diacetoxytridecane, like that of 2-acetoxytridecane (Hillbur et al., 2000), does not seem to affect male behavior. For use in monitoring traps, the French results clearly show that the lowest dose, 1 μg, is the most attractive one (Figure 4a). The fact that no significant differences were seen between the doses in the UK (Figure 4b) could be explained by factors such as differences in temperature that would affect the emission rate of the compounds from the dispensers or the comparatively low population densities at the experimental sites in the UK. Adult pea midges have been shown to be highly aggregated in emergence fields (Wall et al., 1991). At low population densities, this could result in variable trap catches.

Acknowledgments—We thank E. Marling and R. Ward for technical assistance. J. Cheminon, D. Van de Veldes, B. Chazel, G. Abit, C. Baguet (France), A. Kitching, and R. Padgett (UK) are acknowledged for kindly letting us use their fields for experiments. We are also grateful to P. Witzgall for valuable suggestions regarding the field experiments, F. Schlyter and T. Meiners for statistical advice, and P. Anderson for comments on an earlier version of this manuscript. This work was supported by Carl Tryggers Stiftelse för Vetenskaplig Forskning, the Faculty of Landscape Planning and Horticulture, SLU, Sweden, Processors and Growers Research Organisation and the Horticultural Development Council, UK.

REFERENCES

BAKER, T. C., FADAMIRO, H. Y., and COSSÉ, A. A. 1998. Fine-grained resolution of closely spaced odor strands by flying moths. *Nature* 393:530.

BIRCH, M. C. 1984. Aggregation in bark beetles, pp. 331–353, *in* W. J. Bell, and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

COSSÉ, A. A., TODD, J. L., and BAKER, T. C. 1998. Neurons discovered in male *Helicoverpa zea* antennae that correlate with pheromone-mediated attraction and interspecific antagonism. *J. Comp. Physiol.* 182:585–594.

CROOK, D. J., and MORDUE (LUNTZ), A. J. 1999. Olfactory responses and sensilla morphology of the blackcurrant leaf midge *Dasineura tetensi*. *Entomol. Exp. Appl.* 91:37–50.

DOANE, J. F., OLFERT, O. O., and MUKERJI, M. I. 1987. Extraction precision of sieving and brine flotation for removal of wheat midge, *Sitodiplosis mosellana* (Diptera: Cecidomyiidae), cocoons and larvae from soil. *J. Econ. Entomol.* 80:268–271.

FOSTER, S. P., HARRIS, M. O., and MILLLAR, J. G. 1991. Identification of the sex pheromone of the Hessian fly, *Mayetiola destructor* (Say). *Naturwissenschaften* 78:130–131.

GRIES, R., GRIES, G., KHASKIN, G., KING, S., OLFERT, O., KAMINSKI, L.-A., LAMB, R., and BENNETT, R. 2000. Sex pheromone of the orange wheat blossom midge, *Sitodiplosis mosellana*. *Naturwissenschaften* 87:450–454.

GUSS, P. L., SONNET, P. E., CARNEY, R. L., TUMLINSON, J. H., and WILKIN, P. J. 1985. Response of northern corn rootworm, *Diabrotica barberi* Smith and Lawrence, to stereoisomers of 8-methyl-2-decyl propanoate. *J. Chem. Ecol*. 11:21–26.

HALLBERG, E., and HANSSON, B. S. 1999. Arthropod sensilla: Morphology and phylogenetic considerations. *Microsc. Res. Tech*. 47:428–439.

HANSSON, B. S., VAN DER PERS, J. N. C., HÖGBERG, H.-E., HEDENSTRÖM E., ANDERBRANT, O., and LÖFQVIST, J. 1991. Sex pheromone perception in male pine sawflies, *Neodiprion sertifer* (Hymenoptera: Diprionidae). *J. Comp. Physiol. A*. 168:533–538.

HARRIS, M. O., and FOSTER, S. P. 1991. Wind tunnel studies of sex pheromone mediated behavior of the Hessian fly (Diptera: Cecidomyiidae). *J. Chem. Ecol*. 17:2421–2435.

HARRIS, M. O., and FOSTER, S. P. 1999. Gall midges, pp. 27–49, *in* J. Hardie, and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Oxford, UK.

HILLBUR, Y., ANDERSON, P., ARN, H., BENGTSSON, M., LÖFQVIST, J., BIDDLE, A. J., SMITT, O., HÖGBERG, H.-E., PLASS, E., FRANKE, S., and FRANCKE, W. 1999. Identification of sex pheromone components of the pea midge, *Contarinia pisi* (Diptera: Cecidomyiidae). *Naturwissenschaften* 86:292–294.

HILLBUR, Y., EL-SAYED, A., BENGTSSON, M., LÖFQVIST, J., BIDDLE, A., PLASS, E., and FRANCKE, W. 2000. Laboratory and field study on the attraction of male pea midges, *Contarinia pisi*, to synthetic sex pheromone components. *J. Chem. Ecol*. 26:1941–1952.

KRYSAN, J. L., WILKIN, P. H., TUMLINSON, J. H., SONNET, P. E., CARNEY, R. L., and GUSS, P. L. 1986. Response of *Diabrotica lemniscata* and *D. longicornis* (Coleoptera: Chrysomelidae) to stereoisomers of 8-methyl-2-decyl-propanoate and studies on the pheromone of *D. longicornis*. *Ann. Entomol. Soc. Am.* 79:742–746.

LEAL, W. S. 1996. Chemical communication in scarab beetles: Reciprocal behavioral agonist-antagonist activities of chiral pheromones. *Proc. Natl. Acad. Sci. U.S.A.* 93:12112–12115.

LÖFSTEDT, C., HANSSON, B. S., DIJKERMAN, H. J., and HERREBOUT, W. W. 1990. Behavioural and electrophysiological activity of unsaturated analogues of the pheromone tetradecyl acetate in the small ermine moth *Yponomeuta rorellus. Physiol. Entomol.* 15:47–54.

LÖFSTEDT, C., HERREBOUT, W. M., and MENKEN, S. B. J. 1991. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology* 2:20–28.

PILLON, O., and THIEULEUX, J. 1995. La cecidomyie du pois (*Contarinia pisi Winn.*) un noveau ravageur champenois. *Med. Fac. Landbouww. Univ. Gent* 60:645–650.

SEYBOLD, S. J. 1993. Role of chirality in olfactory-directed behavior: Aggregation of pine engraver beetles in the genus *Ips* (Coleoptera:Scolytidae). *J. Chem. Ecol.* 19:1809–1831.

SLIFER, E. H., and SEKHON, S. S. 1971. Circumfila and other sense organs on the antenna of the sorghum midge (Diptera, Cecidomyiidae). *J. Morphol.* 133:281–302.

SOLINAS, M., and NUZZACI, G. 1987. Antennal sensilla of *Mycodiplosis erysiphes* Ruebs. (Cecidomyiidae, Diptera). *Boll. Ist. Entomol. Guido Grandi Univ. Bologna* 41:173–194.

STATISTICA. 1993. Version 4.5. Statsoft Inc., Tulsa, Oklahoma.

SYSTAT. 1992. Systat Statistics, Version 5.2 edition. Systat, Inc., Evanston, Illinois.

SZÖCS, G., TÓTH, M., FRANCKE, W., SCHMIDT, F., PHILIPP, P., KÖNIG, W. A., MORI, K., HANSSON, B. S., and LÖFSTEDT, C. 1993. Species discrimination in five species of winter-flying geometrids (Lepidotera) based on chirality of semiochemicals and flight season. *J. Chem. Ecol*. 19:2721–2735.

TODD, J. L., and BAKER, T. C. 1999. Function of peripheral olfactory organs, pp. 67–96, *in* B. S. Hansson (ed.). Insect Olfaction. Springer-Verlag, Berlin.

WALL, C., PICKETT, J. A., GARTHWAITE, D. G., and MORRIS, N. 1985. A female sex pheromone in the pea midge, *Contarinia pisi. Entomol. Exp. Appl.* 39:11–14.

WALL, C., BIDDLE, A. J., BLOOD SMYTH, J., FOOT, G. E., GARTHWAITE, D. G., MORRIS, N., OWEN, C. R., STURGEON, D. M., and WEYMAN, G. 1991. Local spatial and temporal distribution of pea midge, *Contarinia pisi*. *Asp. Appl. Biol.* 27:355–360.

WOJTASEK, H., HANSSON, B. S., and LEAL, W. S. 1998. Attracted or repelled? A matter of two neurons, one pheromone binding protein, and a chiral center. *Biochem. Biophys. Res. Commun.* 250:217–222.

ZHANG, A., ROBBINS, P. S., LEAL, W. S., LINN, C. E., VILLANI, G., and ROELOFS, W. L. 1997. Essential amino acid methyl esters: Major sex pheromone components of the cranberry white grub, *Phyllophaga anxia* (Coleoptera: Scarabidae). *J. Chem. Ecol.* 23:231–245.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

SEX PHEROMONE COMPONENTS OF THE BUCK MOTH *Hemileuca maia*

J. STEVEN MCELFRESH,[1] ABNER M. HAMMOND,[2] and JOCELYN G. MILLAR[1,*]

[1]*Department of Entomology*
University of California
Riverside California 92521

[2]*Department of Entomology*
Louisiana State University
Agricultural Center
Baton Rouge, Louisiana 70803

(Received December 20, 2000; accepted March 13, 2001)

Abstract—The sex attractant pheromone blend of *Hemileuca maia* (Lepidoptera: Saturniidae) from the vicinity of Baton Rouge, Louisiana, has been identified. The major component of the blend is (*E*10,*Z*12)-hexadeca-10,12-dienal (*E*10,*Z*12–16:Ald), in combination with the minor components (*E*10,*Z*12)-hexadeca-10,12-dien-1-ol (*E*10,*Z*12–16:OH), and (*E*10,*Z*12)-hexadeca-10,12-dien-1-yl acetate (*E*10,*Z*12–16:Ac). Ratios of the compounds in extracts of female pheromone glands varied around a mean of 100:7.4:6.3. None of the three components were attractive to male moths when tested as single components. Several other compounds were tentatively identified from female pheromone gland extracts, including *E*10,*E*12–16:Ald, *E*10,*E*12–16:OH, and *E*10,*E*12–16:Ac, but addition of these components, either alone or in combination, at biologically relevant rates, did not significantly increase the attractiveness of lures. The saturated analogs, hexadecanal, hexadecanol, and hexadecyl acetate, also were identified in gland extracts, but had no apparent effect as pheromone components.

Key Words—*Hemileuca maia*, (*E*10,*Z*12)-hexadecadienal, (*E*10,*E*12)-hexadecadienyl acetate, (*E*10,*Z*12)-hexadecadienol, sex pheromone.

INTRODUCTION

The buck moth, *Hemileuca maia* (Drury) (Lepidoptera: Saturniidae), is widely distributed throughout the eastern half of the United States (Tuskes et al., 1996).

* To whom correspondence should be addressed.

0098-0331/01/0700-1409$19.50/0

It is an occasional defoliating pest of oaks, with localized outbreaks appearing sporadically and unpredictably (Martinat et al., 1997). The mature caterpillars are about 6.2 cm in length, and each body segment contains numerous tufts of compound urticating spines. When the caterpillars are handled or crushed, these spines break off in the skin, causing a severe burning sensation. Reddish welts may persist at the site of contact for weeks. Stinging also may produce anaphylactic shock reactions in sensitive individuals. During outbreaks, the larvae can be a severe nuisance and a health hazard, and the problem is exacerbated by the larval habit of wandering off the host plant. As colorfully described by Earle (1966), "Their presence greatly diminishes the joy of walking barefoot in the springtime." The problem can be severe enough that trees in public places are sprayed with insecticide during the spring caterpillar emergence in attempts to reduce populations in urban and suburban areas.

The basic biology and distribution of *H. maia* have been summarized (Earle, 1966; Tuskes et al., 1996; Martinat et al., 1997). Eggs are laid in late fall in rings around oak twigs, and hatch the following spring. The early instars are gregarious and exhibit processionary behavior, whereas later instars are solitary. In the southern United States, pupation occurs in late May to early June, and pupae go through a summer diapause before emerging in late November to early December. The adult moths are diurnal and are easily seen because of their distinct black and white markings and their size.

Earle (1966) reported that caged virgin female moths attracted numerous male moths with a sex attractant pheromone, but to date, there has been no further investigation of the pheromone components. As part of a continuing study of sex pheromones of the North American Saturniidae, we report here the identification and field testing of the female-produced sex pheromone blend of *H. maia*.

METHODS AND MATERIALS

Insects. Late-instar caterpillars, which feed primarily on live oak, *Quercus virginianus* (Mill), were collected at Burden Research Plantation, Baton Rouge, Louisiana, in late May and early June of 1995–1997. In the laboratory, the caterpillars were held in screen-covered 160-liter plastic containers at 27°C and 65% relative humidity. Fresh-cut oak foliage was placed in the containers daily until pupation. The larvae pupated in the 3 to 5-cm layer of vermiculite covering the bottom of the container. Approximately 150 pupae were collected from the vermiculite in September and October, sexed by using dimorphic genital characters, and sent to University of California, Riverside. Upon arrival, the sexed pupae were held separately in 4-liter paper cans with screen lids and damp vermiculite until the moths emerged. Additional humidity was provided by moistening paper towels and placing them on the screen lid, with a sheet of clear plastic draped over the lid

to maintain high humidity. Upon emergence, males were allowed to expand and dry their wings before being placed in labeled glassine envelopes, then in sealed plastic bags with a damp towel, and held in a refrigerator (10–15°C) until needed for electroantennographic studies. Females were allowed to expand their wings and usually began to call within 1–2 hr of emergence inside the paper can, where they were allowed to remain until excision and extraction of the sex pheromone glands.

Extraction of Sex Pheromone Glands. Female sex pheromone glands were removed from virgin calling females (1–2 days old) by extruding the gland by applying gentle pressure to the tip of the abdomen and removing the everted gland and terminal abdominal segments with microscissors. The excised glands from individuals, or from several females combined, were placed into a 0.25-ml conical glass vial insert containing approximately 30 μl pentane. Care was taken to remove as much of the meconium as possible with clean Kimwipes to minimize contamination of the extract. The glands were extracted for 20 min, then the extract was transferred to a clean insert and concentrated by passive evaporation as needed. If not used immediately, extracts were stored at −20°C.

Gas Chromatography. Gas chromatography (GC) analyses were conducted in splitless mode with Hewlett-Packard 5890A or 5890 Series II gas chromatographs (H-P, Palo Alto, California), that used helium carrier gas. Columns and programs used to analyze extracts included: DB-1701 (30 m × 0.32 mm ID, 0.25 μm film, J&W Scientific, Folsom California; 100°C/0 min, 15°C/min to 275°C, hold 60 min), DB-5 (30 m × 0.32 mm ID, 0.25 μm film, 100°C/0 min, 10 or 15°C/min to 275°C, hold 30 min), and DB-WAX (30 m × 0.32 mm ID, 0.25 μm film, 100°C/0 min, 15°C/min to 250°C, hold 20 min).

Coupled Gas Chromatography–electroantennography (GC-EAD). Aliquots of female extracts and synthetic standards were analyzed by coupled GC-EAD with an H-P 5890 series II GC, with either DB-1701 or DB-5 columns operated under the conditions described above. Details of the set-up and operation of the system, and the preparation of the moth antennae for GC-EAD analyses, have been described previously (McElfresh and Millar, 1999a–d).

Coupled Gas Chromatography–mass spectrometry. Electron impact (EI) mass spectra (70 eV) were taken with an H-P 6890 gas chromatograph interfaced to an H-P 5973 mass selective detector. An HP5-MS column (30 m × 0.25 mm ID) was used in splitless mode, programmed from 50°C at 1 min, 10°/min to 250°C, hold 20 min, injector 275°C, transfer line 280°C. Compounds in the insect extract (an aliquot of a composite sample from five females) were identified by comparison of retention times and mass spectra with those of synthetic standards (see below).

Approximately 2 female equivalents (FE) of a composite extract of five females obtained in July 1997 were concentrated under a gentle stream of N_2, then taken up in 20 μl CH_2Cl_2 and treated with 2.5 μl of a solution of

4-methyl-1,2,4-triazoline-3,5-dione (MTAD, 1.2 mg/ml; Aldrich Chemical, Milwaukee, Wisconsin) (Young et al., 1990; McElfresh and Millar, 1999a). The resulting faint pink solution was concentrated to <5 μl, and a 1-μl aliquot was analyzed by GC-MS, by using an injector temp of 300°C, a temperature program of 100°C/0 min, 15°/min to 300°C, hold 20 min, and a solvent delay time of 8 min. Identifications were confirmed by analyses of adducts prepared in identical fashion from synthetic pheromone standards.

Synthesis of Pheromone Components. Standards used in chromatographic work and for making lures for field trials were synthesized and purified as described in McElfresh and Millar (1999a). All compounds used in field trials were a minimum of 98% chemically and isomerically pure. GC standards of all four 10,12–16:Ac isomers also were obtained from Darwin Reed, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada.

Field Trials. Lures consisted of 11-mm red rubber septa (Wheaton Scientific, Millville, New Jersey), individually labeled with a treatment number and loaded with heptane solutions (100 μl) of test blends, plus 2 drops of 10 mg/ml butylated hydroxytoluene (BHT) in heptane as an antioxidant. Once the treatment solutions had soaked into the septa, the lures were stored in 20-ml glass screw-cap vials for transport. Lures were stored in a freezer or an ice-chest when not in use, except during shipping. A particular set of lures was not used for >11 days.

Field trials with blends of synthetic pheromone components were conducted in December of 1995–1998. Most field trials were conducted at the Burden Research Plantation, Baton Rouge, Louisiana, in a mixed hardwood forest bordered by open fields. Pherocon 1C traps (Trécé Inc., Salinas, California) were used, placed on the ground at least 30 m apart. In 1997, two replications of a trial were conducted in New Orleans, Louisiana, and these traps were placed approx. 1 m off the ground in two separate locations. Treatments were usually tested in three to four replicate blocks spaced 100–270 m apart. Blocks consisted of six to nine treatments, including a trap baited with a solvent-treated control. Each block was counted a minimum of three and a maximum of eight times. Trap bottoms were changed as necessary, as they became covered with accumulations of moth scales. Trap counts were conducted before 09:00 hr or after 15:00 hr when flight had ceased, and traps were rerandomized after each count. All moths were removed from the traps at each count.

Data Analysis. To eliminate the day factor, which was irrelevant for our purposes, the daily counts for each treatment in a given block were pooled. This sum was then transformed ($\sqrt{x} + 0.5$) prior to conducting a two-way ANOVA followed by a Student-Neuman-Keuls (SNK) test to separate means when appropriate (SAS Institute, 1996). Treatments that failed to capture any moths were not included in the ANOVA because of their lack of variance.

RESULTS

Analysis of Female Pheromone Gland Extracts. Analyses of gland extracts by coupled GC-EAD demonstrated that male moth antennae responded to at least six components in the extracts (Figure 1). These compounds, and several others present in the extracts, were identified by GC and GC-MS, and the identifications were confirmed by comparison of retention times on at least two capillary GC columns of differing polarities and of mass spectra with those of standards.

The mass spectrum of the component eliciting the largest antennal response (Figure 1, peak **2**) was consistent with that of a conjugated 16-carbon diene aldehyde, with a comparatively strong molecular ion (*m*/*z* 236, 19% of base peak at *m*/*z* 67), with a possible molecular formula $C_{16}H_{28}O$. The relatively large size of the molecular ion, coupled with a retention time considerably longer than that of a hexadecanal standard, provided support for a conjugated diene being present somewhere in the structure, as did a set of ions at *m*/*z* 67, 81, 95, 109, and 123. Comparison of the mass spectrum and retention time with those of a standard of *E*10,*Z*12–16:Ald provided an exact match. The position of the diene was corroborated by formation of the Diels-Alder adduct with the powerful dienophile 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) (Young et al., 1990; McElfresh and Millar, 1999a). The adduct gave a molecular ion as expected at *m*/*z* 349 (9%) and

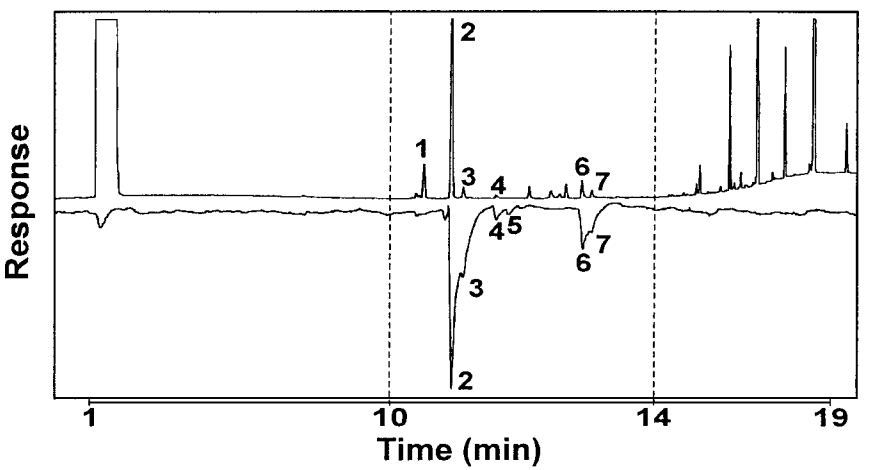


FIG. 1. Coupled gas chromatogram–electroantennogram of male *H. maia* antenna stimulated by a female pheromone gland extract. Column: DB-5 (30 m × 0.32 mm ID, 0.25-μm film, temperature program 100°C/0 min, 10°C/min to 275°C for 30 min). Identifications of peaks: 1, 16:Ald; 2, *E*10,*Z*12–16:Ald; 3, *E*10,*E*12–16:Ald; 4, *E*10,*Z*12–16:OH; 5, *E*10,*E*12–16:OH; 6, *E*10,*Z*12–16:Ac; and 7, *E*10,*E*12–16:Ac.

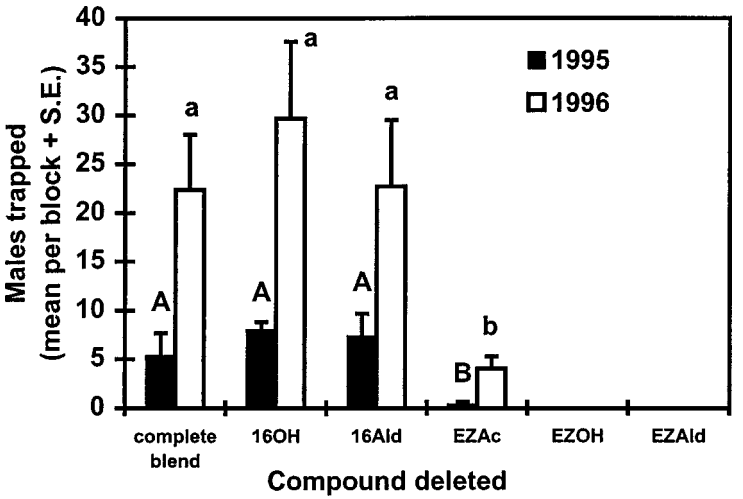


FIG. 2. Trap catches of male *Hemileuca maia* in field trials near Baton Rouge, Louisiana, December 12–15, 1995, and December 7–13, 1996, testing a five-component blend that mimicked the ratios found in a pheromone gland extract from females versus four-component blends with single compounds deleted. The complete blend consisted of: *E*10,*Z*12–16:Ald, *E*10,*Z*12–16:OH, *E*10,*Z*12–16:Ac, 16:Ald, and 16:OH (100:8:4:35:21 μg/septum, respectively). Total number of moths trapped = 63 in 1995 (solid bars), and 236 in 1996 (open bars). Significant differences among treatments are indicated by different letters surmounting the bars (1995, capital letters, 1996, lowercase letters; SNK test, $\alpha = 0.05$). Two-way ANOVA for blend effect in 1995, $F = 9.30$, $df = 3, 6$, $P = 0.0113$; for block effect. $F = 3.75$, $df = 2, 6$, $P = 0.0879$. Two-way ANOVA for blend effect in 1996, $F = 18.64$, $df = 3, 6$, $P = 0.0019$; for block effect, $F = 19.71$, $df = 2, 6$, $P = 0.0023$.

strong peaks at *m*/*z* 208 (100%) and 306 (48%) from cleavage of the alkyl chains in the three and six positions of the adduct, unequivocally demonstrating that the diene in the original structure was in the 10,12 position. The geometry of the diene was corroborated by comparison of retention times on four GC columns with those of synthetic standards of all four 10,12–16:Ald isomers. Only the *E*10,*Z*12–16:Ald isomer matched the insect-produced compound on all four columns.

A minor component of the extract (peak **3**, Figure 1) eluting shortly after the main component also elicited a strong EAD response from male antennae, seen as a shoulder on the main peak. The mass spectrum of this component was similar to that of the main component, *E*10,*Z*12–16:Ald, and these data, in conjunction with retention time matches with a standard on four columns, confirmed the minor component to be *E*10,*E*12–16:Ald.

Two later-eluting minor components (peaks **6** and **7**, Figure 1) eliciting medium intensity responses from male moth antennae were identified as *E*10,*Z*12–

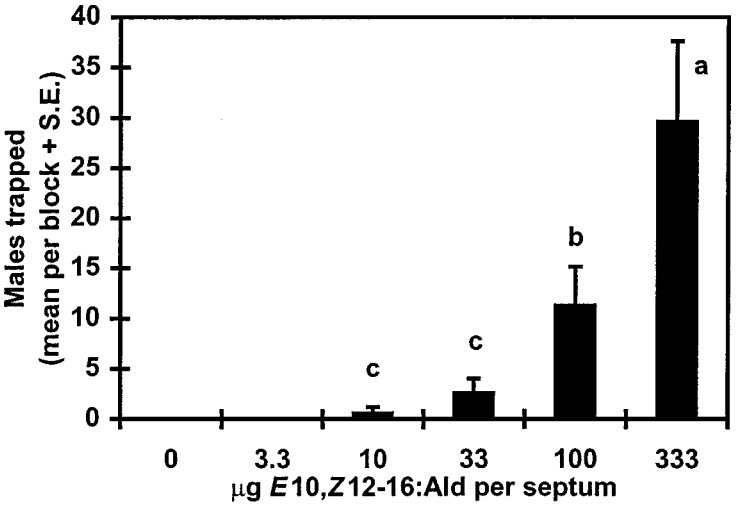


FIG. 3. Trap catches of male *Hemileuca maia* in field trials testing lures with various doses of *E*10,*Z*12–16:Ald, with *E*10,*Z*12–16:OH and *E*10,*Z*12–16:Ac held constant at 2.6 and 1.3 μg/septum, respectively. Trial conducted December 12–17, 1996; total number of moths trapped = 133. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16:Ald dose effect, $F = 34.72$, $df = 3, 6$, $P = 0.0003$; for block effect, $F = 11.91$, $df = 2, 6$, $P = 0.0082$.

and *E*10,*E*12–16:Ac, respectively. The mass spectra of both were similar, although only a partial spectrum of the later-eluting compound, present in smaller amount, was obtained. The mass spectrum of the more abundant compound was characterized by a molecular ion at *m*/*z* 280 (16%; possible molecular formula $C_{18}H_{32}O_2$), and a small but distinct fragment at *m*/*z* 220 (2%, loss of acetic acid). The relatively large size of the molecular ion, coupled with retention time somewhat longer than that of a hexadecyl acetate standard, supported the presence of a conjugated diene rather than two isolated double bonds. Comparison with a database spectrum of *E*10,*Z*12–16:Ac (NIST/EPA/NIH Mass Spectral Database, 1992) also resulted in a close match. The diene position was confirmed by GC-MS analysis of the MTAD adduct, with the adducts showing diagnostic fragments at *m*/*z* 350 (38%) and *m*/*z* 208 (100%) from cleavages of the alkyl groups attached to the 3 and 6 positions of the six-membered ring of the adduct, definitively placing the conjugated diene in the parent molecule at the 10,12 position. Determination of the double bond stereochemistries was made by comparison of retention times of the insect-produced compound with those of authentic standards on four GC columns as described above. Only the *E*10,*Z*12–16:Ac isomer matched the retention time of the insect-produced compound on all four columns.

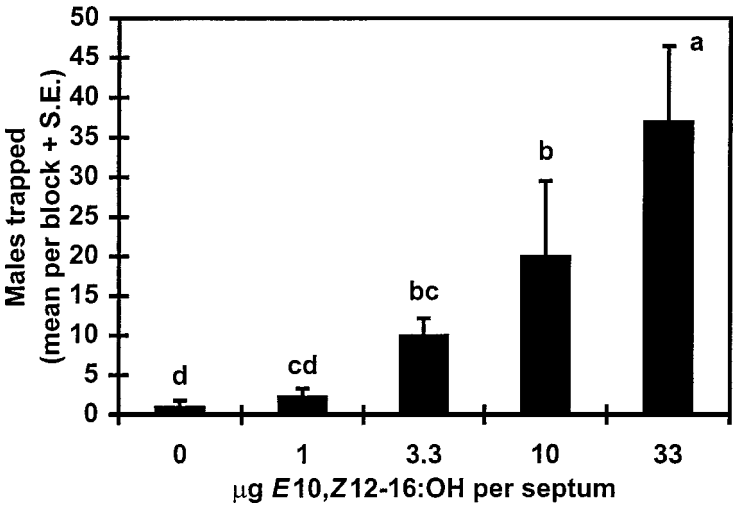


FIG. 4. Trap catches of male *Hemileuca maia* in field trials testing lures with various doses of *E*10,*Z*12–16:OH, with *E*10,*Z*12–16:Ald and *E*10,*Z*12–16:Ac held constant at 100 and 4 μg/septum, respectively. Trial conducted December 7–20, 1997; total number of moths trapped = 211. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16:OH dose effect, $F = 15.78$, $df = 4, 8$, $P = 0.0007$; for block effect, $F = 8.70$, $df = 2, 8$, $P = 0.0098$.

The partial mass spectrum of the less abundant of the two compounds (peak 6, Figure 1) did not exhibit a molecular ion or the weaker *m/z* 220 ion, but the fragmentation pattern in the lower mass ranges, and particularly the characteristic *m/z* 67, 81, 95 ion pattern, was similar to that of the *E*10,*Z*12–16:Ac. This partial mass spectrum, in conjunction with retention time matches on four columns with those of a standard, and the fact that an *E*10,*E*12–16:Ac standard elicits strong signals from male moth antennae, strongly supports the identification of this compound as *E*10,*E*12–16:Ac.

Two further minor components (peaks **4** and **5**, Figure 1) that elicited small responses from male antennae were tentatively identified as *E*10,*Z*12– and *E*10, *E*12–16:OH, respectively. Because of the trace amounts present in the extracts, mass spectra could not be obtained. Nevertheless, retention time matches with standards on several columns, elicitation of responses from male antennae with standards, and the fact that the corresponding aldehydes and acetates are present in the extracts renders these identifications virtually certain.

In addition to these six components, a number of other compounds were identified in extracts from pheromone glands from females, including hexadecanal (peak **1**, Figure 1), docosane, tricosane, tetracosane, and hexacosane, all

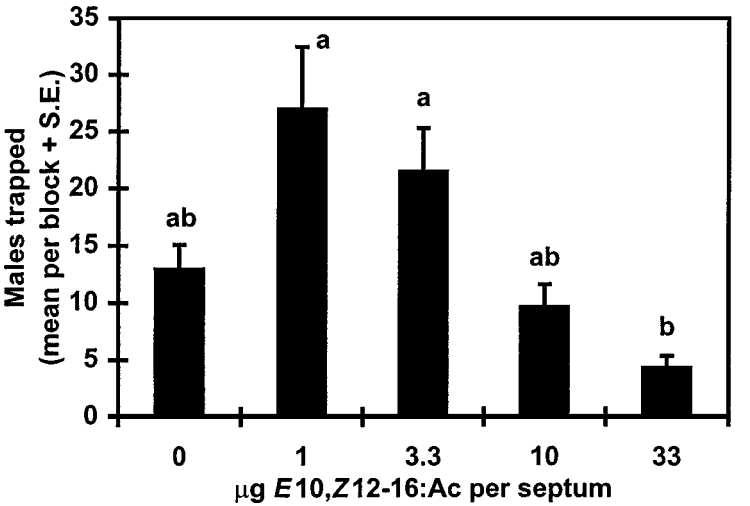


FIG. 5. Trap catches of male *Hemileuca maia* in field trials testing lures with various doses of *E*10,*Z*12–16:Ac, with *E*10,*Z*12–16:Ald and *E*10,*Z*12–16:OH held constant at 100 and 10 μg/septum, respectively. Trial conducted December 7–20, 1997, total number of moths trapped = 205. Treatments with different letters are significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for *E*10,*Z*12–16:Ac dose effect, $F = 6.44$, $df = 4, 7$, $P = 0.0169$; for block effect, $F = 0.86$, $df = 2, 7$, $P = 0.4649$. (Note: The 3.3 dose was lost or stolen from block 2 and could not be included in the analysis).

tentatively identified from mass spectra and mass spectral matches with database spectra. Identifications were confirmed by comparison of retention time and mass spectral data with those of the corresponding standards on multiple GC columns. In addition, hexadecanol and hexadecyl acetate were tentatively identified in extracts by retention time match-ups with standards.

There was considerable variability among extracts in the amounts of compounds present. For example, of 34 extracts from individual females, only four extracts contained measurable amounts of compounds, with two extracts containing about 20 ng of the major component, *E*10,*Z*12–16:Ald, and a further two extracts containing about 10 ng each. In these four extracts, the ratio of *E*10,*Z*12–16:Ald to *E*10,*Z*12–16:OH to *E*10,*Z*12–16:Ac was $100{:}7.4 \pm 3.3{:}6.3 \pm 1.7$. The quantities of hexadecanal and hexadecanol were estimated as $23 \pm 9\%$ and $11 \pm 10\%$ of the major component, respectively. The amounts of the other components were too low to be quantified with any degree of accuracy.

Male antennal responses were also quantified, both with extracts and with standards, with responses being expressed as a percentage of the response to *E*10,*Z*12–16:Ald. The quantities of *E*10,*Z*12–16:OH and *E*10,*Z*12–16:Ac in

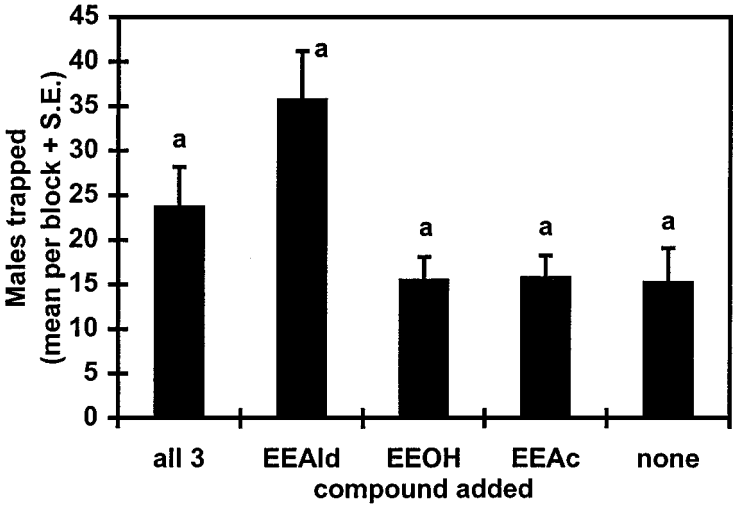


FIG. 6. Trap catches of male *Hemileuca maia* in field trials testing lures with a single dose of *E*10,*E*12–16:Ald, *E*10,*E*12–16:OH, and *E*10,*E*12–16:Ac, either as single components or as a combined mixture, at a dose of 10:2.5:1 μg/septum, respectively, added to the basic blend of *E*10,*Z*12–16:Ald, *E*10,*Z*12–16:OH, and *E*10,*Z*12–16:Ac (100:10:1 μg/septum, respectively). Trial conducted December 17–23, 1997; total number of moths trapped = 424. Treatments were not significantly different, despite a significant ANOVA (SNK, $\alpha = 0.05$). Two-way ANOVA for additive effect, $F = 3.63$, $df = 4, 12$, $P = 0.0369$; for block effect, $F = 1.51$, $df = 3, 12$, $P = 0.2619$.

extracts elicited responses of $17 \pm 7\%$ and $25 \pm 10\%$, respectively, when each of three male antennae were challenged with aliquots of four female extracts. Male antennae ($N = 5$), each challenged six times with mixtures of equal amounts of *E*10,*Z*12–16:Ald, *E*10,*Z*12–16:OH, and *E*10,*Z*12–16:Ac, gave mean responses of 100%, $41 \pm 11\%$, and $47 \pm 19\%$, respectively, indicating that the antennae were most highly tuned to *E*10,*Z*12–16:Ald.

Field Trials. The first field trial conducted in 1995 and repeated in 1996 tested the importance of individual components of a five-component blend mimicking the female blend (Figure 2). Four-component blends, in which either hexadecanol or hexadecanal had been dropped out, were as attractive as the full five-component mixture, indicating that these two components were not of significant importance to the attractiveness of the mixture. However, removal of *E*10,*Z*12–16:Ac from the blend resulted in a sharp drop in attraction of male moths, whereas blends missing either *E*10,*Z*12–16:Ald or *E*10,*Z*12–16:OH were completely unattractive, clearly indicating that these three compounds were critical components of the blends. The next field trial (Figure 3) tested blends of these three components,

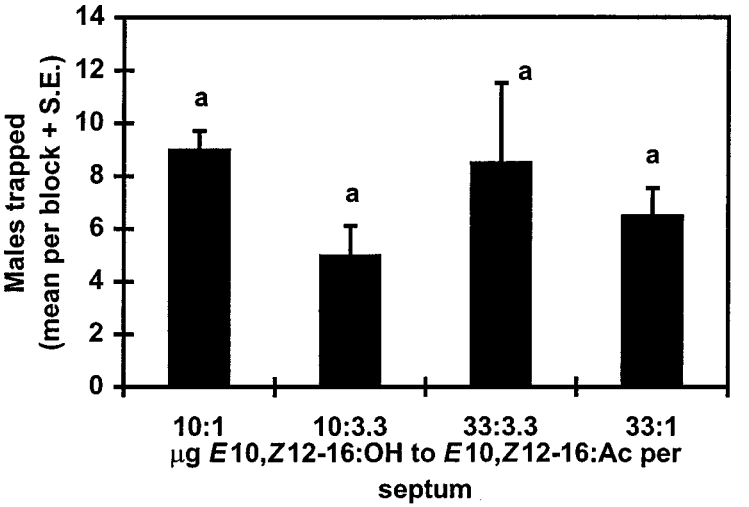


FIG. 7. Trap catches of male *Hemileuca maia* in field trials testing lures with varied amounts of different mixtures of $E10,Z12$–16:OH, and $E10,Z12$–16:Ac, with $E10,Z12$–16:Ald held constant at 100 μg/septum. Trial conducted December 17–23, 1997, total number of moths trapped = 113. Treatments were not significantly different (SNK, $\alpha = 0.05$). Two-way ANOVA for dose effect, $F = 1.06$, $df = 3, 9$, $P = 0.4115$; for block effect, $F = 1.67$, $df = 3, 9$, $P = 0.2426$.

with fixed amounts of $E10,Z12$–16:OH and $E10,Z12$–16:Ac (1.3 and 2.6 μg, respectively) and variable amounts of $E10,Z12$–16:Ald (0-333 μg). Trap captures increased with increasing amounts of $E10,Z12$–16:Ald, the major component of the pheromone. A third field trial (Figure 4) tested blends of the three components in which the amounts of $E10,Z12$–16:Ald and $E10,Z12$–16:Ac were held fixed at 100 and 4 μg, respectively, while the doses of $E10,Z12$–16:OH were varied from 0 to 33 μg (approximately 10 times higher ratio than found in gland extracts from females). Trap catches were directly proportional to the amount of $E10,Z12$–16:OH, with the highest dose used (33 μg) resulting in the largest trap catches. An analogous trial conducted the following year tested the effects of variable amounts of $E10,Z12$–16:Ac (0–33 μg), with fixed amounts of $E10,Z12$–16:Ald and $E10,Z12$–16:OH (100 and 10 μg/septum, respectively) . There were no significant differences in numbers of moths captured in traps baited with 0–10 μg of $E10,Z12$–16:Ac (Figure 5), whereas the highest dose of 33 μg resulted in significantly fewer moths caught than the optimal doses of 1 and 3.3 μg.

A further trial was conducted to determine the possible importance of the $E10$, $E12$–16:Ald, -OH, and -Ac isomers seen in the female gland extracts (Figure 6). Addition of any one of $E10,E12$–16:Ald, $E10,E12$–16:OH, or $E10,E12$–16:Ac,

or the mixture of all three (in ratios similar to those seen in pheromone gland extracts), to the three-component base mixture of *E*10,*Z*12–16:Ald, *E*10,*Z*12–16:OH, and *E*10,*Z*12–16:Ac (100:10:1) had no effect on the attractiveness of lures; all treatments attracted similar numbers of moths. A final trial tested different ratios of *E*10,*Z*12–16:OH to *E*10,*Z*12–16:Ac, with *E*10,*Z*12–16:Ald held constant at 100 μg/lure (Figure 7). There were no significant differences among treatments, indicating that the presence of the two components may be more important than their exact ratio, either in relation to each other or in relation to *E*10, *Z*12–16:Ald.

DISCUSSION

Pheromone components based on (*E*10,*Z*12)-hexadienyl compounds have now been identified in several species in the genus *Hemileuca* and two species in the sister genus *Coloradia* (McElfresh and Millar, 1999a–d; McElfresh et al., 2001). However, *E*10,*Z*12–16:Ald is the major component of the pheromone blend of *H. maia*, whereas *E*10,*Z*12–16:Ac constitutes the major component for the other species. As was found with the other species, a blend of the main component with at least one of the minor components (*E*10,*Z*12–16:OH) is crucial for attraction of male *H. maia*; blends must contain at least these two components to attract any male moths at all, and trap catches increased monotonically with increasing amounts of each of these compounds in blends, indicating their importance in the overall attractiveness. Surprisingly, the highest dose and ratio of *E*10,*Z*12–16:OH tested (33 μg, 33% of *E*10,*Z*12–16:Ald, the major component) was most attractive, even though this ratio is approximately 10 times the ratio found in the pheromone gland extracts.

The role of the third component, *E*10,*Z*12–16:Ac, is less clear. In one trial, (Figure 2), deletion of *E*10,*Z*12–16:Ac from the complete blend resulted in a significant decrease in attraction, whereas in a subsequent trial testing different ratios of *E*10,*Z*12–16:Ac to the other two components, lures containing no *E*10,*Z*12–16:Ac were as attractive as lures containing 1, 3.3, or 10 μg of *E*10,*Z*12–16:Ac. However, possible differences among treatments may have been obscured by the large variability in trap catches in that trial; lures containing 1 μg of *E*10,*Z*12–16:Ac caught almost twice as many moths as the lures without this compound, but the difference was not statistically significant. However, the highest dose of *E*10,*Z*12–16:Ac tested in this trial resulted in significantly fewer moths captured than the optimal doses (1–3.3 μg), clearly indicating that this compound can affect males' behavior.

Other minor components identified in female gland extracts, such as hexadecanal, hexadecanol, and the *E*10,*E*12–16:OH and -Ac isomers, do not appear to be crucial for the attraction of conspecific males; addition of these components in

biologically relevant amounts had no significant effect on trap captures. From the GC-EAD studies, it is clear that at least the latter two compounds are detected by male antennae, but their biological roles remain to be determined.

The possible role of *E*10,*E*12–16:Ald may warrant further investigation. Lures containing this compound, although not statistically different than the base blend without it, attracted more than twice as many male moths as the base blend. As in a previous trial, differences may have been obscured by the relatively small number of replicates and the relatively large standard errors. It is also clear that male moth antennae detect this compound because the EAD traces showed a distinct and reproducible peak (as a shoulder on the peak corresponding to the response to the main component, *E*10,*Z*12–16:Ald) in response to stimulation by this component of the pheromone gland extracts.

For practical purposes, the pheromone is already finding use in monitoring and control programs in those areas of the United States in which *H. maia* outbreaks result in nuisance and human health problems. For example, since 1997, the New Orleans Mosquito and Termite Control Board has used pheromone-baited traps to survey parks, oak-lined boulevards, and the city zoo area, with high trap catches alerting the Control Board to potential high larval populations the following spring.

Acknowledgments—We thank Darwin Reed for reference standards used in this study and Carol Adams for assistance with the statistical analyses. We also thank Stephen R. Sackett for assistance with field trials and placement of survey traps.

This manuscript is approved for publication by the Louisiana State Agricultural Experiment Station as manuscript # 01-17-0009.

REFERENCES

EARLE, N. W. 1966. Demonstration of a sex attractant in the buck moth, *Hemileuca maia* (Lepidoptera: Saturniidae). *Ann. Entomol. Soc. Am.* 60:277.

MARTINAT, P. J., SOLOMON, T. D., and LEININGER, T. D. 1997. Survivorship, development, and fecundity of buck moth (Lepidoptera: Saturniidae) on common tree species in the Gulf Coast urban forest. *J. Entomol. Sci.* 32:192–203.

MCELFRESH, J. S., and MILLAR, J. G. 1999a. Sex pheromone of the common sheep moth, *Hemileuca eglanterina*, from the San Gabriel Mountains of California. *J. Chem. Ecol.* 25:687–709.

MCELFRESH, J. S., and MILLAR, J. G. 1999b. Sex pheromone of Nuttall's sheep moth, *Hemileuca nuttalli*, from the eastern Sierra Nevada Mountains of California. *J. Chem. Ecol.* 25:711–726.

MCELFRESH, J. S., and MILLAR, J. G. 1999c. Sex pheromone of saturniid moth, *Coloradia velda*. *J. Chem. Ecol.* 25:1067–1078.

MCELFRESH, J. S., and MILLAR, J. G. 1999d. Geographic variation in sex pheromone blend of *Hemileuca electra* from southern California. *J. Chem. Ecol.* 25:2505–2525.

MCELFRESH, J. S., CHEN, X., ROSS, D. W., and MILLAR, J. G. 2001. Sex pheromone blend of the Pandora moth *Coloradia* (Lepidoptera: Saturniidae), an outbreak pest in pine forests (Pinaceae). *Can. Entomol.* 132:1–13.

NIST/EPA/NIH MASS SPECTRAL DATABASE. 1992. Licensed to Hewlett-Packard Co., Palo Alto California.

SAS Institute. 1996. SAS System for Personal Computers, Release 6.12. SAS Institute Inc., Cary, North Carolina.

TUSKES, P. M., TUTTLE, J. P., and COLLINS, M. M. 1996. The Wild Silk Moths of North America: A Natural History of the Saturniidae of the United States and Canada. Cornell University Press, Ithaca, New York.

YOUNG, D. C., VOUROS, P., and HOLICK, M. F. 1990. Gas chromatography–mass spectrometry of conjugated dienes by derivatization with 4-methyl-1,2,4–triazoline-3,5-dione. *J. Chromatogr.* 522:295–302.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

THE NATURE OF THE GREGARIZING SIGNAL RESPONSIBLE FOR MATERNAL TRANSFER OF PHASE TO THE OFFSPRING IN THE DESERT LOCUST *Schistocerca gregaria*

ATEM G. MALUAL,[1] AHMED HASSANALI,[1,*] BALDWYN TORTO,[1] YOUSIF O. H. ASSAD,[2] and PETER G. N. NJAGI[1]

[1]*International Centre of Insect Physiology and Ecology*
P.O. Box 30772, Nairobi, Kenya

[2]*University of Gezira*
P.O. Box 20, Wad Medani, Sudan

(Received December 1, 2000; accepted March 4, 2001)

Abstract—We examined aggregative behavior of hatchlings of the desert locust *Schistocerca gregaria* emerging from solitaria egg pods that had been incubated in sand previously used for consecutive ovipositions by gregarious females. Hatchlings derived from such eggs were significantly more gregarious than those derived from pods laid in clean sand. The gregarizing effect of the sand-associated factor originating from 3, 5, or 10 ovipositions by gregarious females increased in a dose-dependent fashion. Washing the sand with organic solvents following such ovipositions, or flushing it with nitrogen gas, led to substantial loss of its gregarizing effect, showing that the active signal is volatile and of medium polarity. The gregarizing activity of the exposed sand correlated with the presence of C-8 unsaturated ketones, (*Z*)-6-octen-3-one and (*E*,*E*)-3,5-octadiene-2-one and its *E*,*Z* isomer, previously shown by us to form part of the releaser pheromone system that mediates group oviposition in *S. gregaria*. These ketones were present in relatively large amounts in the eggs obtained from egg pods of gregarious females and were also detected in the extracts of accessory glands of gregarious females, a candidate source of the gregarization factor. It is proposed that the pheromone is secreted at the onset of oviposition. This would account for its distribution predominantly within the eggs and surrounding sand at the site of oviposition. The study sheds new light on the pheromonal mechanism associated with transgenerational transmission of gregarious characters in crowded *S. gregaria* populations and provides yet another case in this insect of dual releaser and primer roles played by the same pheromone blend.

* To whom correspondence should be addressed.

0098-0331/01/0700-1423$19.50/0

Key Words—*Schistocerca gregaria*, gregarization, oviposition, pheromone parsimony, primer pheromone, 3,5-octadien-2-ones, 6-octen-2-one.

INTRODUCTION

Locusts are characterized by a pronounced ability to transform reversibly between two extreme phases, solitaria and gregaria, that differ in physiology, biochemistry, behavior, and morphology (Uvarov, 1966; Pener and Yerushalmi, 1998; Applebaum et al., 1997). Phase change is predicated on locust density, and crowding or uncrowding can lead to rapid (hours or days) changes in some of the phase-related traits, such as aggregation behavior (Roessingh and Simpson, 1994) and the production of mediating pheromones (Deng et al., 1996). Gregarious characters may spread horizontally through recruitment of solitary individuals into gregarizing groups (Njagi et al., 1996) and accrue across generations through the eggs (Pener, 1991; Islam et al., 1994a,b; Bouaïchi et al., 1995). Egg pods laid by gregarious females produce gregarious hatchlings, and those laid by solitary females produce solitary hatchlings (Pener, 1991; Islam et al., 1994a,b).

Recent studies on the desert locust, *Schistocerca gregaria*, have tried to elucidate the pheromonal mechanisms that underlie transgenerational transmission of gregarious characters. Two sets of pheromonal effects have been shown to operate. One is mediated by volatile signals that promote group oviposition behavior, thus ensuring spatial cohesiveness of the offspring. These include benzene derivatives, acetophenone and veratrole, associated with egg-froth (Saini et al., 1995; Rai et al., 1997), unsaturated ketones [(*Z*)-6-octene-2-one and (*E*,*E*)-3,5-octadien-2-one and its *E*,*Z*-isomer] secreted into the sand during oviposition (Torto et al., 1999), and a saturated aldehyde emitted by ovipositing females that attract other gravid females (Njagi et al., unpublished data). Since oviposition is generally a mixed-sex activity, the male-produced adult aggregation pheromone may also play a role (Saini et al., 1995).

The second pheromonal effect is mediated by a primer gregarizing factor associated with gregarious and gregarizing gravid females, including those that experience crowding at mating or oviposition (Islam et al., 1994a,b; Bouaïchi et al., 1995). McCaffery et al. (1998) considered the soil previously used by gregarious ovipositing females and foam plugs of egg pods as possible sources of the primer pheromone. A soil-borne factor was ruled out since egg pods of solitary females laid into sand previously used for oviposition by gregarious females gave hatchlings that were not different from solitary controls. On the other hand, there was significant evidence of gregarization in hatchlings derived from solitary eggs incubated with froth from egg pods laid by gregarious females. Saline aqueous extracts of the froth were similarly effective in inducing shifts towards the gregarious phase of hatchlings from solitary egg pods. Ultra-filtration at 3 kDa gave a filtrate that

essentially retained the activity of the original extract. The authors then concluded that the pheromone was a polar substance of low molecular weight (<3 kDa), released by the egg pod.

In our studies of soil-borne oviposition signals, the relevant compounds (C-8 unsaturated aliphatic ketones) were found to adhere strongly to dry soil and could only be desorbed under relatively high humidities (Torto et al., 1999). Interestingly, these compounds were also found in the eggs obtained from egg pods of gregarious females. This and the parsimonious functions of some of the desert locust pheromones as releaser and primer signals (Mahamat et al., 1993; Assad et al., 1997; Hassanali and Torto, 1999) led us to hypothesize that the ketones associated with soil used by gregarious females for oviposition might also function as the primer gregarization signal. The present study was undertaken to test this hypothesis. Since accessory glands of gregarious gravid females were recently implicated in the production, release, or activation of the primer factor (Hägele et al., 2000), we also examined extracts of the glands for the presence of the ketones.

METHODS AND MATERIALS

Insects

Desert locusts were reared under crowded (gregarious) or isolated (solitary) conditions as described by Ochieng-Odero et al. (1994). The stocks originated from egg pods supplied by the Desert Locust Control Organisation for Eastern Africa (DLCO-EA), Addis Ababa, Ethiopia, and have been periodically supplemented with collections from ICIPE Field station near Port Sudan. The insects were fed on fresh wheat seedlings, *Triticum* spp., and wheat bran.

Solitary-reared locusts had six nymphal instars, largely green in coloration; the sixth instar had an extra eye stripe. Adults of both sexes were greyish brown and had F/C ratios (length of posterior femur/maximum width of the head) >3.75, typical of solitary adults (Ochieng-Odero et al., 1994). Crowd-reared insects had five nymphal stages. Hoppers had black color patterns on a yellow background; immature adults were pink, while mature adults were bright yellow (males) or pale yellow on the head and pronotum (females). The F/C ratios of adults were <3.15, characteristic of gregarious locusts (Ochieng-Odero et al., 1994). In addition to morphometric and pigmentary differences, the two cultures differed markedly in pheromonal characteristics (Njagi et al., 1996; Torto et al., 1994, 1996).

Effect of Incubating Solitaria Egg Pods in Sand Exposed to Oviposition by Gregarious Females on Aggregation Behavior of Hatchlings

Preparation of Sand. Sand sieved through a wire mesh (2 mm^2 mesh size) was washed sequentially with hot water, methanol, acetone and dicholoromethane to

remove inorganic and organic contaminants and heat-sterilized at 205°C for 24 hr. After cooling to room temperature, it was moistened with distilled water to give a moisture content of ~15% (15 ml water to 100 g of sand) (Norris, 1968). The purity of the sand with respect to volatile contaminants was checked by aeration through activated charcoal and analyzing any trapped material by gas chromatography as described below. Standard aluminum oviposition cups (10 × 4 cm ID) were filled with the moistened sand and placed at the front of the false floor of standard aluminum locust rearing cages (50 × 50 × 50 cm) containing gregarious gravid females (Saini et al., 1995). After 24 hr, deposited egg pods were removed, the sand, thus exposed, was allowed to dry overnight in an aluminum tray in a temperature-controlled (30 ± 2°C) room. The sand was then sifted through a wire mesh (2 mm^2) to remove any remnants of debris of eggs and froth, and its moisture content restored to 15% by adding the appropriate amount of water.

Experimental Treatments of Solitaria Egg Pods. Egg pods from solitary females were transferred into oviposition cups with successively exposed sand prepared as described above or further treated, as outlined below: (i) exposed sand batches with 15% moisture in which gregarious females had previously laid successively 3, 5, or 10 egg pods, respectively; (ii) exposed sand batches as in (i) that were sequentially washed with methanol, acetone, and dichrolomethane, then spread out on a metal tray and left to dry at 30 ± 2°C for 24 hr, and remoistened to 15%; and (iii) exposed sand batches as in (i) that were flushed with gaseous nitrogen for 24 hr with the sand spread out thin in a glass chamber (24 cm long × 5 cm ID) to facilitate elution of volatile compounds, and then remoistened to 15%.

The following treatments served as controls: (iv) egg pods laid by solitary females in clean (unexposed), moistened sand; and (v) egg pods laid by gregarious females also in clean, moistened sand.

The egg pods thus prepared were then separately incubated at 30°C and 60–70% relative humidity. After seven days, they were checked repeatedly for hatching, and additional water was added to restore the moisture level in the sand, until hatching (12–15 days).

Behavioral Assays. Aggregation bioassays were conducted in a uniform ring-shaped aluminum arena with the floor divided into 18 equal segments (Ellis, 1953; Weisel et al., 1996). The diameter of the inner wall was 14.5 cm, the outer wall 29.0 cm, and the height 10 cm. The arena was covered by a glass plate and placed under an exhaust hood. The hood was fitted with two 60-cm diffused-light tubes (60 W) 40 cm above the glass plate to provide uniform illumination at the arena. The temperature of the room was maintained at 30 ± 2°C.

On hatching from oviposition cups, 12 neonate nymphs (<12 hr old) were released randomly into the bioassay arena and observed for 20 min. At the end of this period, the distribution of the insects (number of divisions that contained 0,1, 2, and ≥3 nymphs) was recorded. [Since groups of 4 and 5 in a given division on the arena were relatively rare, even for hatchlings that had emerged from crowd-reared

insects (see also Weisel et al., 1996), these were grouped into the ≥ 3 category]. The insects were then disturbed by tapping the glass top of the cage several times, after which they were allowed to settle before the next recording 20 min later. The procedure was repeated for 2 hr, which resulted in six readings per replicate. Each treatment was replicated five times, between which the arena was cleaned with detergent, rinsed with water and acetone, and dried in an oven at 100°C for 15 min. The level of aggregation was expressed in terms of the proportions of single insects and those that were in groups of ≥ 2 or ≥ 3. These were compared with a random theoretical distribution calculated from Poisson function (Sokal and Rohlf, 1981).

Analyses of Treated Sand and Eggs

Sands associated with the following three treatments were analyzed for the presence of volatiles (candidate primer pheromone): (i) cleaned, sterilized, and moistened sand (untreated control); (ii) sand in which gregarious locusts had oviposited for five successive times and moistened; and (iii) as in (ii), but flushed with nitrogen for 24 hr, and remoistened. Batches of sand from these treatments were put in glass chambers (10 cm long $\times$ 3.5 cm ID) and aerated as previously described (Torto et al., 1999). A charcoal-filtered airstream (106 ml/min) from a compressed air cylinder was passed over the sand for 24 hr in a room maintained at 30 ± 2°C. The effluent passed through activated charcoal (60 mg) contained in Pyrex glass tubes. The trapped volatiles were eluted with pure dicholomethane (2 ml, Aldrich Ltd., HPLC grade), each set pooled together, and stored at -15°C until analyzed. In addition, volatiles were also collected from eggs derived from five $\sim$1-day old egg pods that had been deposited in treatment (ii) above. The eggs were crushed in sterilized sand, moistened with water (to 15%), and volatiles collected as above.

For analyses, the volatiles were concentrated under a stream of nitrogen at 0°C to $\sim$100 μl. Five microliters of the extracts were analyzed by gas chromatography on a Hewlett-Packard (HP) 5890 Series II instrument equipped with a flame ionization detector (FID) and a HP capillary column (Carbowax 20 M, 50 m $\times$ 0.2 mm ID $\times$ 0.2 μm with nitrogen as the carrier at a flow rate of 0.35 ml/min (Torto et al., 1999). The oven was programmed as follows: 60°C (10 min) to 180°C at 5°C/min, 180°C (5 min) to 220°C at 10°C/min. Peaks were integrated by using a HP 3396 Series II integrator.

Analysis of Accessory Glands

Accessory glands (located at the anterior ends of the ovaries) were removed from crowd-reared (gregarious) gravid adult female locusts (40) and transferred into a vial containing saturated sodium chloride solution (10 ml). The suspension

was sonicated for 2 hr, dichloromethane or hexane (20 ml) added, and the mixture thoroughly shaken. The aqueous and organic layers were allowed to separate (2–3 min) and the organic layer collected. This was shaken with aqueous sodium hydroxide (20 ml, 5%) to remove the large amounts of C_1–C_{16} fatty acids present. The layers were allowed to separate (2–3 min), the organic phase collected and dried by passing through anhydrous sodium sulfate and then concentrated under a stream of nitrogen at 0°C to ~200 μl. Samples of the extracts (10 μl) were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph and by GC-MS on a Fisons Instruments 8060 Series II chromatograph coupled to a Fisons Instruments VG Platform MS (EI, 70eV), employing the same chromatographic conditions as described above. A synthetic mixture of (*E*,*Z*) and (*E*,*E*)-3,5-octadien-2-one (donated by Professor T. C. Baker, Iowa State University, Ames, Iowa) allowed the confirmation of the identities of these ketones in the extracts of the accessory glands and contaminated sand.

Data Analyses

The behavioral data were subjected to analysis of variance (SAS Institute Inc.; Cary, North Carolina). Means were compared by the least significance (LSD) test.

RESULTS

Aggregation Assays. Aggregative behavior of newly hatched first-instar nymphs was influenced by the mother's phase state and by the type of sand surrounding the eggs. As expected, solitary mothers produced nonaggregative nymphs and gregarious mothers produced gregarious nymphs (Figure 1A, i and v). However, hatchlings derived from egg pods placed in sand contaminated by substances associated with gregarious egg pods were more gregarious (Figure 1A, ii–iv). On the other hand, when sands from gregarious pods were washed with organic solvents or flushed with streams of nitrogen, they lost their gregarizing activities (Figure 1B, ii–iv, and 1C, ii–iv, respectively).

The aggregation behavior of hatchlings from all treatments compared with those of solitary and gregarious controls is summarized in Figure 1D, where the proportion of single insects on the arena in different treatments is plotted against the proportion in groups of 2, 3, and higher. Pairs of insects were also included in the latter category in this plot, since solitary locusts avoid each other, and pairing constitutes an expression of grouping behavior (Ellis, 1963; Roessingh et al., 1993). The gregarizing effect of sand-associated factors originating from successive ovipositions by gregarious females is seen clearly to increase in a dose dependent fashion. Washing the sand with organic solvents or flushing it with nitrogen leads to loss or substantial decrease in the activity associated with the sand.

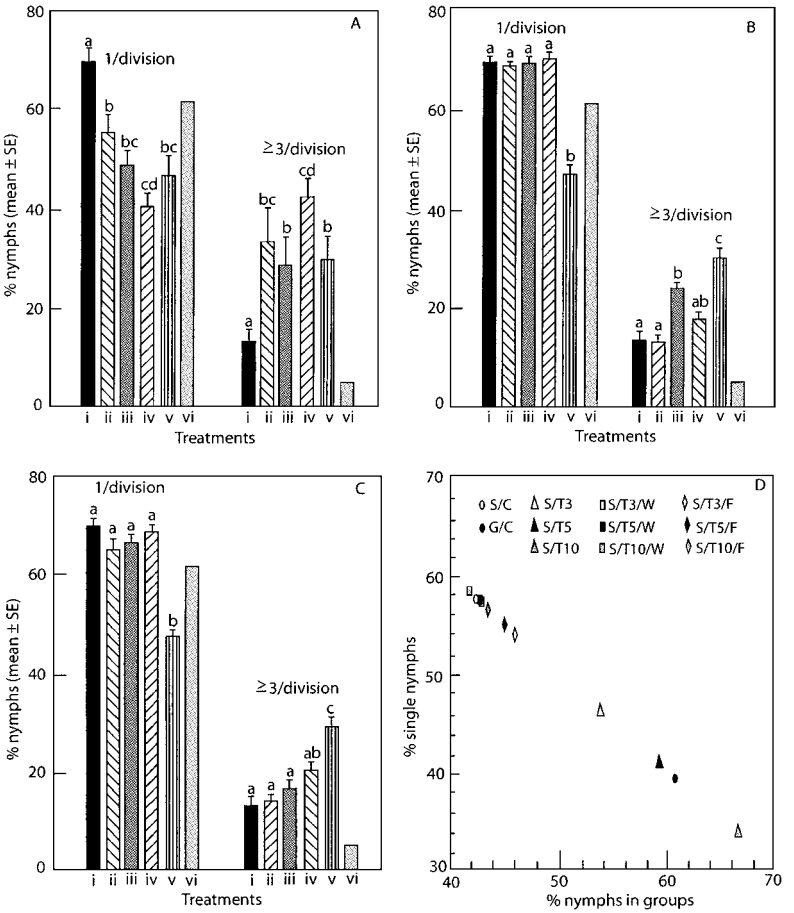


FIG. 1. Aggregation tendency of *S. gregaria* hatchlings from egg pods treated as follows: A, B, C i and v, untreated *solitaria* and *gregaria*, respectively; A ii, iii, and iv, solitaria egg pods incubated in sand exposed to 3,5, and 10 ovipositions by gregarious females, respectively; B ii, iii, and iv, solitaria egg pods incubated in sand exposed to 3, 5, and 10 ovipositions and then washed with organic solvents; C ii, iii, and iv, solitaria egg pods incubated in sand exposed to 3, 5, and 10 ovipositions and then flushed with nitrogen; A, B, C vi, represent values from Poisson function. D summarizes the results from all treatments: *solitaria* (S/C) and *gregaria* (G/C); S/T3, S/T5, and S/T10 represent hatchlings from *solitaria* egg pods exposed to sand contaminated by 3, 5, and 10 ovipositions, respectively; S/T3/W, S/T5/W, S/T10/W represent insects from *solitaria* egg pod from sand similarly contaminated but subsequently washed with solvents; and S/T3/F, S/T5/F, and S/T10/F represent insects from *solitaria* egg pods from contaminated sand but subsequently flushed with nitrogen. $N = 30$ for all data sets; bars in A, B, and C with the same letter are not significantly different at $P < 0.05$ (LSD test).

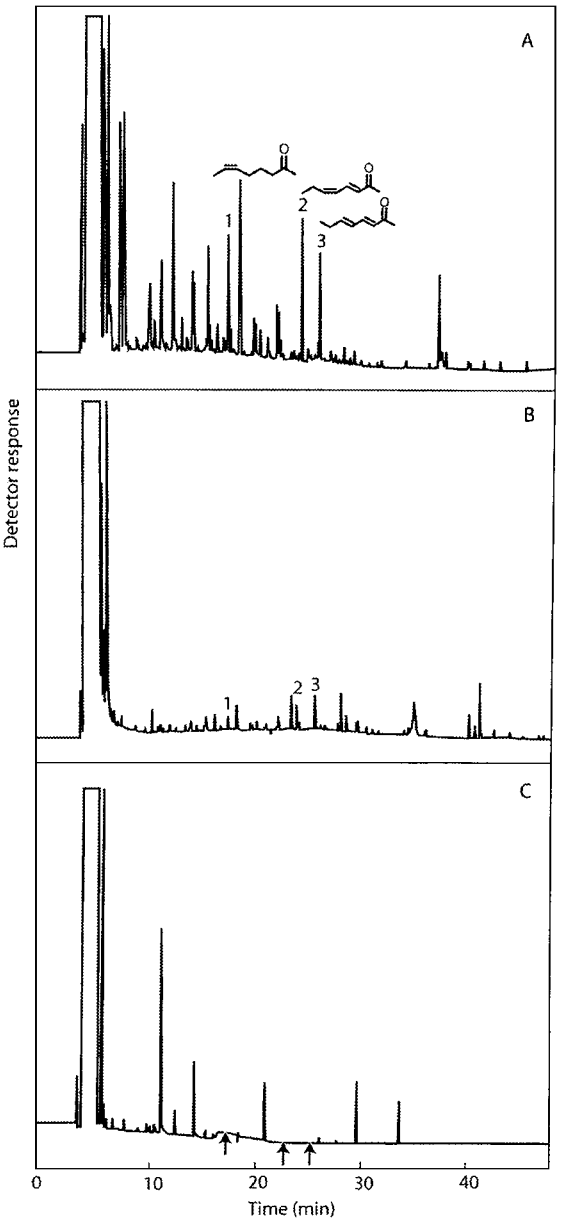


FIG. 2. Gas chromatograms of volatiles collected from (A) eggs from five egg pods crushed in clean sand, (B) sand into which five successive ovipositions by gregarious females had taken place, and (C) as (B) but after the sand had been flushed with nitrogen for 24 hr.

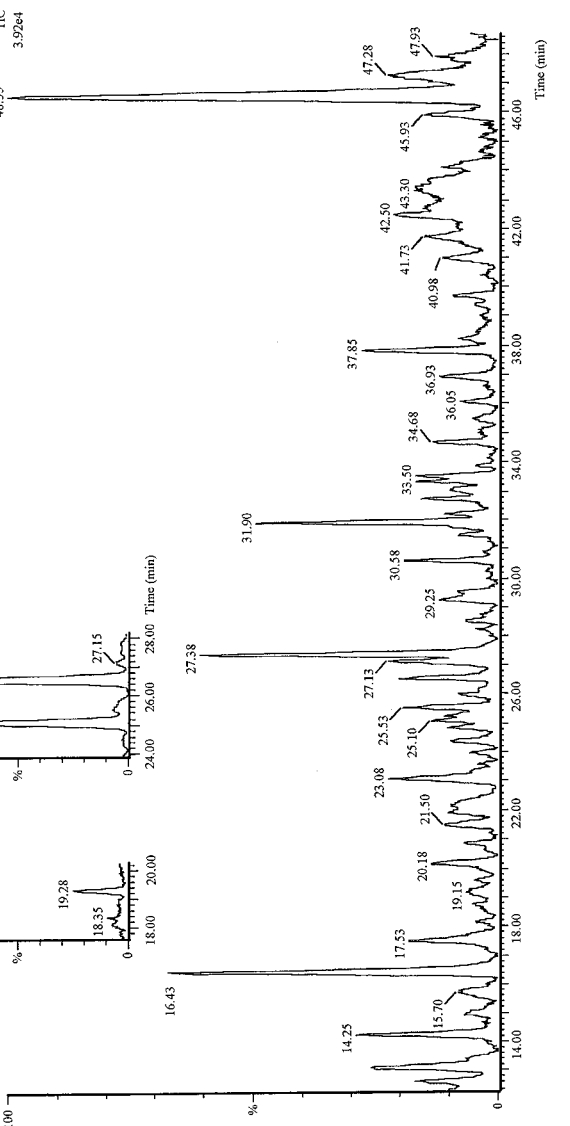


FIG. 3. Total ion MS chromatogram of an extract of accessory glands of *S. gregaria* females showing the presence of (*Z*)-6-octen-2-one ($R_t = 19.28$ min), (*E*,*Z*)-3,5-octadien-2-one ($R_t = 25.10$ min), and (*E*,*E*)-3,5-octadien-2-one ($R_t = 26.55$ min). Insets show single ion monitoring at m/z corresponding to the molecular ions of the compounds.

Analyses of Sand Batches and Eggs. Crushed eggs and sand in which five egg pods had been laid produced (*Z*)-6-octen-2-one and (*E*,*E*)-3,5-octadiene-2-one and its *E*,*Z* geometric isomer (Figure 2A, B). In contrast, these compounds were absent from sand aerated with nitrogen for 24 hr (Figure 2C).

Analysis of Accessory Gland Extracts. Figure 3 gives the total ion GC-MS chromatogram of the nonacidic fraction of accessory gland extracts. (*E*,*E*)-3,5-Octadiene-2-one and its *E*,*Z* geometrical isomer were relatively prominent, with mass spectra comparable to library data (NIST Registry, 1995) and to those of synthetic compounds. *E*,*Z*-isomer: *m*/*z* (%) at 95(100), 43(70), 81(55), 79(45), 124(34), 41(32), 53(28), 39(25), 109(15); *E*,*E*-isomer: 95(100), 81(70), 79(58), 124(50), 53(33), 43(32), 109(22), 65(18), 77(15). (*Z*)-6-Octene-2-one was present in lesser amounts and merged with another minor component. It was detected by single-ion monitoring at *m*/*z* 126, 108, 97, 58, 69, 55 and 43.

DISCUSSION

In a recent communication, we reported that crowd-reared *S. gregaria* oviposited preferentially in sand previously laid in by conspecifics relative to clean sand (Torto et al., 1999). Volatiles collected from sand previously exposed to gregarious egg pods also elicited a similar preference. Female responses toward batches of sand successively laid in by gravid females increased in a dose-dependent manner and correlated with the amounts of trapped volatiles from such batches. GC-EAD and GC-MS studies of the volatiles led to the identification of three EAG-active C_8 ketones, (*Z*)-6-octen-3-one and (*E*,*E*)-3,5-octadiene-2-one and its *E*,*Z* geometric isomer.

The results of the present study show that the aggregative behavior of hatchlings from solitary egg pods that were incubated in sand previously laid in by gregarious females represents a significant shift towards the gregarious phase (Figure 1A). This shift increases in a dose-dependent manner, as evidenced by increasing grouping behavior of hatchlings derived from egg pods incubated in sand batches that had been exposed to 3, 5, and 10 ovipositions, respectively, by gregarious females (Figure 1D). Washing the exposed sand with organic solvents or flushing it with nitrogen leads to loss of its gregarizing effect (Figure 1B,C), consistent with the relative polarity and volatility of the ketones. The presence of these compounds in relatively large amounts in the eggs of gregarious females (Figure 2) suggests that they serve an important function there. Moreover, their detection in the extracts of the accessory glands (Figure 3) is consistent with a recent study by Hägele et al. (2000) that demonstrated that the gregarizing factor probably originates from these glands.

How do we reconcile these findings with those of McCaffery et al. (1998), who found no significant activity in sand that had been exposed to oviposition by

gregarious locusts and who identified foam plugs as the source of the gregarizing factor in the egg pods? First, in our studies we have found that trapping by aeration of the ketones from exposed sand was dependent on the sand's moisture level; at low humidity, the ketones adhered strongly to sand (Torto et al., 1999). Thus, the failure by McCaffery et al. (1998) to observe any significant shift to the gregarious phase in hatchlings from solitaria egg pods that were incubated in exposed sand may have been due to insufficient moisture content of the sand used by these workers. Second, our analyses have shown substantially less ketones in froth plugs than in sand into which the gregaria oviposited (Torto et al., 1999). This is consistent with Szopa's (1981) finding that the accessory glands are not the primary source of the froth material, which appears to be produced mainly by the genital ducts (egg *calyces* and lateral oviducts). The ketone blend is probably secreted at the onset of oviposition, which would account for their relatively high titers in the eggs. The froth forming the plug likely picks up residual compounds, as it is later exuded from the oviduct.

The results of our experiments strongly implicate the C-8 ketones, previously identified as part of the releaser oviposition pheromone system of gregarious-phase desert locusts, as the principal primer signal responsible for maternal transfer of gregarious character and for predisposing solitaria eggs to produce gregarious hatchlings. However, other minor volatile components associated with sand and eggs may also contribute to the signal. The use of this relatively volatile set of compounds secreted during oviposition by crowded locusts would appear to be appropriate for its parsimonious functions. It can form an effective component of the oviposition attractant system of gregarious locusts, thus enhancing group oviposition and spatial cohesiveness of the resulting offspring (Saini et al., 1995; Rai et al., 1997; Torto et al., 1999). Since solitaria gravid females are also strongly attracted, the system also serves to recruit this phase of the insect into gregarizing groups, important during the early stages of phase transformation (Bashir et al., 2000). As a primer signal, we propose that the volatile nature of these substances would allow them to diffuse between the eggs and the surrounding soil and mix with similar vapors from neighboring oviposition sources to create a relatively homogeneous stimulus cloud at the communal oviposition site. This would promote a more uniform phase shift of the developing eggs at the site, the extent of shift being dependent on stimulus intensity. Further studies in the field and under controlled conditions are needed to explore the validity of these speculations.

In summary, the present study provides evidence of yet another case of pheromone parsimony in the desert locust. The blend of C-8 unsaturated ketones, probably produced in the accessory glands and secreted at onset of oviposition by crowded females, found earlier to be part of the oviposition attracting pheromone systems of the desert locust, is a strong candidate for the signal associated with maternal transfer of gregarious characters to the progeny. In our earlier studies, we showed that the nymphal and adult aggregation pheromones, respectively,

sequentially inhibit and accelerate the maturation of young gregarizing or gregarious adults, resulting in a more synchronous development of the insects (Richards and El Mangoury, 1968; Mahamat et al., 1993; Assad et al., 1997). The mechanisms by which these pheromones exert their primer effects remain to be elucidated. Studies with synthetic C-8 ketones of both the releaser and primer effects are in progress.

Acknowledgments—This work was supported by funds from IFAD (International Fund for Agricultural Development) and ADB (African Development Bank), to whom we are most grateful. A.G.M. is grateful to the African Regional Postgraduate Programme in Insect Science (ARPPIS) managed by ICIPE for an award of a fellowship.

REFERENCES

APPLEBAUM, S. W., AVISAR, E., and HEIFITZ, Y. 1997. Juvenile hormone and locust phase. *Arch. Insect Biochem. Physiol.* 35:395–391.

ASSAD, Y. O. H., HASSANALI, A., TORTO, B., MAHAMAT, H., BASHIR, N. H. H., and EL BASHIR, S. 1997. Effects of fifth instar volatiles on the sexual maturation of adult desert locust *Schistocerca gregaria* (Forskal) (Orthoptera:Acrididae). *J. Chem. Ecol.* 23:1373–1388.

BASHIR, M. O., HASSANALI, A., RAI, M. M., and SAINI, R. K. 2000. Changing oviposition preferences of the desert locust, *Schistocerca gregaria*, suggest a strong species predisposition for gregarization. *J. Chem. Ecol.* 26:1721–1733.

BOUAÏCHI, A., ROESSINGH, P., and SIMPSON, S. J. 1995. Analysis of the behavioral effects of crowding and re-isolation on solitary-reared adult desert locusts (*Schistocerca gregaria*) and their offspring. *Physical Entomol.* 20:199–208.

DENG, A. L., TORTO, B., HASSANALI, A., and ALI, E. E. 1996. Effects of shifting to crowded or solitary conditions on pheromone release and morphometrics of the desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera:Acrididae). *J. Insect Physiol.* 42:771–776.

ELLIS, P. E. 1953. Social aggregation and gregarious behavior in hoppers of *Locusta migratoria migratorioides* (R&F). *Behavior* 5:17–258.

ELLIS, P. E. 1963. Changes in the social aggregation of locust hoppers with changes in rearing conditions. *Anim. Behav.* 11:152–160.

HÄGELE, B. F., OAG, V., BOUAÏCHI, A., MCCAFFERY, A. R., and SIMPSON, S. J. 2000. The role of female accessory glands in maternal inheritance of phase in the desert locust *Schistocerca gregaria. J. Insect Physiol.* 46:275–280.

HASSANALI A., and TORTO B. 1999. Grasshoppers and locusts, pp. 305–328, *in* J. HARDIE, and A. K. Minks (eds.). Pheromones of Non-lepidopteran Insects Associated with Agricultural Plants. CABI Publishing, Wallingford, Oxon, UK.

ISLAM, M. S., ROESSINGH, P., SIMPSON, S. J., and MCCAFFERY, A. R. 1994a. Parental effects on the behavior and colouration of nymphs of the desert locust *Schistocerca gregaria. J. Insect Physiol.* 40:173–181.

ISLAM, M. S., ROESSINGH, P., SIMPSON, S. J., and MCCAFFERY, A. R. 1994b. Effects of population density experienced by parents during mating and oviposition on the phase of hatching desert locusts, *Schistocerca gregaria*. *Proc. R. Soc. B* 257:93–98.

MAHAMAT, H., HASSANALI, A., ODONGO, H., TORTO, B., and EL BASHIR, S. 1993. Studies on the maturation-accelerating pheromone of the desert locust, *S. gregaria* (Orthoptera:Acrididae). *Chemoecology* 4:159–164.

McCaffery, A. R., Simpson, S. J., Saiful, Islam, M., and Roessingh, P. 1998. A gregarising factor present in the egg pod foam of the desert locust *Schistocerca gregaria. J. Exp. Biol.* 201:347–363.

Njagi, P. G. N., Torto, B., Obeng-Ofori, D., and Hassanali, A. 1996. Phase-independent responses to phase-specific aggregation pheromone in adult desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera:Acrididae). *Physiol. Entomol.* 21:131–137.

Norris, M. J. 1968. Laboratory experiments on oviposition responses of the desert locust, *Schistocerca gregaria* (Forskål). *Anti-Locust Bull.* 43:1–47.

Ochieng-Odero, J. P. R., Ndugo, S. M., El Bashir, S., and Capstick, P. B. 1994. A method of rearing crowded (gregarious) and isolated (solitary) locusts (Orthoptera: Acrididae) in the laboratory, pp. 33–44, *in* P. G. N. Njagi, and M. F. B. Chaudhury (eds.). Proceedings of Workshop on Effective Networking of Research and Development on Environmentally Sustainable Locust Control Methods among Locust Affected Countries. ICIPE Science Press, Nairobi, Kenya.

Pener, M. P. 1991. Locust phase polymorphism and its endocrine relations. *Adv. Insect Physiol.* 23:1–79.

Pener, M. P., and Yerushalmi, Y. 1998. The physiology of locust phase polymorphism: An update. *J. Insect Physiol.* 44:365–377.

Rai, M. M., Hassanali, A., Saini, R. K. Odongo, H., and Kahoro, H. 1997. Identification of components of the oviposition aggregation pheromone of the gregarious desert locust *Schistocerca gregaria. J. Insect Physiol.* 43:83–97.

Richards, M. J., and El Mangoury, M. A. 1968. Further experiments on the effects of social factors on the rate of sexual maturation in the desert locust. *Nature* 219:865–866.

Roessingh, P., and Simpson, S. J. 1994. The time-course of behavioral phase change in nymphs of the desert locust, *Schistocerca gregaria. Physiol. Entomol.* 19:191–197.

Roessingh, P., Simpson, S. J., and James, S. 1993. Analysis of phase-related changed in the behaviour of desert locust nymphs. *Proc. R. Soc. B* 252:43–49.

Saini, R. K., Rai, M. M., Hassanali, A., Wawiye, J., and Odongo, H. 1995. Semiochemicals from froth of egg pods attract ovipositing female *Schistocerca gregaria. J. Insect Physiol.* 41:711–716.

Sokal R. R., and Rohlf, F. J. 1981. Biometry, 2nd ed. Freeman, New York.

Szopa, T. M. 1981. The role of the accessory reproductive glands and genital ducts in egg pod formation in female *Schistocerca gregaria. J. Insect Physiol.* 27:23–29.

Torto, B., Obeng-Ofori, D., Njagi, P. G. N., Hassanali, A., and Amiani, H. 1994. Aggregation pheromone system of adult gregarious desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera:Acrididae). *J. Chem. Ecol.* 20:1749–1762.

Torto, B., Njagi, P. G. N., Hassanali, A., and Amiani, H. 1996. Aggregation pheromone system of nymphal gregarious desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera:Acrididae). *J. Chem. Ecol.* 22:2273–2281.

Torto, B., Assad, Y. O. H., Njagi, P. G. N., and Hassanali, A. 1999. Evidence for additional pheromonal components mediating oviposition aggregation in *Schistocerca gregaria. J. Chem. Ecol.* 25:835–845.

Uvarov, B. P. 1966. Grasshoppers and Locusts, Vol. 1. Cambridge University Press, London, 481 pp.

Weisel G., Tappermann S., and Dorn A. 1996. Effects of juvenile hormone and juvenile hormone analogues on the phase behaviour of *Schistocerca gregaria* and *Locusta migratoria. J. Insect Physiol.* 42:385–395.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

(*Z*)-3-HEXENYL (*R*)-3-HYDROXYBUTANOATE: A MALE SPECIFIC COMPOUND IN THREE NORTH AMERICAN DECORATOR WASPS *Eucerceris rubripes*, *E. conata* AND *E. tricolor*

S. R. CLARKE,[1] F. R. DANI,[1,3] G. R. JONES,[1,*]
E. D. MORGAN,[1] and J. O. SCHMIDT[2]

[1]*Chemical Ecology Group, School of Chemistry and Physics*
Lennard-Jones Laboratories, Keele University
Staffordshire, ST5 5BG, U.K.

[2]*Southwestern Biological Institute*
Tucson, Arizona 85745

(Received December 15, 2000; accepted March 6, 2001)

Abstract—The males of the decorator wasps *Eucerceris* have been observed to display abdomen-dragging behavior on plants surrounding their nest. It is thought that this applies a territorial-marking sex pheromone that serves to alert females to the males' territory for courtship and mating. The extracts of three species *E. rubripes*, *E. conata*, and *E. tricolor* have been analyzed by GC-MS. The gas chromatograms revealed the presence, in large quantities, of one common volatile compound in the male head extracts. By analogy with the closely related bee wolves *Philanthus*, we believe the pheromone is produced in the mandibular glands. Utilizing mass spectrometry, microreactions, FT-IR, and ^{1}H and ^{13}C NMR spectroscopy the structure of the pheromone was proposed to be (*Z*)-3-hexenyl 3-hydroxybutanoate, which was confirmed by synthesis. The absolute configuration of the chiral center was determined to be *R* for the three species by preparing the Mosher esters of the insect samples and comparing their GC retention times with a synthetic sample of known absolute configuration. In addition 2- and 3-hexenoic acid and some aromatic compounds were also found in varying quantities in both males and females along with hydrocarbons and fatty acids, although no species-specific profiles emerged.

Key Words—Mosher esters, absolute configuration, territorial marking sex pheromone, (*Z*)-3-hexenyl (*R*)-3-hydroxybutanoate, decorator wasp, *Eucerceris*.

* To whom correspondence should be addressed.
[3] Present address Dipartimento di Biologia Animale e Genetica, via Romana 17, 50129, Firenze, Italy.

0098-0331/01/0700-1437$19.50/0

INTRODUCTION

Eucerceris is a genus of digger wasps whose population is limited in distribution to North America (Scullen, 1968). These wasps, which we call decorator wasps because of their elaborate and decorative body sculpture and color patterns, build underground nests in sandy areas. They are members of the family Sphecidae within the subfamily Philanthinae (Alexander, 1992) and are closely related to the beewolves *Philanthus*. Both *Eucerceris* and *Philanthus* prey upon other insects which they sting into a state of paralysis and transport back to the nest. Multiple prey are then placed in cells, an egg is laid in each cell, and the female seals the cell. Development to adulthood is completed in the cell, with the prey consumed by the hatched larva.

Eucerceris and *Philanthus* also have a relatively unusual mating system among wasps and Hymenoptera in general (Thornhill and Alcock, 1983). Males of these species aggregate into groups, sometimes comprising leks, and establish territories on different plants. The males then display a walking behavior, during which they apply a pheromone by dragging their abdomen on the substrate. The pheromone is used for territorial marking and to attract receptive females to the territory (Alcock, 1975; Evans and O'Neill, 1985; O'Neill, 1983, 1992). Other males are also attracted to the territory and may try to usurp the established male's territory or mate with the approaching females.

The origin of this territorial marking pheromone is believed to be the mandibular glands, which has been confirmed by chemical analysis in several *Philanthus* species (Schmidt et al., 1985, 1990; McDaniel et al., 1987, 1992). The glandular secretion spreads from the mandibular glands to the brush of hairs present on the clypeus and from there is passed onto hairs arranged along the ventral part of the abdomen.

The purpose of this study was to identify and characterize the territorial-marking pheromones of four species of *Eucerceris: E. conata, E. montana, E. rubripes*, and *E. tricolor*. Males of *E. rubripes* and *E. tricolor* display the abdomen dragging behavior and aggressively defend their perches. Morphological examination of the males of all these species revealed the presence of rows of abdominal hairbrushes (Evans and O'Neill, 1985; Alcock, 1975).

METHODS AND MATERIALS

Biological Samples. Wasps were collected live, cooled on ice, and placed either intact or as heads and thorax–abdomens in dichloromethane (Burdick & Jackson, Muskegon, Michigan). Extracts (Table 1) were maintained at -25°C until analyzed. Wasps were obtained from the following locations, all within Cochise County, Arizona, U.S.A.: *Eucerceris conata*—5 km ESE of Willcox, September 2,

TABLE 1. EXTRACTS ANALYZED

Species	Male head	Male body	Female intact
Eucerceris conata	11	7	7
Eucerceris montana	5		
Eucerceris rubripes	5	5	1
Eucerceris tricolor	12		

1989; *E. montana*—7 km NW of Huachuca City, June 26, 1993; *E. rubripes*—7 km NNE of Tombstone, June 26, 1992; and *E. tricolor*—7 km NNE of Tombstone, June 26, 1993.

GC-MS Analysis. Generally gas chromatographic injections of 1 μl of a solution were performed in a splitless mode on a 5890 Hewlett Packard gas chromatograph coupled with a 5970B quadrupole mass spectrometer (70 eV electron impact ionization). The system was controlled by a 59970 ChemStation. For most samples a SGE/BPX5, 5% phenylpolyphenylenesiloxane was used (column: 12 m $\times$ 0.32 mm; 0.5 μm). The GC oven was programmed from an initial temperature of 40°C to a final temperature of 250°C heating at a rate of 10°C/min. Injection port and transfer line were set at 250°C and helium (1 ml/min) was used as carrier gas. Compounds were identified by comparison of their retention time and mass spectrum against synthetic standards.

Microreactions. Microhydrolysis of a sample was carried out using sodium hydroxide, following a similar procedure to one previously described (Pomonis et al., 1993), and the GC-MS of the resulting ether extract was recorded. Dimethyl-thiolation was carried out on the ester as previously described (Billen et al., 1986), but under an atmosphere of nitrogen, and the chromatogram and mass spectrum recorded.

Spectroscopic Measurements. FT-IR, spectroscopy was carried out on a Perkin Elmer Paragon 1000 FT-IR, and NMR spectra were recorded on a Bruker Avance DPX 300-MHz spectrometer. Samples were prepared for NMR by first passing the solution through a short silica column, eluting with deuterated chloroform, followed by repeated microdistillation of the dichloromethane solution gradually replacing the solvent with more deuterated chloroform.

Synthesis of (Z)-*3-Hexenyl* (R)-*3-hydroxybutyrate*. The compound was prepared using an adaptation of a method by Seebach et al. (1996). Sodium (*R*)-3-hydroxybutanoate (0.65 g, 6.2 mmol) in chloroform (9 ml) was added slowly to a stirred solution of (*Z*)-3-hexen-1-ol (0.85 g, 8.5 mmol), dicyclohexylcarbodiimide (DCC, 1.29 g, 6.2 mmol), 4-dimethylaminopyridine (DMAP, 1.13 g, 9.3 mmol), and 4-dimethylaminopyridine hydrochloride salt (1.96 g, 12.4 mmol) in chloroform (12 ml) under nitrogen. After 16 hr, the reaction mixture was filtered through cotton wool, washed with ether (20 ml), and evaporated on a rotary evaporator. The resulting residue was purified by flash chromatography (SiO_2, 7:3

petroleum ether–ethyl acetate) to give an oil, which by ^{1}H NMR was found to contain a mixture of the desired ester and (*Z*)-3-hexen-1-ol. Distillation under reduced pressure using a Kugelrohr apparatus (125–127°C, 0.2 mm Hg) gave the desired ester as an oil (0.45 g, 38.8% yield). FT-IR (CH_2Cl_2)/cm^{-1} 3449 (O-H), 1737 (C=O); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.73 (3H, t, *J* 7.6, C$\underline{H}_3$-CH_2), 0.99 (3H, d, *J* 6.3, C$\underline{H}_3$-CH), 1.83 (2H, quintet, *J* 7.6, C$\underline{H}_2$-CH_3), 2.16 (2H, q, *J* 7.0, C$\underline{H}_2$-CH=C), 2.18 (1H, dd, *J* 8.4 and 16.5, C$\underline{H}_a$H$_b$-CO), 2.27 (1H, dd, *J* 3.7 and 16.5, CH$_a\underline{H}_b$-CO), 2.80 (1H, d, *J* 3.7, -O$\underline{H}$), 3.89 (2H, t, *J* 6.9, C$\underline{H}_2$-O), 3.90–4.30 (1H, m, C$\underline{H}$-OH), 5.07 (1H, ddt, *J* 1.4, 7.3 and 10.8, C$\underline{H}$=CH-CH_2), 5.26 (1H, ddt, *J* 1.4, 7.2 and 10.8, CH=C$\underline{H}$-CH_2); ^{13}C NMR (75 MHz, $CDCl_3$, off-resonance decoupled and DEPT) δ 14.23 ($\underline{C}H_3$-CH_2), 21.08 ($\underline{C}H_2$-CH_3), 22.30 ($\underline{C}H_3$-CH), 26.63 ($\underline{C}H_2$-CH=C), 42.67 ($\underline{C}H_2$-C=O), 64.08 ($\underline{C}$H-OH), 64.20 ($\underline{C}H_2$-O), 123.49 ($\underline{C}$H=CH-CH_2), 134.83 (CH=$\underline{C}$H-CH_2), 173.30 (O-$\underline{C}$=O).

Assignment of Absolute Configuration. (*Z*)-3-Hexenyl 3-hydroxybutyrate (insect sample or 1 mg, 0.95 μmol of synthetic samples) in chloroform (0.1 ml) was added slowly to a small vial containing a mixture of (*R*)-3-(3,3,3-trifluoro)-2-methoxy-2-phenylpropionic acid (9 mg, 3.9 μmol), DCC (10 mg, 4.8 μmol) and DMAP (1 crystal) in chloroform (0.1 ml). The reaction mixture was stirred for 16 h at room temperature. A gas chromatogram of the mixture was then recorded to ensure that all the (*Z*)-3-hexenyl 3-hydroxybutyrate had reacted. GC resolution of the resulting diastereoisomers was achieved using a Restek Stabilwax column with a polyethylene glycol phase (15 m $\times$ 0.32 mm; 0.25 μm) with the following temperature program: the injector port was set at 250°C, the initial temperature of the oven of 40°C was held for 3 min and then increased at 10°C/min to 250°C. The transfer line was at 250°C and helium was used as the carrier gas with a flow of 1 ml/min. Absolute configuration of the insect samples was established by comparison of retention times and co-injection with the synthetic (*Z*)-3-hexenyl (*R*)-3-hydroxybutyrate of known absolute configuration.

RESULTS

Male and female whole body extracts of *E. conata* and *E. rubripes* were analyzed by GC-MS and the resulting chromatograms compared (Table 2). For both species the main difference observed between the gas chromatograms was the presence of peak 7, which was found only in males. Both male and female body extracts contained similar mixtures of fatty acids and hydrocarbons (peaks 13 and above) plus 4-phenyl-3-hydroxybutan-2-one (peak 8) and its 2-hydroxy analog (peak 9).

GC-MS analysis was then undertaken on the male head extracts of all four species of *Eucerceris* (Table 2). It was found that head extracts of *E. conata*, *E. rubripes*, and *E. tricolor* contained the compounds giving rise to peak 7 in high

TABLE 2. COMPOUNDS IN MALE AND FEMALE EXTRACTS OF *E. conata*, *E. rubripes*, AND *E. tricolor*[a]

Peak	Compounds	*E. conata*			*E. rubripes*			*E. tricolor*
		MH	MB	F	MH	MB	F	male
1	3-Hexenyl acetate	—	—	—	—	—	—	++
2	3-Hexenoic acid	++	—	—	—	—	—	++
3	2-Hexenoic acid	++	—	—	—	—	—	t
4	Octanoic acid	t	t	—	t	t	—	—
5	Unknown	—	—	t	—	—	—	—
6	4-Phenylbutan-2, 3-dione	—	++	t	—	t	—	—
7	(*Z*)-3-Hexenyl (*R*)-3 -hydroxybutyrate	+++	+++	—	+++	+++	—	+++
8	4-Phenyl-3-hydroxy-butan-2-one	—	++	++	++	++	++	—
9	4-Phenyl-2-hydroxy-butan-3-one	—	++	++	—	++	++	—
10	Pentadecene	—	++	—	—	—	—	—
11	Dodecyl acetate	—	++	—	—	—	—	—
12	9-Hexadecenoic acid	—	—	t	—	t	t	t
13	Hexadecanoic acid	++	++	++	++	++	++	++
14	Linoleic acid	++	++	+++	++	+++	+++	+++
15	Oleic acid	t	t	++	t	++	++	++
16	Stearic acid	t	t	++	—	++	t	++
17	Tricosane	—	—	—	—	—	++	—
18	Pentacosene	—	—	—	—	++	—	t
19	Pentacosane	t	t	++	++	t	+++	—
20	Heptacosene	++	++	++	—	++	+++	++
21	Heptacosane	t	t	t	++	++	++	—
22	Unidentified hydrocarbon	—	—	t	—	t	—	—
23	Unidentified compound	—	t	—	—	—	—	—
24	Squalene	t	t	t	t	t	t	t
25	Nonacosene	++	++	++	++	++	t	—
26	Nonacosane	t	t	—	t	t	++	—
27	Hentriacontene	t	—	—	t	t	—	—
28	Hentriacontane						t	++
29	Cholest-5-en-3-ol	—	—	—	—	t	—	—

[a] MH = male head; MB = male body; F = female; +++ = major; ++ = minor; t = trace.

amounts, while the gas chromatograms of *E. montana* samples did not contain any peaks apart from those from fatty acids that could be identified on the polar column (data not shown). The abundance of the compounds giving rise to peak 7 in relation to the fatty acids and hydrocarbons in the male heads was greater than

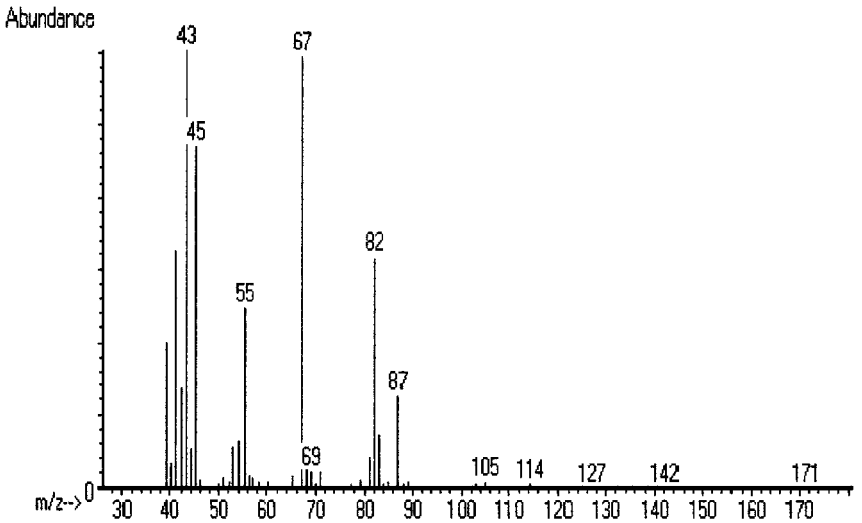


FIG. 1. Mass spectrum of (*Z*)-3-hexenyl (*R*)-3-hydroxybutanoate.

that found in the body extracts. There were also other minor compounds in the head extracts of all three species.

The mass spectrum corresponding to peak 7 had a base peak of 43, strong ions at 45, 55, 67, 82, and 87, no molecular ion and the highest ion at 171 (Figure 1). As we were unable to fully interpret the spectrum at the time we decided to undertake microreactions in order to elucidate the structure (Figure 2). Microhydrolysis under basic conditions followed by acidification and GC-MS of a hexane extract revealed the presence of an alcohol, (*Z*)-3-hexen-1-ol. Surprisingly no carboxylic acid could be found, suggesting it had either decomposed or it was soluble in the aqueous layer. The spectrum of the alcohol did explain the presence of the ion at 82 in the original spectrum resulting from alkyl–oxygen fission and also the 67 from further fragmentation. Methylthiolation using dimethyl disulfide (DMDS) followed by GC-MS confirmed the presence of only one double bond with a M^+ at 280 and two distinctive peaks at *m/z* 89 and 191 (Figure 2) resulting from cleavage adjacent to the methylthio groups.

The high concentration and purity of the compound corresponding to peak 7 in the *E. rubripes* head extract allowed FT-IR, ^{1}H NMR, and ^{13}C NMR spectra to be taken on the insect sample. FT-IR of the dichloromethane male head extract of *E. conata* revealed two strong absorptions at 3522 cm^{-1} and 1720 cm^{-1}, suggesting the presence of a hydroxyl group and an ester group. Substitution of the dichloromethane solvent for deuterated chloroform by careful microdistillation allowed a ^{1}H NMR spectrum of good quality to be acquired. This spectrum also

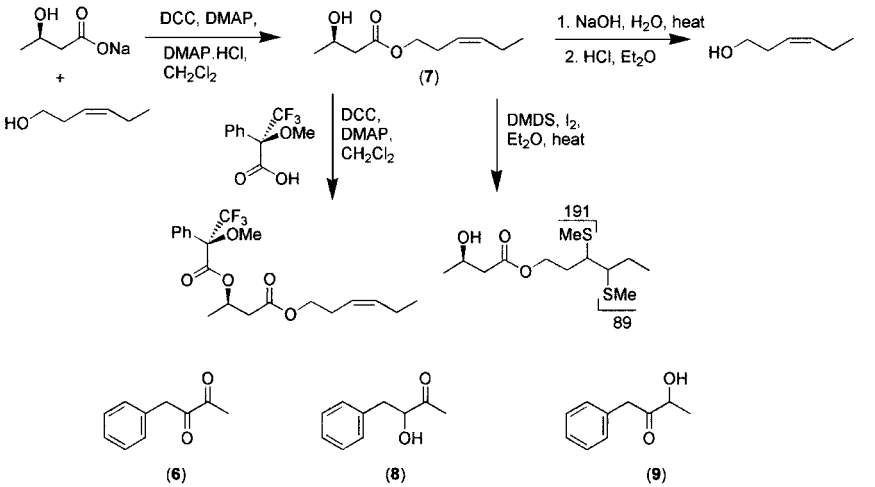


FIG. 2. Chemical procedures undertaken for the analysis and synthesis of (*Z*)-3-hexenyl (*R*)-3-hydroxybutanoate (7) and the structures of 4-phenylbutan-2,3-dione (6), 4-phenyl-3-hydroxybutan-2-one (8), and 4-phenyl-2-hydroxybutan-3-one (9).

confirmed the presence of a *Z* double bond ($J = 10.8$ Hz), and the signal at δ 3.8 ppm suggested a CH_2 adjacent to an oxygen. The ^{13}C NMR decoupled spectrum showed peaks at 123 and 135 ppm, which corresponded to the alkene carbons and an absorption at 173 ppm due to the ester carbonyl carbon. ^{13}C NMR DEPT spectra further confirmed the assignment of the structure. All of this evidence clearly pointed to the structure being (*Z*)-3-hexenyl 3-hydroxybutyrate. This was confirmed by coinjection onto the GC-MS of the natural extract with an authentic synthetic sample, which also had identical IR and NMR spectra.

The absolute configuration of the (*Z*)-3-hexenyl 3-hydroxybutyrate was determined by derivatization with (*R*)-Mosher acid (Dale et al., 1969) and comparison of the retention times of the resulting diastereomeric esters (Figure 3). Injection of the Mosher esters of the racemic mixture onto a 15-m Carbowax column showed two well-resolved peaks corresponding to the two diastereoisomers. Derivatization and analysis of the head extracts of *E. conata*, *E. rubripes*, and *E. tricolor* showed that these samples contained only one diastereoisomer. Preparation and derivatization of an enantiomerically pure sample of (*R*)-hydroxybutyric acid revealed that the absolute configuration of the (*Z*)-3-hexenyl 3-hydroxybutyrate in all of the *Eucerceris* samples was *R*.

A number of minor compounds were found within the *Eucerceris* extracts that belonged to four groups: aromatic ketones, unsaturated acids, acetates and hydrocarbons. Decomposition products of the ester, 3-hexenoic acid and 2-hexenoic acid, via plausible retroaldol, hydrolysis, oxidation, and isomerization reactions

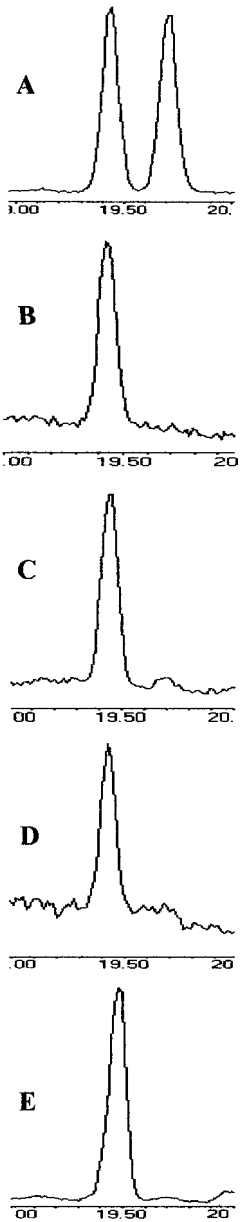


FIG. 3. Gas chromatograms of the Mosher esters of (*Z*)-3-hexenyl-3-hydroxybutanoate from: (A) a synthetic racemic sample, (B) *E. rubripes*, (C) *E. conata*, (D) *E. tricolor*, and (E) synthetic (*Z*)-3-hexenyl (*R*)-3-hydroxybutanoate.

were found to be among the minor compounds in *E. conata* and *E. tricolor* together with 3-hexenyl acetate in the latter species.

More interestingly were three related compounds that corresponded to peaks 6, 8, and 9. Peaks 8 and 9 had the same molecular ion M^+ 164 and a loss of 18 mass units suggesting they were isomeric alcohols and peak 6 had an M^+ of 162 suggesting that it was the corresponding ketone. GC-MS analysis followed by synthesis (Cocker and Grayson, 1975) confirmed their structures as 4-phenyl-3-hydroxybutan-2-one (8), its isomer 4-phenyl-2-hydroxybutan-3-one (9), and their oxidation product 4-phenylbutan-2,3-dione (6).

DISCUSSION

As the territorial marking sex pheromone is produced and released only by *Eucerceris* males and (*Z*)-3-hexenyl (*R*)-3-hydroxybutyrate is only found in extracts of males, then it is highly probable that this is the pheromone in *E. conata*, *E. rubripes*, and *E. tricolor*. We do not know why no other major components were detected, although there is a precedent for predominantly one compound from *Philanthus triangulum*, which contains 89% (*Z*)-11-eicosen-1-ol (Schmidt et al., 1990). We can only speculate that the lack of hydrocarbons or (*Z*)-3-hexenyl (*R*)-3-hydroxybutyrate in the chromatograms of *E. montana* is due to either decomposition of the sample or sampling at the wrong time of the season.

The chemical composition of the mandibular gland secretion of a number of species of *Philanthus*, a sister genus to *Eucerceris*, have been reported to consist of mixtures of long-chain alcohols, aldehydes, ketones and esters (Schmidt et al., 1985, 1990; McDaniel et al., 1987, 1992). In the five North American species of *Philanthus* previously studied, each was shown to have a species-specific blend of pheromones. It was suggested that as the species nest sympatrically in similar habitats, these pheromone blends help prevent species interbreeding. If (*Z*)-3-hexenyl (*R*)-3-hydroxybutyrate is the pheromone in all three congeneric species then this suggests that *Eucerceris* has evolved a different mechanism of species differentiation, possibly involving some of the minor compounds identified, even though our data are insufficient for us to distinguish between species. We also suspect that the minor aromatic compounds found in the male body and female extracts of *E. conata* and *E. rubripes* probably originate from a gland on the abdomen and are not cuticular compounds.

This is the first report of (*Z*)-3-hexenyl (*R*)-3-hydroxybutyrate being found in nature. Its odor was described by some as similar to that of old rose petals and its moderate volatility makes it highly suitable for an airborne pheromone that will degrade with time. (*R*)-3-Hydroxybutanoic acid is commonly found in microorganisms (Doi, 1990) and the compound itself and its dimer have been identified as the sex pheromones of the spider *Linyphia triangularis* (Schulz and Toft, 1993).

3-Hydroxy derivatives of other longer-chain acids have also been isolated from the leaf-cutting ant *Atta sexdens* (C_6, C_8, C_{10}) (Schildkneckt and Koob, 1971) and the mandibular glands of the bumble bee *Bombus terrestris* (C_6, C_8, C_{10}, C_{12}, C_{14}) (Hefetz et al., 1996). In the former the absolute configuration for the different 3-hydroxy acids varied; 3-hydroxydecanoic acid, the major compound was assigned the *S* configuration, whereas 3-hydroxytetradecanoic acid was racemic.

Methyl esters of the 3-hydroxy-substituted octanoic, decanoic, and dodecanoic acids have been found in the cephalic secretions of the solitary wasp *Campsocolia ciliata* (Borg-Karlson et al., 1987). Finally another similar ester to the one found in *Eucerceris*, (*Z*)-3-hexenyl (*R*)-2-hydroxy-3-methylbutanoate has been found to be the male produced aggregation pheromone of the assassin bug *Pristhesancus plagipennis* (James et al., 1994).

The finding that the highest concentrations of (*Z*)-3-hexenyl (*R*)-3-hydroxybutyrate are in the male head extracts suggests that, by analogy with *Philanthus*, the compound originates from the mandibular glands. Although accurate quantification was not possible at the time, what was most surprising were the considerable quantities of the ester present in *E. rubripes* head extracts. We found it remarkable that the extract of only seven heads was sufficient to take a ^{13}C NMR spectrum of good resolution with approximately 11,500 scans, suggesting there was over 1 mg of ester in that sample. Further studies are required in order to establish whether the wasps biosynthesize the compound themselves or sequester part or all of it from elsewhere.

REFERENCES

ALCOCK, J. 1975. Male mating strategies of some philanthine wasps (Hymenoptera: Sphecidae). *J. Kans. Entmol. Soc.* 48:532–545.

ALEXANDER, B. A. 1992. A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Syst. Entomol*. 17:91–108.

BILLEN, J. P. J., EVERSHED, R. P., ATTYGALLE, A. B., MORGAN, E. D., and OLLETT, D. G. 1986. Contents of the Dufour glands of the workers of three species of *Tetramorium* (Hymenoptera: Formicidae). *J. Chem. Ecol*. 12:669–685.

BORG-KARLSON, A.-K., BERGSTRÖM, G., and KULLENBERG, B. 1987. Chemical basis for the relationship between *Ophrys* orchids and their pollinators. *Chem. Scr.* 27:303–311.

COCKER, W., and GRAYSON, D. H. 1975. Reactions of some dicarbonyl compounds, Part III, Oxidation of some β-diketones with alkaline hydrogen peroxide. *J. Chem. Soc. Perkin Trans. I.* 1975:1347–1352.

DALE, J. A., DULL, D. L., and MOSHER, H. S. 1969. α-Methoxy-α-trifluoromethylphenylacetic acid a versatile reagent for the determination of enantiomeric composition of alcohols and amines. *J. Org. Chem.* 34:2543.

DOI, Y. 1990. Microbial Polyesters. VCH, New York.

EVANS, H. E., and O'NEILL, K. M. 1985. Male territorial behavior in 4 species of the tribe Cercerini (Sphecidae, Philanthinae). *J.N.Y. Entomol. Soc.* 93:1033–1040.

HEFETZ, A., TAGHIZADEH, T., and FRANCKE, W. 1996. The exocrinology of the queen bumble bee *Bombus terrestris* (Hymenoptera: Apidae, Bombini). *Z. Naturforsch.* 51c:409–442.

JAMES, D. G., MOORE, C. J., and ALDRICH, J. R. 1994. Identification, synthesis and bioactivity of the male-produced aggregation pheromone in the assassin bug *Pristhesancus plagipennis* (Hemiptera: Reduviidae). *J. Chem. Ecol.* 20:3281–3295.

McDANIEL, C. A., HOWARD, R. W., O'NEILL, K. M., and SCHMIDT, J. O. 1987. Chemistry of male mandibular gland secretions of *Philanthus basilaris* (Cresson) and *Philanthus bicinctus* (Mickel) (Hymenoptera, Sphecidae). *J. Chem. Ecol.* 13:227–235.

McDANIEL, C. A., SCHMIDT, J. O., and HOWARD, R. W. 1992. Mandibular gland secretions of the male beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera, Sphecidae). *J. Chem. Ecol.* 18:27–37.

O'NEILL, K. M. 1983. The significance of body size in territorial interactions of male beewolves (Hymenoptera, Sphecidae, *Philanthus*). *Anim. Behav.* 31:404–411.

O'NEILL, K. M. 1992. Territoriality, body size, and spacing in males of the beewolf *Philanthus basilaris* (Hymenoptera, Sphecidae). *Behavior* 86:295–321.

PONOMONIS, J. G., HAMMACK, L., and HAKK, H. 1993. Identification of compounds in an HPLC fraction from female extracts that elicit mating responses in male screwworm flies, *Cochliomyia hominivorax. J. Chem, Ecol.* 19:985–1007.

SCHILDKNECHT, H., and KOOB, K. 1971. Myrmicacin, das erste Insekten-Herbicid. *Angew. Chem.* 83:110.

SCHMIDT, J. O., O'NEILL, K. M., FALES, H. M., McDANIEL, C. A., and HOWARD, R. W. 1985. Volatiles from mandibular glands of male beewolves (Hymenoptera, Sphecidae, *Philanthus*) and their possible roles. *J. Chem. Ecol.* 11:895–901.

SCHMIDT, J. O., McDANIEL, C. A., and SIMON-THOMAS, R. T. 1990. Chemistry of male mandibular gland secretions of *Philanthus triangulum. J. Chem. Ecol.* 16:2135–2143.

SCHULZ, S., and TOFT, S. 1993. Identification of a sex pheromone from a spider. *Science* 260:1635–1637.

SCULLEN, H. A. 1968. A revision of the genus *Eucerceris* Cresson (Hymenoptera: Sphecidae). *U.S. Nat. Mus. Bull.* 268:1–97.

SEEBACH, D., CICERI, P. E., OVERHARD, M., JAUN, B., and RIGO, D. 1996. Probing the helical secondary structure of short-chain beta-peptides. *Helv. Chim. Acta* 79:2043–2066.

THORNHILL, R., and ALCOCK, J. 1983. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge, Massachusetts.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

CORRESPONDENCE OF SOLDIER DEFENSE SECRETION MIXTURES WITH CUTICULAR HYDROCARBON PHENOTYPES FOR CHEMOTAXONOMY OF THE TERMITE GENUS *Reticulitermes* IN NORTH AMERICA

LORI J. NELSON,[1,*] LAURENCE G. COOL,[2] BRIAN T. FORSCHLER,[3] and MICHAEL I. HAVERTY[1]

[1]*Chemical Ecology of Forest Insects, Pacific Southwest Research Station*
USDA Forest Service
P.O. Box 245, Berkeley, California 94701

[2]*Forest Products Laboratory, University of California*
Richmond, California 94804

[3]*Department of Entomology, University of Georgia*
Athens, Georgia 30602

(Received July 27, 2000; accepted March 14, 2001)

Abstract—Soldier defense secretions from samples of *Reticulitermes* collected in California, Nevada, Arizona, New Mexico, and Georgia were characterized and correlated with cuticular hydrocarbon phenotypes. Twenty-seven cuticular hydrocarbon phenotypes have been defined, and soldier defense secretion (SDS) phenotypes have been described for 25 of these. Forty-five terpenoid compounds were found, including monoterpenes, sesquiterpenes, and a few diterpenes. The monoterpenes include (−)-α-pinene, (−)-β-pinene, (−)-camphene, myrcene, (*Z*)- and (*E*)-ocimene, and (−)-limonene. The major sesquiterpenes produced are (+)-γ-cadinene, (+)-γ-cadinene aldehyde, (−)-germacrene A, germacrene B, γ-himachalene, and β-bisabolene. Some SDS phenotypes pair with more than one cuticular hydrocarbon phenotype; however, with two exceptions, each hydrocarbon phenotype is associated with only one SDS phenotype. These chemical characterizations lend support to the conclusion that there are numerous undescribed species of *Reticulitermes* in North America.

Key Words—*Reticulitermes*, Isoptera, Rhinotermitidae, subterranean termites, monoterpenes, sesquiterpenes, diterpenes, geranyl linalool, soldier defense secretions, cuticular hydrocarbons.

* To whom correspondence should be addressed. e-mail: lnelson@fs.fed.us

0098-0331/01/0700-1449$19.50/0

INTRODUCTION

We have been studying the foraging ecology of *Reticulitermes* (Isoptera: Rhinotermitidae) from sites in the western and southeastern United States, but have encountered difficulty identifying our collections to species in many locations. According to accepted biogeographical information, only one species, *R. hesperus* Banks, should be present at our California sites; *Reticulitermes* from New Mexico, Arizona, and Nevada should all be *R. tibialis* Banks; and the Georgia sites could be inhabited by the sympatric species *R. flavipes* (Kollar), *R. virginicus* Banks, and/or *R. hageni* Banks (Weesner, 1965, 1970; Haverty and Nelson, 1997; Haverty et al., 1996, 1999b,c, 2000).

The available keys to soldiers were unreliable in identifying many of our samples. For example, all California colonies keyed to *R. tibialis*, not *R. hesperus*, regardless of their geographic origin (Haverty and Nelson, 1997). Soldier samples from Georgia keyed to one of the three extant species that inhabit the southeastern United States (Haverty et al., 1996, 1999b). Alates rarely occur in foraging groups, thus seldom could be used for species determination. When alate specimens were present, however, species determination was often equivocal, especially for colonies determined to be *R. hageni* by soldier morphology. In short, determination of *Reticulitermes* species based on morphology can be uncertain. Because of this, we have routinely examined chemical characters as an aid in determining taxonomic identity.

Chemical characters, such as cuticular hydrocarbons and soldier defense secretions, have proven useful for distinguishing taxa of *Reticulitermes* (Bagnères et al., 1988, 1990, 1991; Baker et al., 1982; Clément and Bagnères, 1998; Clément et al., 1985, 1986; Haverty and Nelson, 1997; Haverty et al., 1996, 1999b; Howard et al., 1978, 1982b; Lange et al., 1989; Parton et al., 1981; Takematsu, 1999; Takematsu and Yamaoka, 1999; Zalkow et al., 1981). Cuticular hydrocarbon biosynthesis is largely genetically determined (Coyne et al., 1994; Kaib et al., 1991; Page et al., 1991), and the resultant mixture of compounds comprises a chemical "signature" enabling recognition at the species, colony, and individual levels, especially important in social insects (Bagnères et al., 1990, 1991; Clément and Bagnères, 1998; Howard, 1993; Howard et al., 1980, 1982a,b; Takahashi and Gassa, 1995). In fact, mimicry of the hydrocarbon signature allows termitophilous beetles to integrate peacefully into *Reticulitermes* colonies (Howard et al., 1980, 1982a). There is also evidence that when two termite species are artificially mixed, there will be some exchange of cuticular hydrocarbons, most likely by passive transfer (Vauchot et al., 1998).

Termite soldier defense secretions are synthesized *de novo* (Prestwich, 1979, 1984, 1988; Prestwich et al., 1981) and are used as weapons against intruders (Mill, 1983; Moore, 1974; Prestwich, 1979, 1983, 1984, 1988; Scheffrahn et al., 1983). Termite soldiers protect the colony against ants and vertebrate

predators, while workers are capable defenders in termite–termite conflicts (Thorne, 1982). Worker termites appear to be devoid of terpenoid compounds, so it is not likely that these chemicals are part of any colony signature (Clément and Bagnères, 1998). Although cuticular hydrocarbons and soldier terpenoid defense secretions probably have very different functions, both are nonetheless useful as taxonomic characters.

We analyzed cuticular hydrocarbons and soldier defense secretions from *Reticulitermes* collected at our experimental sites in California and Georgia, at various other sites in these states, and in Nevada, Arizona, and New Mexico. We report here the results of this chemotaxonomic study, and the complementary nature of these two sets of chemical characters.

METHODS AND MATERIALS

Insects. Termites were collected from monitoring stations at study sites in California and Georgia (Haverty and Nelson, 1997; Haverty et al., 1996, 1999b) or from infested wood in California, Georgia, Nevada, Arizona, and New Mexico (Haverty et al., 1999b) (Table 1). Termite workers were used for hydrocarbon analysis and soldiers from the same collection were used for terpene analysis.

Cuticular Hydrocarbons. Hydrocarbons were extracted from 100–200 dried workers and identified by gas chromatography–mass spectrometry (GC-MS) (Haverty et al., 1996; Page et al., 1997). Termite samples were sorted, separately for each state, on the basis of cuticular hydrocarbon composition and assigned to a hydrocarbon phenotype (Haverty and Nelson, 1997; Haverty et al., 1996, 1999b). Phenotypes are designated by the two-letter state abbreviation, followed by a letter, for example GA-A. Letters were assigned to phenotypes in the order they were discovered. There is no relationship between phenotypes from different states that have the same letter.

Soldier Defense Secretions (SDS). One to 10 soldiers from single collections were covered in *n*-pentane (ca. 200–600 μl) and held below -15°C until analysis. Monoterpenes, sesquiterpenes, and diterpenes extracted from soldiers were identified and quantified by GC-MS. Analysis was by splitless (0.7 min) injection on a 0.25 mm ID $\times$ 30 m 5% phenyl–95% methylpolysiloxane capillary column, helium carrier gas, temperature program 35°C (0.7 min isothermal) to 280°C at 6°C/min, with EI detection at 70 eV. Most compounds were identified by retention time and MS comparison with authentic materials or components of well-characterized essential oils run under identical conditions. In some cases, identification was by comparison with published GC-MS data only and considered tentative (compound name in parentheses). Unknowns were designated by apparent molecular weight and an identifying suffix letter.

Quantities of each terpenoid were calculated as a percentage of the total from integrated peak areas of the total-ion chromatograms without correction by

TABLE 1. COLLECTION LOCALITIES FOR *Reticulitermes* SAMPLES USED TO CHARACTERIZE SOLDIER DEFENSE SECRETIONS

Hydrocarbon phenotype	Localities	County	State	Terpenes (>10%)[a]	Species identification[b]	
					Soldier	Alate
CA-A	Placerville	El Dorado	California	γ-Cadinene	*R. tibialis*	NA
	Kentfield	Marin	California			
CA-A′	Kentfield; Novato	Marin	California	γ-Cadinene	*R. tibialis*	NA
	Cloverdale	Sonoma	California			
CA-B	Placerville	El Dorado	California	γ-Cadinene, Geranyl linalool, γ-Himachalene	*R. tibialis*	NA
CA-C	Placerville	El Dorado	California	Germacrene A	*R. tibialis*	NA
CA-D	Oakland	Alameda	California	Geranyl linalool, Germacrene A, γ-Cadinene	*R. tibialis*	NA
	Kentfield; Novato	Marin	California			
NV-A	Lockwood	Storey	Nevada	Geranyl linalool		NA
	Pyramid Lake; Wadsworth	Washoe	Nevada			
NV-B	Pyramid Lake	Washoe	Nevada	Germacrene A		NA
AZ-A	Hwy 40 ca. 30 mi E of Flagstaff; Intersection of Hwys 277 and 377	Coconino	Arizona	Geranyl linalool, γ-Cadinene		NA
	Second Mesa	Navajo	Arizona			
AZ-B	Second Mesa	Navajo	Arizona	Geranyl linalool, γ-Cadinene		NA
	Prescott NF near: Camp Verde, Jerome, Mingus Mtn., Prescott	Yavapai	Arizona			

AZ-C(I)	Kaibab NF near Williams	Coconino	Arizona	β-Bisabolene, γ-Cadinene, Geranyl linalool, Myrcene		NA
	Prescott NF near: Camp Verde, Jerome, Mingus Mtn., Prescott	Yavapai	Arizona			
AZ-C(II)	Chiracahua Mts., Coronado, NF	Cochise	Arizona	Geranyl linalool		NA
	Pinoleno Mts., Coronado NF	Graham	Arizona			
NM-A	Albuquerque	Bernalillo	New Mexico	Geranyl linalool		NA
GA-A(I)	Athens	Clarke	Georgia	Geranyl linalool, β-Pinene	*R. flavipes*	*R. flavipes*
	Brasstown Bald; Vogel St. Park	Union	Georgia			
GA-A(II)	Barnesville	Lamar	Georgia	γ-Cadinene, γ-Cadinenal, β-Pinene	*R. flavipes*	*R. flavipes*
	Bledsoe Farm; Westbrook Farm	Spalding	Georgia			
	Plains	Sumter	Georgia			
	Blairsville; Brasstown Bald; Mtn. Branch Expt. Stn.; Vogel St. Park	Union	Georgia			
GA-AB	Bamboo Exp. Stn.	Chatham	Georgia	γ-Cadinenal, γ-Cadinene	*R. flavipes*	*R. flavipes*
	Sapelo Island	McIntosh	Georgia			
GA-C	Bamboo Exp. Stn.	Chatham	Georgia	γ-Cadinenal, γ-Cadinene	*R. virginicus*	*R. virginicus*

CONTINUED

TABLE 1. CONTINUED

Hydrocarbon phenotype	Localities	County	State	Terpenes (>10%)[a]	Species Identification[b]	
					Soldier	Alate
	Athens	Clarke	Georgia			
	Westbrook Farm	Spalding	Georgia			
	Sapelo Island	McIntosh	Georgia			
GA-D	Aldora; Barnesville	Lamar	Georgia	γ-Cadinene	*R. hageni*	*R. hageni*
GA-E	Barnesville	Spalding	Georgia	γ-Cadinene	*R. hageni*	*R. virginicus*
	Brasstown Bald; Mtn. Branch Expt. Stn.	Union	Georgia			
GA-F	Sapelo Island	McIntosh	Georgia	γ-Cadinene	*R. hageni*	*R. virginicus*
GA-G	Sapelo Island	McIntosh	Georgia	γ-Cadinene	*R. hageni*	NA
GA-H	Plains	Sumter	Georgia	γ-Cadinene	*R. hageni*	NA
GA-I	Plains	Sumter	Georgia	γ-Cadinene	*R. hageni*	NA
GA-J	Sapelo Island	McIntosh	Georgia	Germacrene A, Germacrene B	*R. hageni*	NA
GA-K	Bledsoe Farm	Spalding	Georgia	γ-Cadinene	*R. hageni*	NA
	Blairsville; Mtn. Branch Expt. Stn.	Union	Georgia	γ-Cadinene		
GA-L	Sapelo Island	McIntosh	Georgia	γ-Cadinene	*R. hageni*	NA
GA-M	Athens	Clarke	Georgia	γ-Cadinene	*R. hageni*	NA
GA-N	Sapelo Island	McIntosh	Georgia	γ-Cadinene	*R. hageni*	NA

[a] Listed in descending order of abundance (see Tables 3–6 for specific quantities).

[b] Termites used for species identification were of the same hydrocarbon phenotype, but not necessarily from the same collection or locality as the samples used for soldier defense secretion analysis (Haverty and Nelson, 1997, Haverty et al., 1996, 1999b). Keys used for identification are from Weesner (1965) or Nutting (1990). Alate samples were collected along with other castes in wood or monitoring stations, otherwise noted as not available (NA).

response factors. Germacrene A, which gradually rearranges to β-elemene under GC conditions, appeared as a broad hump following the β-elemene peak. Its quantity was determined as the sum of the areas of the β-elemene peak plus the hump, minus the areas of the other peaks superimposed on the hump. Germacrene B behaved similarly (rearranging to γ-elemene) and was quantified by the same technique.

(+)-γ-Cadinene from pooled samples from various collections was purified by silica gel liquid chromatography and preparative GC as described elsewhere (Kim et al., 1994) and positively identified by diffuse-reflectance Fourier-transform infrared spectroscopy of ca. 50 μg of the neat material on powdered KBr in a microcup. Its optical rotation (α_D) was measured in hexane at 20°C.

The enantiomeric identity of a number of the monoterpenes and sesquiterpenes was determined by chiral GC. Conditions were: 10% permethylated β-cyclodextrin–90% OV-1701 capillary column, 0.25 mm ID × 30 m; 1°C/min program (0.5°C/min for β-elemene), 60°C–80°C for monoterpenes, 90°C–140°C for sesquiterpenes; splitless injection (0.7 min); flame-ionization detection; internal retention time reference standard was *n*-undecane for monoterpenes and *n*-tetradecane, *n*-pentadecane, and *n*-hexadecane for sesquiterpenes. Chiral analysis was done on one soldier collection from the hydrocarbon phenotype that was richest in the relevant terpene(s). Before analysis, oxygenated components were removed by passing the pentane solution through activated alumina. Enantiomer identification (indicated by sign of rotation in Tables 2–6 below) was by retention time comparison with authentic materials run under identical GC conditions. In the case of (−)-germacrene A, pooled samples were purified as for γ-cadinene, although under preparative GC conditions only β-elemene was recovered. This was then analyzed by chiral GC and compared to authentic (−)-β-elemene. The Kovats retention indices of the various sesquiterpene enantiomers are presented in Table 2.

RESULTS

Terpenoid Identification. γ-Cadinene and its aldehyde have often been reported from European and American *Reticulitermes* species, but there is some confusion in the literature as to the precise identity of these compounds. The original report (Zalkow et al., 1981) determined them to be the rare (+)-γ_1-cadinene [cadina-4(14),9-diene] and the corresponding C-15 aldehyde, but later literature from these and other authors variously report the hydrocarbon as "γ_1-cadinene" or just "γ-cadinene." We found the GC-MS data and IR spectrum of the hydrocarbon to be identical to that of authentic γ-cadinene [cadina-4,10(15)-diene], and the value of its optical rotation ($+100° \pm 10°$) was the same as that of the common enantiomer found in higher plants. Moreover, its retention time on the chiral GC

TABLE 2. KOVATS RETENTION INDICES OF *Reticulitermes* SESQUITERPENES AND ENANTIOMERS ON PERMETHYLATED β-CYCLODEXTRIN GC COLUMN[a]

Compound and source	Kovats retention index
(−)-β-Elemene, *Abies concolor* cortical oleoresin	1423
(+)-β-Elemene, *Reticulitermes* sp. (GA-J)[b]	1424
(−)-α-Himachalene, *Cedrus deodara* wood	1470
(+)-α-Himachalene, *Reticulitermes* sp. (AZ-B, CA-B) or *Scapania undulata*	1478
(−)-α-Amorphene, *Pinus edulis* oleoresin	1506
(+)-α-Amorphene, *Reticulitermes* sp. (AZ-B) or *Scapania undulata*	1527
(+)-α-Selinene, *Abies concolor* cortical oleoresin	1531
(−)-α-Selinene, *Reticulitermes* sp. (CA-D)	1537
(+)-β-Selinene, *Abies concolor* cortical oleoresin	1532
(−)-β-Selinene, *Reticulitermes* sp. (CA-D)	1540
(+)-γ-Cadinene, *Reticulitermes* sp. (GA, CA, AZ) or *Pinus edulis* oleoresin	1565

[a] 10% permethyl-β-cyclodextrin, 90% OV-1701, 0.25 mm ID × 30 m silica; temperature program 1°/min (β-elemene: 0.5°/min).
[b] Rearrangement product of (−)-germacrene A.

column was identical to that of authentic (+)-γ-cadinene. As for the aldehyde, the NMR data reported by Zalkow et al. (1981) are identical to those given in the literature for cadina-4,10(15)-dien-14-al (Kaiser and Lamparsky, 1983). Thus, these ubiquitous *Reticulitermes* sesquiterpenoids are shown to be (+)-γ-cadinene and its C-14 aldehyde (hereinafter called γ-cadinenal), rather than (+)-γ_1-cadinene and its C-15 aldehyde.

The CA-B collections had a sesquiterpene whose mass spectrum was nearly identical to germacrene A, but which had a slightly different GC retention time. It also did not rearrange to β-elemene as does germacrene A. This compound is tentatively identified as the *cis*-double bond isomer (*Z*,*E*)-germacrene A, also known as helminthogermacrene. It has been reported as the major SDS component in *Amitermes wheeleri* (Scheffrahn et al., 1986). In all examined cases (with the possible exception of β-elemene, the enantiomers of which were poorly resolved), the terpenoids were found to be optically pure (>90%). It is striking that except for (+)-γ-cadinene and its aldehyde, *Reticulitermes* sesquiterpenes are enantiomeric to those found in higher plants (Table 2), many having heretofore been reported only from fungi, liverworts, or marine organisms. This is further evidence that these termites are producing the SDS terpenoids *de novo* rather than sequestering them from ingested terpene-containing plants.

In this study, we identified 20 SDS terpenoids that have not yet been reported from termite soldiers of any family. The names of these compounds appear in bold face in Tables 3–6 below. In addition, we report nine unknowns that may also be

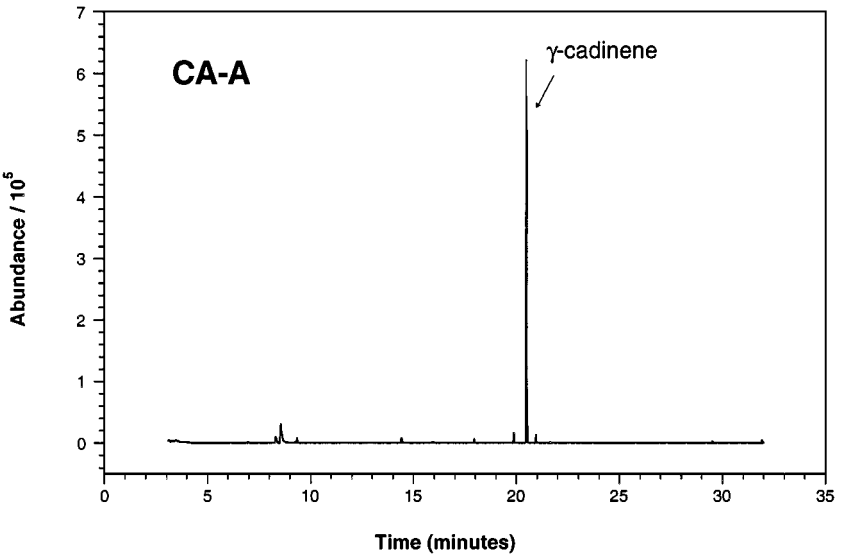


FIG. 1. Total ion chromatogram of defense secretions extracted from soldiers of cuticular hydrocarbon phenotype CA-A.

previously unreported. For brevity in the following discussion, signs of rotation will not be specified, although they are given in Tables 3–6 below.

Reticulitermes *from California.* So far we have characterized five cuticular hydrocarbon phenotypes of *Reticulitermes* in northern California: CA-A, CA-A′, CA-B, CA-C, and CA-D (Haverty and Nelson, 1997). CA-A′ is nearly identical to CA-A, distinguished only by the presence of two fairly abundant pentacosatrienes not found in CA-A, and it appears to be the most common phenotype (Haverty and Nelson, 1997).

Termite collections with cuticular hydrocarbon phenotypes CA-A or CA-A′ produce very similar SDS profiles: γ-cadinene predominates ($\geq$90.0%) (Table 3; Figure 1). A few compounds appear in small amounts ($\leq$2.0%) in some samples of CA-A that were not seen in CA-A′: myrcene, unknown (unk.) 204c, δ-cadinene, (E)-γ-bisabolene, and unk. 238a. CA-B samples produced α-himachalene (5.0%), γ-himachalene (13.4%), γ-humulene (1.2%), β-himachalene (4.9%), (Z,E)-germacrene A (6.8%), δ-amorphene (8.5%), γ-cadinene (39.6%), unk. 204e (1.4%), geranyl linalool (16.7%), and a few other minor components. The SDS mixtures of hydrocarbon phenotype CA-C contain predominantly germacrene A (92.8%), and small amounts of γ-cadinene (3.7%) and α-selinene (2.0%). α-Selinene (9.1%) was a larger component in CA-D, along with germacrene A (33.0%), γ-cadinene (17.4%), and geranyl linalool (38.0%) (Table 3).

TABLE 3. TERPENOID PERCENT COMPOSITION OF SOLDIER DEFENSE SECRETIONS FROM 5 CUTICULAR HYDROCARBON PHENOTYPES OF *Reticulitermes* FROM NORTHERN CALIFORNIA

	CA-A (N = 16)					CA-A′ (N = 10)					CA-B (N = 6)					CA-C (N = 6)					CA-D (N = 10)				
Terpenoid[a]	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%
(−)-α-Pinene	0.1	0.19	0.0	0.6	0.63	0.0	0.08	0.0	0.2	0.80	0.0	0.01	0.0	0.0	1.00						0.0	0.01	0.0	0.0	1.00
(−)-Camphene																									
(−)-β-Pinene	0.1	0.56	0.0	2.2	0.94	0.1	0.21	0.0	0.7	0.90	0.0	0.02	0.0	0.1	1.00	0.0	0.03	0.0	0.1	1.00	0.0	0.03	0.0	0.1	0.90
Myrcene	0.1	0.25	0.0	1.0	0.94						0.0	0.02	0.0	0.0	1.00	0.0	0.02	0.0	0.0	1.00	0.0	0.02	0.0	0.1	1.00
3-Carene																									
(−)-Limonene	0.1	0.12	0.0	0.4	0.69	0.0	0.08	0.0	0.2	0.80						0.0	0.00	0.0	0.0	1.00	0.0	0.00	0.0	0.0	1.00
Terpinolene																									
(*Z*)-Ocimene																									
(*E*)-Ocimene																									
δ-Elemene																									
Unk. 204a																									
Longifolene																									
Unk. 204b											0.7	0.23	0.3	1.0	0.00										
(+)-α-Himachalene											5.0	1.02	4.1	6.5	0.00										
(*E*)-β-Farnesene																0.2	0.22	0.0	0.6	0.33	0.2	0.19	0.0	0.7	0.20
α-Humulene											0.4	0.12	0.2	0.5	0.00										
***cis*-Muurola-4(14), 5-diene**	0.0	0.10	0.0	0.3	0.81	0.1	0.13	0.0	0.4	0.80	0.0	0.03	0.0	0.1	1.00						0.0	0.03	0.0	0.1	0.90
Unk. 204c	0.0	0.10	0.0	0.3	0.88																0.0	0.03	0.0	0.1	1.00
γ-Himachalene											13.4	3.19	9.7	19.0	0.00										
γ-Humulene	1.6	1.02	0.0	2.7	0.19	1.8	0.97	0.0	2.9	0.10	1.2	0.07	1.1	1.3	0.00	0.0	0.11	0.0	0.3	0.83	0.7	0.79	0.0	2.5	0.40
(+)-α-Amorphene																									

(γ-Curcumene)																										
(−)-β-Selinene																0.2	0.40	0.0	1.0	0.67	0.9	0.85	0.0	2.1	0.40
(−)-α-Selinene																2.0	3.38	0.0	8.6	0.50	9.1	7.43	0.0	21.5	0.10
Unk. 204d																										
β-Himachalene										4.9	1.58	2.8	6.7	0.00												
(Zingiberene)																										
β-Bisabolene	1.1	2.15	0.0	7.9	0.69	0.9	1.62	0.0	4.8	0.70																
(*Z*,*E*-Germacrene A)	0.1	0.39	0.0	1.2	0.88	0.2	0.64	0.0	2.0	0.90	6.8	2.05	4.8	10.5	0.00											
(−)-Germacrene A	1.3	0.02	0.0	5.1	0.38	1.0	1.71	0.0	4.4	0.70						92.8	4.54	87.0	98.4	0.00	33.0	14.68	1.8	53.0	0.00
δ-Amorphene											8.5	1.87	6.2	10.9	0.00											
(+)-γ-Cadinene	90.0	7.37	71.3	100.0	0.00	94.3	3.84	87.1	100.0	0.00	39.6	6.74	31.1	50.6	0.00	3.7	3.54	0.5	9.4	0.00	17.4	9.87	8.2	40.1	0.00
δ-Cadinene	0.2	0.36	0.0	1.2	0.81						0.7	0.15	0.5	0.9	0.00											
10-Epicubebol																										
Unk. 204e											1.4	0.33	0.9	1.9	0.00											
α-Cadinene	1.6	1.23	0.0	5.0	0.19	0.8	1.06	0.0	2.5	0.60	0.8	0.14	0.7	1.0	0.00	0.0	0.06	0.0	0.2	0.83	0.3	0.20	0.1	0.7	0.00
Germacrene B																										
[(*E*)-γ-Bisabolene)]	0.3	0.72	0.0	2.0	0.81																					
(*E*)-α-Bisabolene																										
(+)-γ-Cadinenal																										
Unk. 218a																										
Unk. 272a											0.0	0.02	0.0	0.0	1.00											
Unk. 238a	0.2	0.36	0.0	1.3	0.69						0.0	0.03	0.0	0.1	1.00	0.1	0.11	0.0	0.3	0.83						
Unk. 290a																					0.3	0.33	0.0	1.1	0.40	
Geranyl linalool	3.1	3.30	0.0	10.0	0.31	0.8	1.62	0.0	4.7	0.70	16.7	12.67	1.4	34.0	0.00	0.7	1.19	0.0	3.1	0.17	38.0	18.28	10.4	72.1	0.00	

[a] Terpenoids are listed in GC elution order. Terpenoids in bold type are newly reported for termite soldiers. Compound name in parentheses or brackets indicates identification is considered tentative. Unknowns are designated by apparent molecular weight and an identifying suffix letter.

[b] Values of 0.0 indicate the compound was detected, but at a level <0.05% of the total SDS.

[c] Values of 0.0 may indicate the compound was not detected, or detected at a level <0.05% of the total SDS.

TABLE 4. TERPENOID PERCENT COMPOSITION OF SOLDIER DEFENSE SECRETIONS FROM 8 CUTICULAR HYDROCARBON PHENOTYPES OF *Reticulitermes* FROM NEVADA, ARIZONA, NEW MEXICO

	NV-A (N = 3)					NV-B	AZ-A (N = 4)					AZ-B (N = 16)					AZ-C(I) (N=18)					AZ-C(II) (N = 1)					NM-A (A = 2)				
Terpenoid[a]	Mean[b]	SD (%)	Min.[c]	Max.[b]	Prop. ≤ 0.1%	(N = 1)[b]	Mean[b]	SD (%)	Min.[c]	Max.[b]	Prop. ≤ 0.1%	Mean[b]	SD (%)	Min.[c]	Max.[b]	Prop. ≤ 0.1%	Mean[b]	SD (%)	Min.[c]	Max.[b]	Prop. ≤ 0.1%	Mean[b]	SD (%)	Min.[c]	Max.[b]	Prop. ≤ 0.1%	Mean[b]	SD (%)	Min.[c]	Max.[b]	Prop. ≤ 0.1%
(−)-α-Pinene	0.0	0.01	0.0	0.0	1.00	0.0	0.0	0.03	0.0	0.1	1.00	0.0	0.04	0.0	0.2	0.94	0.1	0.22	0.0	0.8	0.89										
(−)-Camphene																															
(−)-β-Pinene	0.0	0.01	0.0	0.0	1.00		0.0	0.01	0.0	0.0	1.00	0.0	0.01	0.0	0.0	1.00	0.0	0.07	0.0	0.3	0.94										
Myrcene						0.1						0.1	0.21	0.0	0.8	0.81	10.2	5.94	1.3	20.3	0.00	0.2	0.23	0.0	0.6	0.55					
3-Carene																	0.0	0.12	0.0	0.5	0.94										
(−)-Limonene	0.0	0.03	0.0	0.1	1.00	0.0						0.0	0.09	0.0	0.3	0.88	1.6	1.04	0.0	3.8	0.11	0.0	0.04	0.0	0.1	0.91					
Terpinoline												0.0	0.10	0.0	0.4	0.94	0.0	0.06	0.0	0.3	0.94										
(*Z*)-Ocimene												0.0	0.7	0.0	0.2	0.88	3.3	2.56	0.0	7.6	0.06	0.2	0.20	0.0	0.7	0.57					
(*E*)-Ocimene												0.0	0.12	0.0	0.4	0.88	5.3	3.66	0.0	12.8	0.11	0.0	0.10	0.0	0.2	0.71					
δ-Elemene																															
Unk. 204a												0.0	0.5	0.0	0.1	0.88															
Longifolene																	1.1	4.28	0.0	18.2	0.83	0.0	0.03	0.0	0.1	1.00					
Unk. 204b																															
(+)-α-Himachalene												2.1	2.38	0.0	6.6	0.25															
(*E*)-β-Farnesene												0.1	0.11	0.0	0.3	0.75	3.4	1.17	1.8	5.9	0.00	1.2	0.76	0.3	3.1	0.00					
α-Humulene																															
***cis*-Muurola-4(14), 5-diene**												0.1	0.07	0.0	0.2	0.38															
Unk. 204c												0.0	0.05	0.0	0.1	0.88	0.1	0.09	0.0	0.3	0.72										
γ-Himachalene												0.5	0.73	0.0	1.8	0.50															
γ-Humulene							0.3	0.11	0.2	0.4	0.00	0.9	0.60	0.5	2.7	0.00	0.2	0.34	0.0	1.0	0.61										
(+)-α-Amorphene												0.1	0.46	0.0	1.8	0.88															
(γ-Curcumene)												0.0	0.01	0.0	0.1	1.00	3.8	1.37	2.1	7.0	0.00	0.0	0.07	0.0	0.2	0.82					

(−)-β-Selinene						1.0																											
(−)-α-Selinene						0.7																											
Unk. 204d																																	
β-Himachalene											0.1	0.13	0.0	0.4	0.81																		
(Zingiberene)																0.2	0.25	0.0	0.7	0.67													
β-Bisabolene											0.2	0.26	0.0	0.7	0.56	37.6	10.35	17.8	57.3	0.00	0.8	0.46	0.3	1.8	0.00	0.1	0.13	0.0	0.2	0.50			
[(*Z*,*E*)-Germacrene A]																																	
(−)-Germacrene A						97.0					0.0	0.03	0.0	0.1	0.94																		
δ-Amorphene											0.6	0.83	0.0	2.5	0.44																		
(+)-γ-Cadinene							16.8	2.82	13.4	19.8	0.00	23.1	15.36	9.7	66.7	0.00	17.0	10.63	1.8	40.5	0.00												
δ-Cadinene											0.1	0.12	0.0	0.4	0.81																		
10-Epicubebol											0.1	0.06	0.0	0.2	0.75																		
Unk. 204e																																	
α-Cadinene							0.3	0.08	0.2	0.4	0.00	0.8	0.55	0.0	1.8	0.06																	
Germacrene B						1.2																											
[(*E*)-γ-Bisabolene]											0.0	0.03	0.0	0.1	0.94	0.9	0.59	0.0	2.0	0.22													
(*E*)-α-Bisabolene											0.3	0.53	0.0	1.9	0.69	3.3	1.24	1.5	7.2	0.00	0.1	0.10	0.0	0.3	0.64							
(+)-γ-Cadinenal							0.3	0.64	0.0	1.3	0.75	6.1	4.20	0.5	13.2	0.00	0.5	1.08	0.0	3.4	0.75											
Unk. 218a																																	
Unk. 272a											0.1	0.44	0.0	1.7	0.94																		
Unk. 238a																																	
Unk. 290a																																	
Geranyl linalool	99.7	0.08	99.6	99.8	0.00	0.0	82.4	2.52	79.5	84.9	0.00	64.4	13.99	26.4	78.3	0.00	11.4	14.08	0.0	50.4	0.28	97.3	1.54	94.7	99.1	0.00	99.8	0.05	99.8	99.8	0.00		

[a] Terpenoids are listed in GC elution order. Terpenoids in bold characters are newly reported for termite soldiers. Compound name in parentheses or brackets indicates identification is considered tentative. Unknowns are designated by apparent molecular weight and an identifying suffix letter.

[b] Values of 0.0 indicate the compound was detected, but at a level <0.05% of the total SDS.

[c] Values of 0.0 may indicate the compound was not detected, or detected at a level <0.05% of the total SDS.

TABLE 5. TERPENOID PERCENT COMPOSITION OF SOLDIER DEFENSE SECRETIONS FROM 14 CUTICULAR HYDROCARBON PHENOTYPES OF *Reticulitermes* FROM GEORGIA

	GA-A(I) (N = 4)					GA-A(II) (N = 28)					GA-AB (N = 25)					GA-C (N = 18)					GA-D-I,K-N (N = 59)					GA-J (N = 5)				
Terpenoid[a]	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ 0.1%	Mean[b]	SD (%)	Min[c]	Max[b]	Prop ≤ (%)
(−)-*α*-Pinene	4.2	1.19	2.5	5.3	0.00	3.7	3.62	0.0	14.1	0.18	0.5	0.22	0.1	0.9	0.00						0.1	0.35	0.0	2.7	0.95					
(−)-Camphene	0.9	0.34	0.5	1.4	0.00	0.7	0.77	0.0	3.2	0.29	0.1	0.05	0.0	0.2	0.52						0.0	0.08	0.0	0.6	0.97					
(−)-*β*-Pinene	11.5	1.56	9.9	13.6	0.00	10.6	10.54	0.1	36.6	0.11	2.0	0.70	0.5	3.5	0.00						0.0	0.16	0.0	1.2	0.97					
Myrcene	0.0	0.05	0.0	0.1	1.00						0.0	0.02	0.0	0.1	1.00						0.0	0.07	0.0	0.6	0.98					
3-Carene																														
(−)-Limonene	2.6	0.88	2.0	3.9	0.00	2.6	2.82	0.0	10.3	0.18	0.5	0.30	0.0	1.6	0.04						0.0	0.08	0.0	0.6	0.98					
Terpinolene	0.0	0.06	0.0	0.1	0.75	0.1	0.10	0.0	0.3	0.75																				
(*Z*)-Ocimene																														
(*E*)-Ocimene																														
***δ*-Elemene**																										3.5	0.84	2.8	4.9	0.00
Unk. 204a																														
Longifolene																														
Unk. 204b	0.0	0.01	0.0	0.0	1.00	0.0	0.10	0.0	0.5	0.96	0.0	0.02	0.0	0.1	1.00															
(+)-*α*-Himachalene	0.3	0.48	0.0	1.0	0.75	1.6	2.92	0.0	15.2	0.11	0.1	0.42	0.0	2.1	0.80															
(*E*)-*β*-Farnesene											0.0	0.05	0.0	0.3	0.96											0.5	0.27	0.0	0.7	0.20
α-Humulene	0.0	0.01	0.0	0.0	1.00	0.0	0.10	0.0	0.5	0.93	0.0	0.02	0.0	0.1	1.00															
***cis*-Muurola-4(14), 5-diene**	0.0	0.01	0.0	0.0	1.00	0.2	0.10	0.0	0.5	0.32	0.3	0.07	0.1	0.4	0.00	0.3	0.10	0.1	0.5	0.00	0.0	0.02	0.0	0.1	0.98					
Unk. 204c	0.0	0.01	0.0	0.0	1.00	0.0	0.04	0.0	0.1	0.93	0.1	0.04	0.0	0.1	0.96	0.0	0.03	0.0	0.1	1.00	0.1	0.25	0.0	1.4	0.93					
γ-Himachalene	0.1	0.10	0.0	0.2	0.75	0.2	0.46	0.0	2.3	0.64	0.0	0.03	0.0	0.2	0.96															
γ-Humulene	0.3	0.34	0.0	0.7	0.50	2.2	0.57	1.0	3.2	0.00	2.6	0.31	2.0	3.3	0.00	1.6	0.51	0.9	2.8	0.00	0.3	0.39	0.0	1.4	0.46					
(+)-*α*-Amorphene																														
(γ-Curcumene)																														

(−)-β-Selinene																															
(−)-α-Selinene																															
Unk. 204d						0.0	0.08	0.0	0.2	0.75	0.1	0.06	0.0	0.2	0.32	0.1	0.05	0.0	0.1	0.72	0.1	0.40	0.0	2.5	0.95						
β-Himachalene	0.1	0.12	0.0	0.3	0.75	0.3	0.43	0.0	1.8	0.57	0.0	0.02	0.0	0.1	1.00	0.0	0.02	0.0	0.1	1.00	0.0	0.08	0.0	0.7	0.98						
(Zingiberene)																															
β-Bisabolene	0.0	0.01	0.0	0.0	1.00																										
[(*Z*,*E*)-Germacrene A]						0.3	0.33	0.0	1.8	0.14	0.2	0.05	0.1	0.3	0.08	0.0	0.01	0.0	0.0	1.00	0.0	0.02	0.0	0.2	0.98						
(−)-Germacrene A																										50.8	8.09	39.9	59.8	0.00	
δ-Amorphene						0.1	0.39	0.0	2.0	0.86																					
(+)-γ-Cadinene	7.9	9.70	0.1	20.4	0.00	56.3	15.38	25.0	80.9	0.00	30.7	6.59	17.1	45.6	0.00	20.1	8.40	11.4	39.9	0.00	95.0	4.13	79.1	100.0	0.00						
δ-Cadinene																					0.4	0.39	0.0	1.2	0.46						
10-Epicubebol	0.0	0.04	0.0	0.1	1.00	0.1	0.08	0.0	0.2	0.64	0.2	0.10	0.1	0.4	0.36	0.0	0.01	0.0	0.0	1.00											
Unk. 204e	0.0	0.03	0.0	0.1	1.00	0.1	0.17	0.0	0.8	0.75																					
α-Cadinene	0.1	0.07	0.0	0.2	0.75	0.2	0.18	0.0	0.8	0.29	0.1	0.06	0.0	0.2	0.76	0.2	0.16	0.0	0.5	0.33											
Germacrene B																										39.9	4.39	34.7	46.6	0.00	
[(*E*)-γ-Bisabolene]																															
(*E*)-α-Bisabolene																															
(+)-γ-Cadinenal	2.5	4.15	0.0	8.7	0.50	17.2	10.51	3.4	49.4	0.00	60.3	6.87	45.2	76.3	0.00	76.7	9.00	55.2	86.2	0.00	0.1	0.44	0.0	2.6	0.90						
Unk. 218a						0.2	0.48	0.0	2.1	0.79	1.2	0.29	0.6	1.9	0.00	0.9	0.41	0.0	1.9	0.06											
Unk. 272a	0.0	0.03	0.0	0.1	1.00	0.3	0.61	0.0	2.8	0.54	0.8	0.63	0.3	3.3	0.00	0.1	0.11	0.0	0.3	0.72	3.8	3.40	0.0	12.4	0.24						
Unk. 238a	1.2	0.57	0.4	1.7	0.00	1.4	1.03	0.0	3.4	0.18																					
Unk. 290a	0.0	0.05	0.0	0.1	1.00																										
Geranyl linalool	68.1	14.86	47.0	80.9	0.00	1.0	0.59	0.0	2.3	0.07	0.0	0.04	0.0	0.2	0.96						0.1	0.70	0.0	5.3	0.95	5.3	4.87	2.2	13.9	0.00	

[a] Terpenoids are listed in GC elution order. Terpenoids in bold type are newly reported for termite soldiers. Compound name in parentheses or brackets indicates identification is considered tentative. Unknowns are designated by apparent molecular weight and an identifying suffix letter.

[b] Values of 0.0 indicate the compound was detected, but at a level <0.05% of the total SDS.

[c] Values of 0.0 may indicate the compound was not detected, or detected at a level <0.05% of the total SDS.

TABLE 6. COMPARISON OF PERCENT COMPOSITION OF SOLDIER DEFENSE SECRETIONS FOR ALL CUTICULAR HYDROCARBON PHENOTYPES ANALYZED[a]

Terpenoid[b]	CA-A (N = 16)	CA-A′ (N = 10)	CA-B (N = 6)	CA-C (N = 6)	CA-D (N = 10)	NV-A (N = 3)	NV-B (N = 1)	AZ-A (N = 4)	AZ-B (N = 16)	AZ-C(I) (N = 18)	AZ-C(II) (N = 11)	NM-A (N = 2)	GA-A(I) (N = 4)	GA-A(II) (N = 28)	GA-AB (N = 25)	GA-C (N = 18)	GA-D-I,K-N (N = 59)	GA-J (N = 5)
(−)-α-Pinene	tr	tr	tr		tr	tr	tr	tr	tr	tr			4.2	3.7	0.5		tr	
(−)-Camphene													0.9	0.7	tr		tr	
(−)-β-Pinene	tr	tr	tr	tr	tr	tr		tr	tr	tr			11.5	10.6	2.0		tr	
Myrcene	tr		tr	tr	tr		tr		tr	10.2	tr		tr		tr		tr	
3-Carene										tr								
(−)-Limonene	tr	tr		tr	tr	tr	tr		tr	1.6	tr		2.6	2.6	0.5		tr	
Terpinolene									tr	tr			tr	tr				
(*Z*)-Ocimene									tr	3.3	tr							
(*E*)-Ocimene									tr	5.3	tr							
δ-Elemene																		3.5
Unk. 204a									tr									
Longifolene										1.1	tr							
Unk. 204b			0.7										tr	tr	tr			
(+)-α-Himachalene			5.0						2.1				0.3	1.6	tr			
(*E*)-β-Farnesene				tr	tr				tr	3.4	1.2				tr			0.5
α-Humulene			0.4										tr	tr	tr			
***cis*-Muurola-4(14),5-diene**	tr	tr	tr		tr				tr				tr	tr	0.3	0.3	tr	
Unk. 204c	tr				tr				tr	tr			tr	tr	tr	tr	tr	
γ-Himachalene			13.4						0.5				tr	tr	tr			
γ-Humulene	1.6	1.8	1.2	tr	0.7			0.3	0.9	tr			0.3	2.2	2.6	1.6	0.3	
(+)-α-Amorphene									tr									
(γ-Curcumene)									tr	3.8	tr							
(−)-β-Selinene				tr	0.9		1.0											

(−)-α-Selinene				2.0	9.1		0.7											
Unk. 204d													tr	tr	tr	tr		
β-Himachalene			4.9						tr				tr	0.3	tr	tr	tr	
(Zingiberene)										tr								
β-Bisabolene	1.1	0.9							tr	37.6	0.8	tr	tr					
[(*Z*,*E*)-Germacrene A]	tr	tr	6.8											0.3	tr	tr	tr	
(−)-Germacrene A	1.3	1.0		92.8	33.0		97.0		tr									50.8
δ-Amorphene			8.5						0.6					tr				
(+)-γ-Cadinene	90.0	94.3	39.6	3.7	17.4			16.8	23.1	17.0			7.9	56.3	30.7	20.1	95.0	
δ-Cadinene	tr		0.7						tr								0.4	
10-Epicubebol									tr				tr	tr	tr	tr		
Unk. 204e			1.4										tr	tr				
α-Cadinene	1.6	0.8	0.8	tr	0.3			0.3	0.8				tr	tr	tr	tr		
Germacrene B							1.2											39.9
[(*E*)-γ-Bisabolene]	0.3								tr	0.9								
(*E*)-α-Bisabolene									0.3	3.3	tr							
(+)-γ-Cadinenal								0.3	6.1	0.5			2.5	17.2	60.3	76.7	tr	
Unk. 218a														tr	1.2	0.9		
Unk. 272a			tr						tr				tr	0.3	0.8	tr	3.8	
Unk. 238a	tr		tr	tr									1.2	1.4				
Unk. 290a					0.3								tr					
Geranyl linalool	3.1	0.8	16.7	0.7	38.0	99.7	tr	82.4	64.4	11.4	97.3	99.8	68.1	1.0	tr		tr	5.3

[a] tr indicates a value <0.3%. All values except those for NV-B are means.

[b] Terpenoids are listed in GC elution order. Terpenoids in bold characters are newly reported for termite soldiers. Compound name in parentheses indicates identification is considered tentative. Unknowns are designated by apparent molecular weight and an identifying suffix letter.

Reticulitermes *from Nevada, New Mexico, and Arizona.* Collections in western Nevada provided us with another cuticular hydrocarbon phenotype, NV-A (Haverty et al., 1999b). The soldiers from these samples produced defense secretions consisting almost exclusively of geranyl linalool (99.7%) (Table 4). One sample with a cuticular hydrocarbon mixture qualitatively and quantitatively nearly identical to phenotype CA-B was also collected in Nevada near Pyramid Lake. However, the soldiers from this colony had a defense secretion comprised of 97.0% germacrene A, 1.2% germacrene B, and 1.0% β-selinene along with traces of myrcene, a composition more similar to soldiers of CA-C than of CA-B (Tables 3 and 4). This sample is referred to here as phenotype NV-B.

Currently, only one species of *Reticulitermes, R. tibialis,* is recognized from Arizona and New Mexico (Weesner, 1970). So far, five cuticular hydrocarbon phenotypes have been identified from this region: AZ-A, AZ-B, AZ-C, AZ-D, and NM-A (Haverty et al., 1999b). The terpene profiles for southwestern *Reticulitermes* sorted into four main SDS phenotypes. Hydrocarbon phenotypes AZ-A and AZ-B had similar terpene profiles, primarily comprised of γ-cadinene and geranyl linalool, although quantities were variable (Table 4). AZ-B generally had more γ-cadinenal (0.5–13.2%), than AZ-A (0–1.3%). Soldier defense secretions from hydrocarbon phenotype AZ-C showed evidence of geographic differences (Table 4; Figure 2). Phenotype AZ-C has only been collected from the Petran Montane Coniferous Forests biotic community zone in southcentral Arizona (Brown and Lowe, 1980; Haverty and Nelson, unpublished observations). Samples from the Mogollon (Interior) biogeographic province (Brown et al., 1979), from Prescott to Springerville, Arizona, had soldier defense secretions consisting mainly of myrcene (1.3–20.3%), (*Z*)- and (*E*)-ocimene (0–19.8%), β-bisabolene (17.8–57.3%), γ-cadinene (1.8–40.5%), and geranyl linalool (0–50.4%). These samples were summarized as one group, AZ-C(I), although they were quite variable. There was much less variability in SDS profiles from samples collected in the higher elevations of the Santa Catalina, Santa Rita, Chiricahua, and Pinaleno mountains of the Sonoran biogeographic province (Brown et al., 1979). Samples of this SDS phenotype, AZ-C(II), all produced $\geq$94.7% geranyl linalool. We have not yet collected live soldiers of AZ-D, thus, the defense secretion profile of this phenotype is unknown. Soldiers of hydrocarbon phenotype NM-A yielded almost exclusively geranyl linalool (99.8%) (Table 4).

Reticulitermes *from Georgia.* Fifteen different cuticular hydrocarbon phenotypes have been reported from Georgia: GA-A, GA-AB, GA-B, GA-C, GA-D, GA-E, GA-F, GA-G, GA-H, GA-I, GA-J, GA-K, GA-L, GA-M, and GA-N (Haverty et al., 1996, 1999b). Soldier defense secretions were characterized for all hydrocarbon phenotypes except for GA-B. Two SDS phenotypes were associated with GA-A. Phenotype GA-A(I) was distinguished by a large quantity of geranyl linalool (47.0–80.9%), while in the second phenotype, GA-A(II), this diterpene alcohol never exceeded 2.3% of the total (Table 5; Figure 3). Within

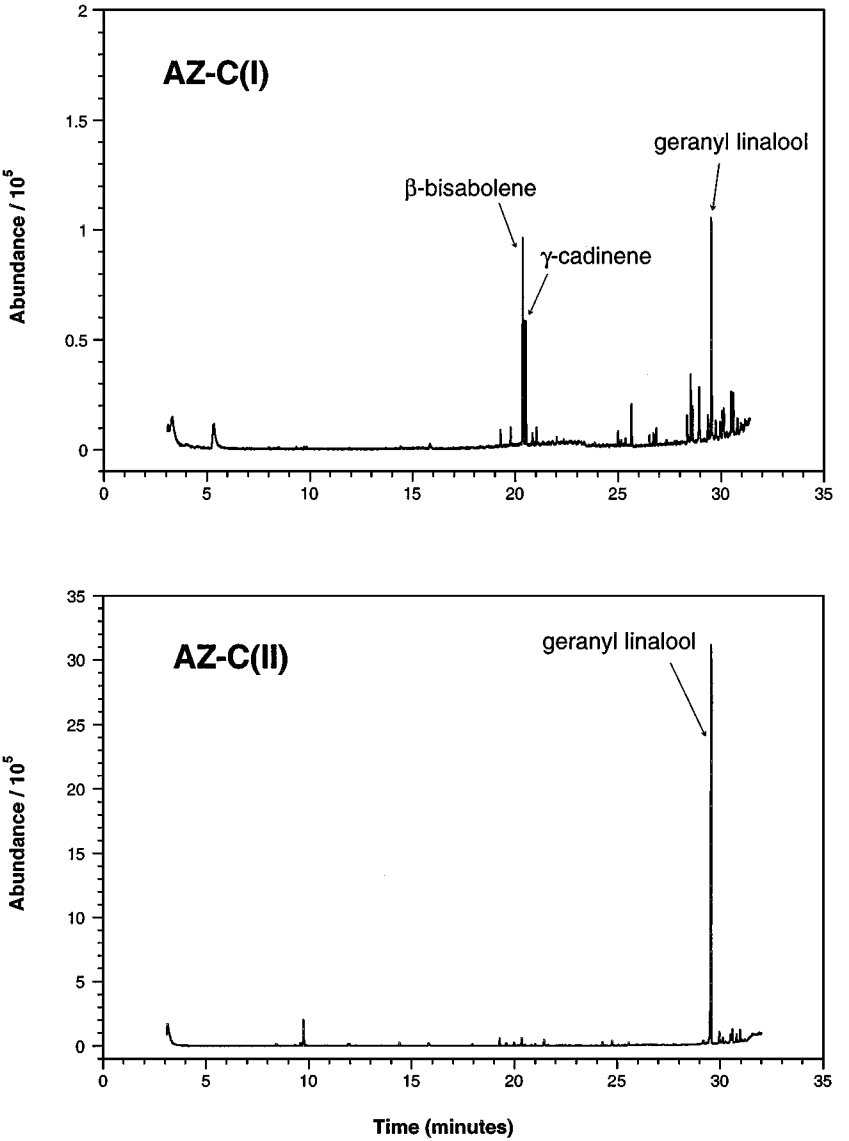


FIG. 2. Total ion chromatograms of defense secretions extracted from soldiers of cuticular hydrocarbon phenotype AZ-C.

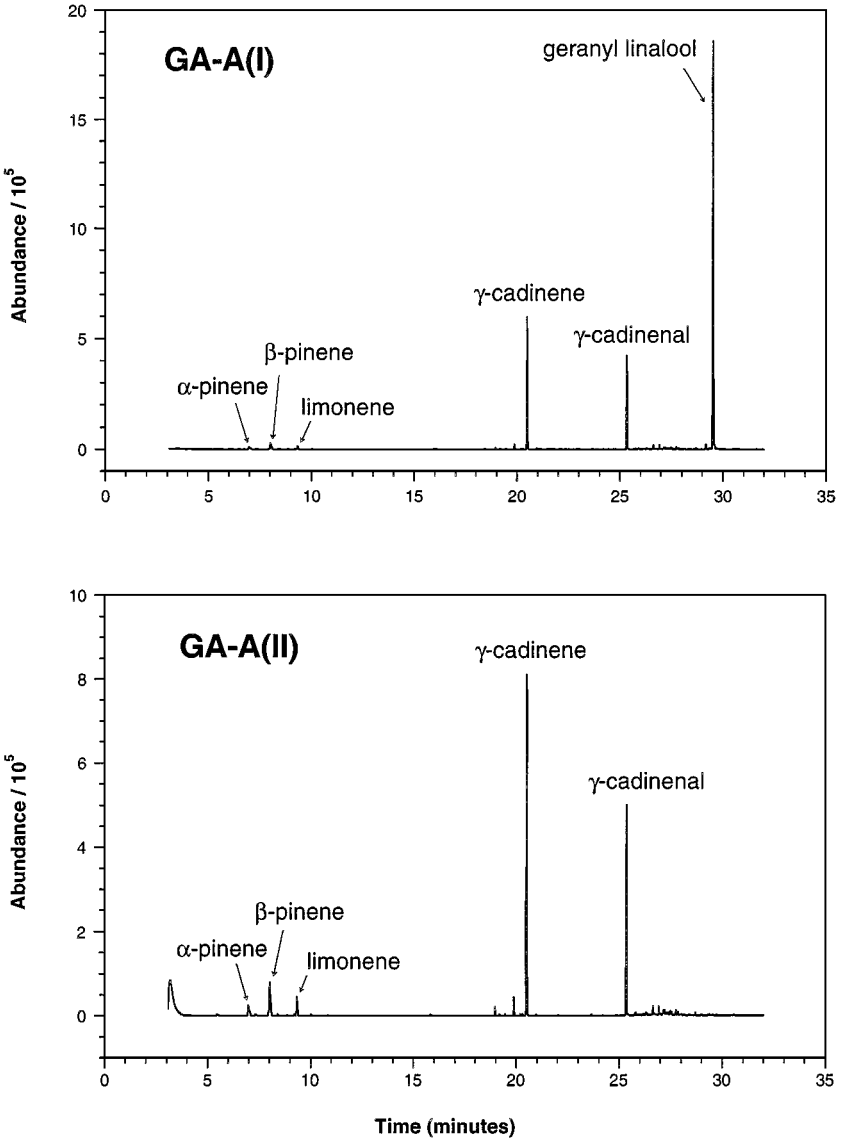


FIG. 3. Total ion chromatograms of defense secretions extracted from soldiers of cuticular hydrocarbon phenotype GA-A.

SDS phenotype GA-A(I), there was marked geographic variation in the amounts of the sesquiterpenes γ-cadinene and γ-cadinenal. Of the four samples making up this phenotype, the two from northern Georgia produced moderate amounts of γ-cadinene (10.7% and 20.4%) and γ-cadinenal (1.3% and 8.7%), while the two from Athens had trace amounts of γ-cadinene ($\leq$0.2%) and no detectable γ-cadinenal. These two sesquiterpenes were the major components for SDS phenotype GA-A(II); γ-cadinene averaged 56.3% of the total, and γ-cadinenal 17.2%. In both GA-A SDS phenotypes, with the exception of the two Athens samples, the ratio of γ-cadinene to its aldehyde was approximately 3:1 (Table 5). These phenotypes both contained monoterpenes (α-pinene, camphene, β-pinene, and limonene) in varying amounts (<0.1–15.9%) (Table 5). All of these GA-A samples keyed to *R. flavipes.*

Hydrocarbon phenotype GA-AB had only one SDS phenotype. This terpene type appeared to be intermediate between those produced by GA-A and GA-C in that it contained a significant monoterpene component, trace amounts of geranyl linalool, and roughly twice as much γ-cadinenal as γ-cadinene (Table 5; Figure 4). All GA-AB samples keyed to *R. flavipes.*

The SDS phenotype produced by GA-C soldiers contained no monoterpenes or geranyl linalool. The predominant components were γ-cadinene (averaging 20.1%) and γ-cadinenal (76.7%); the proportion of the latter was about three

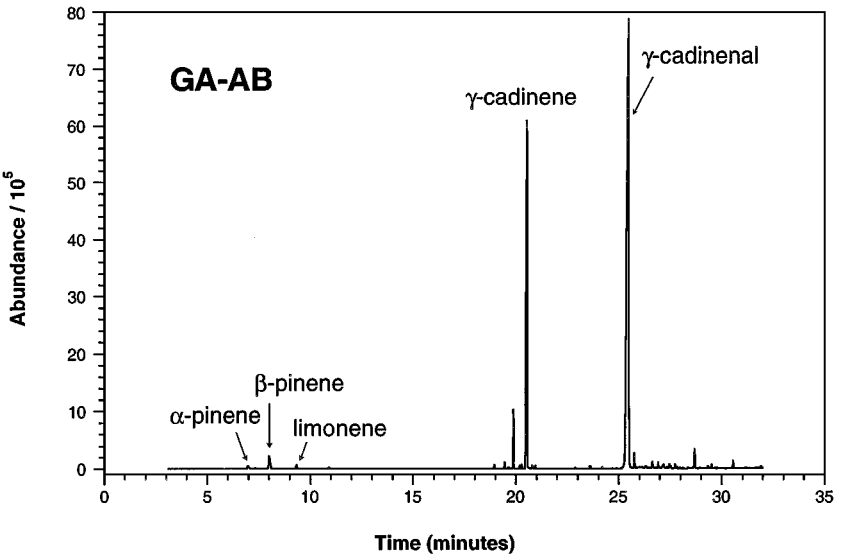


FIG. 4. Total ion chromatogram of defense secretions extracted from soldiers of cuticular hydrocarbon phenotype GA-AB.

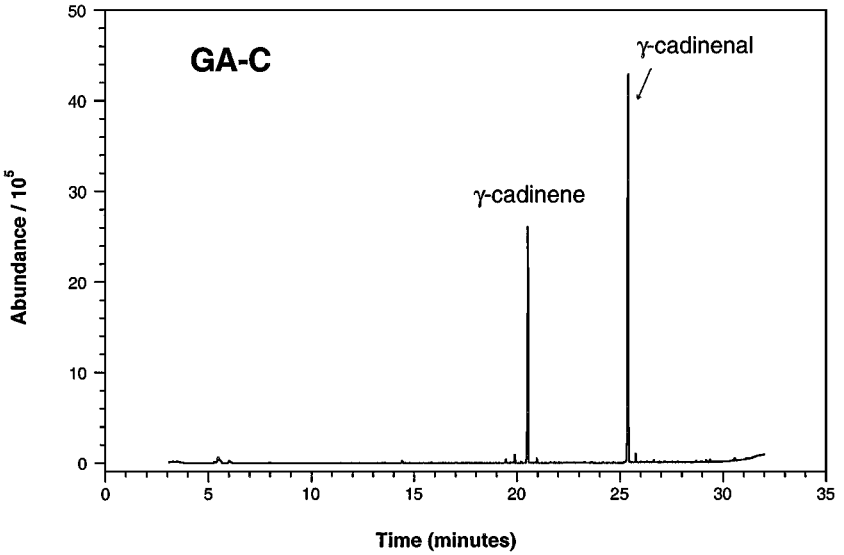


FIG. 5. Total ion chromatogram of defense secretions extracted from soldiers of cuticular hydrocarbon phenotype GA-C.

times that of the former, the opposite of SDS phenotypes produced by GA-A (Table 5; Figure 5). All GA-C samples keyed to *R. virginicus.*

Numerous hydrocarbon phenotypes, specifically those with a predominance of unsaturated hydrocarbon components (GA-D-I, -K to -N) produced nearly identical soldier defense secretions. This terpene mixture consisted primarily of γ-cadinene (79.1–100%) with only a trace of γ-cadinenal (Table 5; Figure 6). An unknown diterpene ($M_r = 272$, with a base peak of 257) was present in amounts ranging from $<0.1\%$ to 12.4%. All these samples keyed to *R. hageni* based on soldier morphology, although GA-D was the only phenotype that keyed to *R. hageni* based on alate morphology. Two of the cuticular hydrocarbon phenotypes, GA-E and GA-F, had alates that keyed to *R. virginicus* (Haverty et al., 1996). Alates were not available for the other phenotypes.

A single, distinct SDS phenotype was associated with hydrocarbon phenotype GA-J and was comprised primarily of germacrene A (50.8%) and germacrene B (39.9%) with lesser amounts of δ-elemene, farnesene, and geranyl linalool (Table 5). These samples all keyed to *R. hageni* based on soldiers.

DISCUSSION

Four SDS phenotypes paired with multiple hydrocarbon phenotypes: CA-C and NV-B produce nearly identical mixtures of soldier defense secretions; as do

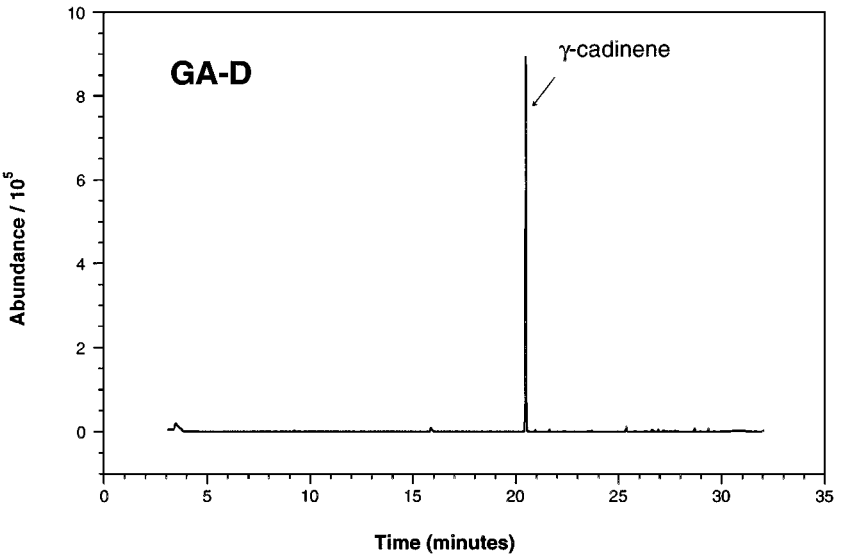


FIG. 6. Total ion chromatogram of defense secretions extracted from soldiers of cuticular hydrocarbon phenotype GA-D.

NV-A, NM-A, and some AZ-C samples; AZ-A and AZ-B; and GA-D-I, -K to -N (Table 6). Conversely, with the exception of AZ-C and GA-A, each hydrocarbon phenotype is correlated with only one SDS phenotype (Table 6).

The results from northern California consistently and repeatedly associate one hydrocarbon phenotype with one distinct soldier defense secretion phenotype (Table 3). We now consider CA-A and CA-A′ to be variations of one hydrocarbon phenotype, as we have recently seen samples from other locations in California that appear to be intermediate between the two (L. J. Nelson, unpublished observations). We believe there are at least four species of *Reticulitermes* represented by hydrocarbon phenotypes CA-A/CA-A′, CA-B, CA-C, and CA-D (Haverty and Nelson, 1997).

The cuticular hydrocarbon composition of the single sample of NV-B is nearly identical to that of CA-B, but we will designate it as a separate phenotype pending further sampling. There was virtually no overlap in terpene composition for these two hydrocarbon phenotypes (Tables 3, 4, and 6).

There are many similarities between NV-A and NM-A in both sets of chemical characters. They could quite possibly represent a single species that occurs in the Great Basin biogeographic province (Brown et al., 1979). AZ-A and AZ-B also likely represent a single taxon, based on their chemical phenotypes. These four phenotypes (NV-A, NM-A, AZ-A, and AZ-B) form a clade when subjected to

phylogenetic (PAUP) analysis based on cuticular hydrocarbons (Page et al., 2001), which may indicate a close taxonomic relationship. AZ-C is a distinct cuticular hydrocarbon phenotype, but exhibits some variability in SDS profiles (Tables 5 and 6; Figure 2). We feel confident that this is a separate species.

Sampling from Georgia has produced 15 cuticular hydrocarbon phenotypes and 6 SDS phenotypes. We consider GA-C (=*R. virginicus*) and GA-J (=undescribed species of *Reticulitermes*) to be distinct species, and we suggest GA-A, GA-AB, and GA-B are either separate taxa or represent intraspecific variation in *R. flavipes*. GA-AB has been found only on Sapelo Island off the Atlantic coast of Georgia, and nearby on the mainland (Chatham County). GA-B has been rarely found in Georgia, despite directed efforts to locate additional samples (Haverty et al., 1996, 1999b).

The remaining 10 hydrocarbon phenotypes from Georgia all possess the same terpene phenotype. Cuticular hydrocarbon phenotypes GA-D, GA-E, GA-F, GA-G, GA-H, GA-I, GA-K, GA-L, GA-M, and GA-N also have a common theme in their abundant production of certain olefins (Haverty et al., 1996, 1999b). Although some phenotypes appear to be restricted to certain geographic localities, they have all been seen from multiple collections. Variation in cuticular hydrocarbon composition due to the influence of seasonal or environmental effects has not been ruled out. However, when mitochondrial DNA genotypes of several colonies of *Reticulitermes* in Georgia were examined, samples with hydrocarbon phenotypes GA-I and GA-L (which were identified as *R. hageni* based on soldier morphology) formed a separate clade from phenotypes GA-D (identified as *R. hageni* by soldiers and alates) (Jenkins et al., 2000). Resolution of this taxonomic conundrum will require additional biological, chemical and genetic information.

The use of state designations for hydrocarbon phenotypes will undoubtedly be revised when we have sampled more widely and the phenotype distributions can be delineated more clearly. There are a few parallels in chemical characters when comparing collections from distant locations. For example, the hydrocarbon profiles of GA-A and GA-AB are qualitatively and quantitatively similar to CA-A/A′ (Haverty and Nelson, 1997; Haverty et al., 1996; Page et al., 2001), the main difference being the quantities of C_{33} methylalkanes present. Their terpene secretions are less similar, although the percent ranges of γ-cadinene and geranyl linalool overlap in some cases (Tables 3, 5, and 6). The hydrocarbon profile of the single sample of AZ-D appeared to be nearly identical to CA-A′. We were unable to collect soldiers from this colony, so the terpene profile remains unknown until more collections can be made in northwestern Arizona as well as other localities in the Great Basin Desert.

Termite collections that keyed to *R. hageni* by soldier morphology presented us with a wide array of hydrocarbon phenotypes (11), but with the exception of GA-J, all had the same SDS phenotype. This terpene phenotype is characterized by the abundance of γ-cadinene (averaging 95% of the total), as is the terpene

phenotype found in CA-A/A′ (Tables 3, 5, and 6; Figures 1 and 6). However, these 10 hydrocarbon phenotypes from Georgia are, as a group, qualitatively quite dissimilar in hydrocarbon components from phenotype CA-A/A′. The former have an abundance of olefins (>50% of total hydrocarbon) and the latter are characterized by a predominance of normal and methylbranched alkanes (Haverty and Nelson, 1997; Haverty et al., 1996, 1999b; Page et al., 2001).

No terpene compound was seen in every sample, although γ-cadinene and geranyl linalool were present in some amount in most phenotypes. The only phenotype with no detectable geranyl linalool was GA-C (Tables 5 and 6). γ-Cadinene was not detected in hydrocarbon phenotypes NV-A, NV-B, NM-A, or GA-J (Tables 4–6). Monoterpenes were seen in most phenotypes, in trace amounts, but were consistently present in cuticular hydrocarbon phenotypes GA-A and GA-AB (Tables 5 and 6).

Our results add to the body of knowledge already accumulated regarding the utility of these chemical characters for distinguishing taxa of North American *Reticulitermes*. Howard et al. (1978, 1982b) first reported the cuticular hydrocarbons of *R. flavipes* and *R. virginicus* collected near Gulfport, Mississippi. "γ_1-Cadinene" and "γ_1-cadinenal" were reported by Zalkow et al. (1981) as the major soldier cephalic secretion components of both *R. flavipes* and *R. virginicus* from Mississippi. Quantities were reported only for *R. flavipes*, and the two sesquiterpenes were present in nearly equal amounts. The identity of these sesquiterpenes is here revised to (+)-γ-cadinene and (+)-γ-cadinen-14-al. A few of our *R. flavipes* samples within groups GA-A(II) and GA-AB had comparable proportions to those reported by Zalkow et al. (1981), however, we found considerable variation in terpene composition within the three *R. flavipes* groups (Tables 5 and 6).

Clément et al. (1985) also examined frontal gland secretions and described four phenotypes of *Reticulitermes* in the southeastern United States: *R. flavipes* 1 and 2, *R. virginicus*, and *R. malletei*. Both *R. flavipes* phenotypes, Rf1 and Rf2, had γ-cadinene, geranyl linalool and small amounts of monoterpenes, but Rf1 also had γ-cadinenal. They also reported γ-cadinene and γ-cadinenal from *R. virginicus* and γ-cadinene alone in what is referred to as the species *R. malletei*. These would correspond to the SDS from our cuticular hydrocarbon phenotypes GA-C and GA-D-I, -K to -N, respectively (Haverty et al., 1996, 1999b). The hydrocarbon phenotype of *R. malletei* as reported in Bagnères (1989) is equivalent to our cuticular hydrocarbon phenotype GA-M (Haverty et al., 1999b).

Bagnères et al. (1990) documented the cuticular hydrocarbons and soldier cephalic secretions of *R. flavipes* in the southeastern United States and *R. santonensis* in France. The hydrocarbons of these two species were found to be qualitatively, but not quantitatively, identical. *R. flavipes* exhibited polymorphism in both sets of chemical characters, while *R. santonensis* did not. Three hydrocarbon phenotypes of *R. flavipes* were defined by multivariate analysis, two of which had unique

defensive secretion phenotypes (A and B), while the third was further divided into four defensive secretion phenotypes (C–F). Terpenes identified were α-pinene, β-pinene, limonene, γ-cadinene, cadinene aldehyde, and geranyl linalool. Phenotype A was defined as having the three monoterpenes and two sesquiterpenes, while B had only the two sesquiterpenes. Phenotype C exhibited all six compounds, D the two sesquiterpenes and geranyl linalool, while E and F had no γ-cadinene, only its aldehyde. Phenotype E also produced geranyl linalool, as did F, and F also had the three monoterpenes.

All but two of our samples identified as *R. flavipes* had γ-cadinene in quantities of at least 10.0% of the total. Those two samples, from Athens, Georgia, showed only traces of γ-cadinene and no γ-cadinenal, their profiles consisting mostly of geranyl linalool ($>$75.0%) and monoterpenes. As for *R. santonensis*, its terpene profile included the monoterpenes, geranyl linalool, and an unidentified sesquiterpene (Bagnères et al., 1990). γ-Cadinene and its aldehyde were absent. These authors suggest that *R. santonensis* and *R. flavipes* are the same species and that the lack of chemical variation in *R. santonensis* results from a founder event of a single colony of *R. flavipes* being successfully introduced to Europe from the United States (Bagnères et al., 1990).

The primary function of the various termite-produced terpenoid compounds has been attributed to their effectiveness in deterring predation by ants, either by toxicity or repellency (Zalkow et al., 1981; Baker et al., 1982; Mill, 1983; Scheffrahn et al., 1983; Clément et al., 1988; Lemaire et al., 1990). There is a strong indication of coevolution between termites and ants: where they are sympatric, the ants are less sensitive to the specific terpenoid compounds and where they are allopatric the ants are more sensitive (Zalkow, et al., 1981; Lemaire et al., 1990). Further behavioral studies are needed involving *Reticulitermes* species' defensive compounds and their effects on predators. Although an untested hypothesis, variation in SDS may reflect adaptation to the local ant fauna and could explain the presence of multiple SDS phenotypes of GA-A and AZ-C.

Howse and Bradshaw (1980) discussed the use of chemical data in ant and termite systematics and felt that the behavioral or ecological roles of the various chemicals should be known in order to be useful in this context. However, Prestwich (1983) suggested that the necessity of understanding the biological or ecological functions of the chemical characters is perhaps less important than knowing their biosynthetic relationships and that the phylogeny of the biosynthetic pathways reflects genetic drift or founder effects, rather than selective pressure. Brand (1978) stated that all available evidence, including chemical data, should be taken into account in describing a taxon and suggested that the biosynthetic homology of chemical characters provides a closer look at genetic relationships than does the similarity of morphological characters. Chemical variation certainly seems as likely to affect behavior as would variation in morphology. This suggests that chemical characters such as cuticular hydrocarbons and soldier defense

secretions are ideal tools for understanding the evolutionary relationships of the taxa in question, particularly so once the full elucidation of their biosynthesis has been achieved.

We believe, and have so stated, that existing keys to species of *Reticulitermes* are grossly inadequate and give a false sense of confidence in identifying species (Haverty and Nelson, 1997; Haverty et al., 1996, 1999b). Taxonomic assessments based on chemical characters and those based on morphological characters are not always in agreement. Takematsu and Yamaoka (1999) recently examined the cuticular hydrocarbons of six species of *Reticulitermes*, including three undescribed species, in Japan, Korea, and Taiwan. They observed nine different hydrocarbon phenotypes and recommended that four subspecies be elevated to species status. Takematsu (1999) then published a revision of *Reticulitermes* species in Japan, which included cuticular hydrocarbon data to supplement the more conventional characters used in taxonomy.

We agree that chemical characters have a place in the description of species. The more differences that exist to distinguish two groups of organisms, the greater the likelihood of their being separate species (Futuyma, 1998). Genetic studies of this genus will provide additional clarification. In studies of European *Reticulitermes*, variation in the composition of soldier frontal gland secretons has been correlated with differences in frequencies of esterase alleles (Clément, 1981; Parton et al., 1981). In future research we will attempt to match chemical characters with DNA sequences for all cuticular hydrocarbon phenotypes, building on previous work in this area (Jenkins et al., 1998, 1999, 2000). Agonistic behavior bioassays using *Reticulitermes* from northern California demonstrated a high degree of correlation between aggression and hydrocarbon phenotype (Haverty et al., 1999a). These chemotaxonomic studies have served to benefit our studies of ecology, behavior, and control of *Reticulitermes* (Getty et al., 1999a,b, 2000a,b; Haverty et al., 1999a,c, 2000). We hope further study will allow us to fully characterize these taxa.

ACKNOWLEDGMENTS

The authors thank Marion Page for his helpful review of this manuscript and Pierre Escoubas for inspiring us to pursue this line of research. Monica Townsend, Sherry Ridgeway, Anna Anisko, Gail Getty, Kirsten Copren, and Mechelle Cochran assisted in collecting and preparing many of the samples used in this study.

REFERENCES

BAGNÈRES, A. G. 1989. Les hydrocarbures cuticulaires des insectes sociaux. Détermination et rôle dans la reconnaissance spécifique coloniale et individuelle. Doctoral thesis. Université Pierre et Marie Curie, Paris, France, 167 pp.

BAGNÈRES, A. G., LANGE, C., CLÉMENT, J. L., and JOULIE, C. 1988. Les hydrocarbures cuticulaires: Variation spècifiques et coloniales. *Actes Colloq. Insect. Soc.* 4:34–42.

BAGNÈRES, A. G., CLÉMENT, J. L., BLUM, M. S., SEVERSON, R. F., JOULIE, C., and LANGE, C. 1990. Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonenesis* (Feytaud): Polymorphism and chemotaxonomy. *J. Chem. Ecol.* 16:3213–3244.

BAGNÈRES, A. G., KILLIAN, A., CLÉMENT, J. L., and LANGE, C. 1991. Interspecific recognition among termites of the genus *Reticulitermes:* Evidence for a role for the cuticular hydrocarbons. *J. Chem. Ecol*. 17:2397–2420.

BAKER, R., PARTON, A. H., and HOWSE, P. E. 1982. Identification of an acyclic diterpene alcohol in the defense secretion of soldiers of *Reticulitermes lucifugus. Experientia* 38:297–298.

BRAND, J. M. 1978. Fire ant venom alkaloids: their contribution to chemosystematics and biochemical evolution. *Biochem. Syst. Ecol*. 6:337–340.

BROWN, D. E., and LOWE, C. H. 1980. Biotic communities of the Southwest. Gen. Tech. Report RM-78, Rocky Mountain Forest and Range Experiment Station, Forest Service, U.S. Department of Agriculture (map).

BROWN, D. E., LOWE, C. H., and PASE, C. P. 1979. A digitized classification system for the biotic communities of North America, with community (series) and association examples for the Southwest. *J. Ariz.-Nev. Acad. Sci.* 14(suppl 1):1–16.

CLÉMENT, J. L. 1981. Enzymatic polymorphism in the European populations of various *Reticulitermes* species (Isoptera), pp. 49–62, *in* P. E. Howse and J. L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London.

CLÉMENT, J. L., and BAGNÈRES, A. G. 1998. Nestmate recognition in termites, pp. 126–155, *in* R. K. Vander Meer, M. D. Breed, M. L. Winston, and K. E. Espelie (eds.). Pheromone Communication in Social Insects, Ants, Wasps, Bees and Termites. Westview Press, Boulder, Colorado.

CLÉMENT, J. L., LANGE, C., BLUM, M. S., and LLOYD, H. 1985. Chimiosystematique du genre *Reticulitermes* (Isoptères) aux U.S.A. et en Europe. *Actes Colloq. Insect. Soc.* 2:123–131.

CLÉMENT, J. L., HOWARD, R. W., BLUM, M. S., and LLOYD, H. 1986. L'isolement spécifique des termites du genre *Reticulitermes* (Isoptera) du sudest des Etats-Unis. Mise en évidence grâce à la chimie et au comportement d'une espèce jumelle de *R. virginicus* = *R. malletei* sp. nov. et d'une semi-species de *R. flavipes. C.R. Acad. Sci. Paris* 302:67–70.

CLÉMENT, J. L., LEMAIRE, M., NAGNAN, P., ESCOUBAS, P., BAGNÈRES, A. G., and JOULIE, C. 1988. Chemical ecology of European termites of the genus *Reticulitermes*: Allomones, pheromones and kairomones. *Sociobiology* 14:165–174.

COYNE, J. A., CRITTENDEN, A. P., and MAH, K. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila. Science* 265:1461–1464.

FUTUYMA, D. J. 1998. Evolutionary Biology, 3rd ed. Sinauer Associates, Sunderland, Massachusetts.

GETTY, G. M., HAVERTY, M. I., COPREN, K. A., and LEWIS, V. R. 1999a. Seasonal foraging behavior of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in northern California, USA, pp. 347–352, *in* W. H. Robinson, F. Rettich, and G. W. Rambo (eds.). Proceedings of the 3rd International Conference on Urban Pests (ICUP), Czech University of Agriculture, Prague, Czech Republic, July 19–22, 1999.

GETTY, G. M., HAVERTY, M. I., LEWIS, V. R., and SBRAGIA, R. J. 1999b. Interfacing basic biology of *Reticulitermes* spp. and the Sentricon Termite Colony Elimination System in northern California, USA, pp. 601–604, *in* W. H. Robinson, F. Rettich, and G. W. Rambo (eds.). Proceedings of the 3rd International Conference on Urban Pests (ICUP), Czech University of Agriculture, Prague, Czech Republic, July 19–22, 1999.

GETTY, G. M., HAVERTY, M. I., and LEWIS, V. R. 2000a. Agonistic behavior between recently collected and laboratory cultured *Reticulitermes* spp. (Isoptera: Rhinotermitidae) from northern California. *Pan-Pac. Entomol.* 76:243–250.

GETTY, G. M., HAVERTY, M. I., COPREN, K. A., and LEWIS, V. R. 2000b. Response of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in northern California to baiting with hexaflumuron with Sentricon Termite Colony Elimination System. *J. Econ. Entomol.* 93:1498–1507.

HAVERTY, M. I., and NELSON, L. J. 1997. Cuticular hydrocarbons of *Reticulitermes* (Isoptera: Rhinotermitidae) from northern California indicate undescribed species. *Comp. Biochem. Physiol.* 118B:869–880.

HAVERTY, M. I., FORSCHLER, B. T., and NELSON, L. J. 1996. An assessment of the taxonomy of *Reticulitermes* (Isoptera: Rhinotermitidae) from the southeastern United States based on cuticular hydrocarbons. *Sociobiology* 28:287–318.

HAVERTY, M. I., COPREN, K. A., GETTY, G. M., and LEWIS, V. R. 1999a. Agonistic behavior and cuticular hydrocarbon phenotypes of colonies of *Reticulitermes* (Isoptera: Rhinotermitidae) from northern California. *Ann. Entomol. Soc. Am.* 92:269–277.

HAVERTY, M. I., NELSON, L. J., and FORSCHLER, B. T. 1999b. New cuticular hydrocarbon phenotypes of *Reticulitermes* (Isoptera: Rhinotermitidae) from the United States. *Sociobiology* 34:1–21.

HAVERTY, M. I., GETTY, G. M., COPREN, K. A., and LEWIS, V. R. 1999c. Seasonal foraging and feeding behavior of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in a wildland and a residential location in northern California. *Environ. Entomol.* 28:1077–1084.

HAVERTY, M. I., GETTY, G. M., COPREN, K. A., and LEWIS, V. R. 2000. Size and dispersion of colonies of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in a wildland and a residential location in northern California. *Environ. Entomol.* 29:241–249.

HOWARD, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp. 177–226, *in* D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *J. Chem Ecol.* 4:233–245.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1980. Chemical mimicry as an integrating mechanism: Cuticular hydrocarbons of a termitophile and its host. *Science* 210:431–433.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1982a. Chemical mimicry as an integrating mechanism for three termitophiles associated with *Reticulitermes virginicus*. *Psyche* 89:157–167.

HOWARD, R. W., MCDANIEL, C. A., NELSON, D. R., BLOMQUIST, G. J., GELBAUM, L. T., and ZALKOW, L. H. 1982b. Cuticular hydrocarbons of *Reticulitermes virginicus* (Banks) and their role as potential species and caste recognition cues. *J. Chem. Ecol.* 8:1227–1239.

HOWSE, P. E. 1984. Sociochemicals of termites, pp. 475–519, *in* W. J. Bell and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

HOWSE, P. E., and BRADSHAW, J. W. S. 1980. Chemical systematics of social insects with particular reference to ants and termites, pp. 71–90, *in* F. A. Bisby, J. G. Vaughan and C. A. Wright (eds). Chemosystematics: Principles and Practice. Academic Press, New York.

JENKINS, T. M., BASTEN, C. J., DEAN, R., MITCHELL, S. E., KRESOVICH, S., and FORSCHLER, B. T. 1998. Matriarchal genetic structure of *Reticulitermes* (Isoptera: Rhinotermitidae) populations. *Sociobiology* 33:239–263.

JENKINS, T. M., BASTEN, C. J., KRESOVICH, S., and FORSCHLER, B. T. 1999. Mitochondrial gene sequence questions *Reticulitermes* sp. social structure (Isoptera: Rhinotermitidae). *Sociobiology* 34:161–172.

JENKINS, T. M., BASTEN, C. J., HAVERTY, M. I., NELSON, L. J., PAGE, M., and FORSCHLER, B. T. 2000. Correlation of mitochondrial genotypes and cuticular hydrocarbon phenotypes for three sympatric *Reticulitermes* species (Isoptera: Rhinotermitidae) from the southeastern United States. *J. Chem Ecol.* 26:1525–1542.

KAIB, M., BRANDL, R., and BAGINE, R. K. N. 1991. Cuticular hydrocarbon profiles: A valuable tool in termite taxonomy. *Naturwissenschaften* 78:176–179.

KAISER, R., and LAMPARSKY, D. 1983. New carbonyl compounds in the high-boiling fraction of lavender oil. *Helv. Chim. Acta* 66:1835–1842.

KIM, Y.-K., COOL, L. G., and ZAVARIN, E. 1994. *cis*-Calamenene-related sesquiterpenoids from *Cupressus bakeri* foliage. *Phytochemistry* 36:961–965.

LANGE, C., BASSELIER, J. J., BAGNÈRES, A. G., ESCOUBAS, P., LEMAIRE, M., LENOIR, A., CLÉMENT, J. L., BONAVITA-COUGOURDAN, A., TRABALON, M., and CAMPAN, M. 1989. Strategy for the analysis of cuticular hydrocarbon waxes from insects using gas chromatography/mass spectrometry with electron impact and chemical ionization. *Biomed. Environ. Spectr.* 18:787–800.

LEMAIRE, M., NAGNAN, P., CLÉMENT, J. L., LANGE, C., PERU, L., and BASSELIER, J. J. 1990. Geranyllinalool (dipterpene alcohol). An insecticidal component of pine wood and termites (Isoptera: Rhinotermitidae) in four European ecosystems. *J. Chem. Ecol.* 16:2067–2079.

MILL, A. E. 1983. Behavioural and toxic effects of termite defensive secretions on ants. *Physiol. Entomol.* 8:413–418.

MOORE, B. P. 1974. Pheromones in the termite societies. pp. 250–265, *in* M. C. Birch (ed.). Pheromones. American Elsevier, New York.

NUTTING, W. L. 1990. Insecta: Isoptera, pp. 997–1032, *in* D. L. Dindal. (ed.). Soil Biology Guide. John Wiley & Sons, New York.

PAGE, M., NELSON, L. J., BLOMQUIST, G. J., and SEYBOLD, S. J. 1997. Cuticular hydrocarbons as chemotaxonomic characters of pine engraver beetles (*Ips* spp.) in the *grandicollis* subgeneric group. *J. Chem. Ecol.* 23:1053–1099.

PAGE, M., NELSON, L. J., FORSCHLER, B. T., and HAVERTY, M. I. 2001. Cuticular hydrocarbons suggest three lineages in *Reticulitermes* (Isoptera: Rhinotermitidae) from North America. *Comp. Biochem. Physiol.* In press.

PAGE, R. E., METCALF, R. A., METCALF, R. L., ERICKSON, E. H, and LAMPMAN, R. L. 1991. Extractable hydrocarbons and kin recognition in honeybee (*Apis mellifera*). *J. Chem. Ecol.* 17:745–756.

PARTON, A. H., HOWSE, P. E., and BAKER, R., and CLÉMENT, J. L. 1981. Variation in the chemistry of the frontal gland secretion of European *Reticulitermes* species, pp. 193–209, *in* P. E. Howse and J. L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London.

PRESTWICH, G. D. 1979. Chemical defense by termite soldiers. *J. Chem Ecol.* 5:459–480.

PRESTWICH, G. D. 1983. Chemical systematics of termite exocrine secretions. *Annu. Rev. Ecol. Syst.* 14:287–311.

PRESTWICH, G. D. 1984. Defense mechanisms in termites. *Annu. Rev. Entomol.* 29:201–232.

PRESTWICH, G. D. 1988. The chemicals in termite societies (Isoptera). *Sociobiology* 14:175–191.

PRESTWICH, G. D., JONES, R.W., and COLLINS, M. S. 1981. Terpene biosynthesis by nasute termite soldiers (Isoptera: Nasutitermitinae). *Insect Biochem.* 11:331–336.

SCHEFFRAHN, R. H., GASTON, L. K., SIMS, J. J., and RUST, M. K. 1983. Identification of the defensive secretion from soldiers of the North American termite *Amitermes wheeleri* (Desneux) (Isoptera:Termitidae). *J. Chem. Ecol.* 9:1293–1305.

SCHEFFRAHN, R. H., SIMS, J. J., LEE, R. K., and RUST, M. K. 1986. Helminthogermacrene, a major component in the defensive secretion of the nearctic termite, *Amitermes wheeleri. J. Nat. Prod.* 49:699–701.

TAKAHASHI, S., and GASSA, A. 1995. Roles of cuticular hydrocarbons in intra- and interspecific recognition behavior of two rhinotermitidae species. *J. Chem. Ecol.* 21:1837–1845.

TAKEMATSU, Y. 1999. The genus *Reticulitermes* (Isoptera:Rhinotermitidae) in Japan, with description of a new species. *Entomol. Sci.* 2:231–243.

TAKEMATSU, Y., and YAMAOKA, R. 1999. Cuticular hydrocarbons of *Reticulitermes* (Isoptera:Rhinotermitidae) in Japan and neighboring countries as chemotaxonomic characters. *Appl. Entomol. Zool.* 34:179–188.

THORNE, B. L. 1982. Termite-termite interactions: workers as an agonistic caste. *Psyche* 89:133–150.

VAUCHOT, B., PROVOST, E., BAGNÈRES, A. G., RIVIERE, G., ROUX, M., and CLÉMENT, J. L. 1998. Differential adsorption of allospecific hydrocarbons by the cuticles of two termite species, *Reticulitermes santonensis* and *R. lucifugus grassei*, living in a mixed colony. *J. Insect Physiol.* 44:59–66.

WEESNER, F. M. 1965. The Termites of the United States. A Handbook. The National Pest Control Association, Elizabeth, New Jersey.

WEESNER, F. M. 1970. Termites of the Nearctic region, pp. 477–525, *in* K. Krishna and F. M. Weesner (eds.). Biology of Termites, Vol. II. Academic Press, New York.

ZALKOW, L. H., HOWARD, R. W., GELBAUM, L. T., GORDON, M. M., DEUTSCH, H. M., and BLUM, M. S. 1981. Chemical ecology of *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) (Rhinotermitidae): Chemistry of the soldier cephalic secretions. *J. Chem. Ecol.* 7:717–731.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

A MODEL FOR PEAK AND WIDTH OF SIGNALING WINDOWS: *Ips duplicatus* AND *Chilo partellus* PHEROMONE COMPONENT PROPORTIONS—DOES RESPONSE HAVE A WIDER WINDOW THAN PRODUCTION?

FREDRIK SCHLYTER,[1,6,*] MATS SVENSSON,[1,7] QING-HE ZHANG,[2,6] MILOS KNÍZEK,[3] PAAL KROKENE,[4] PER IVARSSON,[5] and GÖRAN BIRGERSSON[5]

[1]*Animal Ecology, Department of Ecology*
Lund University, SE-223 62 Lund, Sweden

[2]*General Station of Forest Pest Control, Ministry of Forestry, No. 58*
N Huanghe Street, Shenyang CN-110034, P.R. China

[3]*Forestry and Game Mngmt Res Inst*
CZ-156 04 PRAHA 5 Zbraslav, Czech Republic

[4]*Norwegian Forest Res Inst*
NO-1432 ÅS-NLH, Norway

[5]*Chemical Ecology, Department of Botany*
Göteborg University, SE-413 19 Göteborg, Sweden

(Received September 22, 1999; accepted February 28, 2001)

Abstract—Pheromone communication systems have a reliable signal with a restricted window of amounts and ratios released and perceived. We propose a model based on a Gaussian response profile that allows a quantification of the response peak (location of optimum) and a measure of the peak width (response window). Interpreting the Gaussian curve, fitted by nonlinear regression (NLR), as a standard normal distribution, the peak location equals the mean (μ) and the window width equals $2\times$ the standard deviation (2σ). The NLR procedure can provide an objective measure for both peak location and width for a wide range of data sets. Four empirical data sets as well as 10 literature data sets were analyzed. The double-spined spruce engraver, *Ips duplicatus*, was field tested in four populations to find the optimum proportion for attraction to the two male aggregation pheromone components, ipsdienol (Id) and (*E*)-myrcenol

* To whom correspondence should be addressed.
[6] Present address: Chemical Ecology, Department of Crop Science, Swedish University of Agricultural Sciences, P.O. Box 44, SE-230 53 Alnarp, Sweden.
[7] Present address: Department of Chemistry Engineering, Lund University, SE-223 62 Lund, Sweden.

0098-0331/01/0700-1481$19.50/0

(EM), ranging from 0 to 100% of Id. Tests in Norway and the Czech Republic confirmed the preference of western populations for a blend between 50 and 90% Id. A population in Inner Mongolia showed a preference for traps with the 10 and 50% Id baits. The NLR fitted values for response peak and width (μ; 2σ) were: Norway 0.64, 0.73; Czech Republic 0.53, 0.73; NE China 0.77, 0.29; and Inner Mongolia 0.33, 0.50. The signal produced by Norwegian field-collected males had a narrower window width ($2\sigma = 0.12$). Males of the maize stem borer, *Chilo partellus*, were tested in a flight tunnel for their response to variation in the two major female sex pheromone gland components, (Z)-11-hexadecenal and the corresponding alcohol (OH). Variation of the alcohol in seven levels from 2 to 29% OH showed the highest male response for 17% OH. For all behavioral steps, the peak of male response was near $\mu = 0.14$, while the window width fell from $2\sigma = 0.5$ to 0.2 for eight sequential behavioral steps from take-off to copulation. Female production had a similar peak location ($\mu = 0.13$) but a narrower width, $2\sigma = 0.14$. Literature data from other moth species showed similar patterns, with a wider male response relative to the female production windows. Literature data on response to enantiomer ratios in a hymenopteran and to pheromone amounts in a dipteran were also described by our model. In a bark beetle population (*Ips pini*), with two hybridizing enantiomeric strains, the production peaks were narrower (0.1) than the response peaks (0.5). Thus, it in general, seems that in the pheromone systems analyzed, the width of the response window ($2\sigma = 0.1$ to 0.8) is larger than that of the production window ($2\sigma = 0.03$ to 0.14), irrespective of the sex of the sender.

Key Words—Response window, Gaussian curve, *Ips*, *Chilo*, individual variation, population variation, *Pectinophora*, *Argyrotaenia*, *Agrotis*, *Spodoptera*, *Neodiprion*, *Dacus*.

INTRODUCTION

The importance of ratios of pheromone components for optimum response and species specificity has long been appreciated for lepidopteran pheromone systems (Roelofs, 1978), and only more recently for coleopteran systems (Teale et al., 1991; Leal et al., 1994). The term "response window" was coined by Roelofs (1978) for a two-dimensional range of amounts (concentrations) and proportions of components evoking an optimum behavioral response, limited by a lower response threshold and an upper arrestment threshold. Later reviews (Löfstedt, 1990; Linn and Roelofs, 1994; Svensson, 1996b) and experimental papers (Linn et al., 1991; Svensson, 1996a) show the importance of variation in ratios for optimum response and the width of the response, determined by genetic (Löfstedt, 1990, 1993) and environmental (Svensson, 1996b and references therein) factors. However, the optimum of response has so far been determined by simply choosing the best experimental value or by visual interpolation between discrete values of the independent axis. The width of the response window has sometimes been set by visual inspection, but usually not quantified at all.

We propose a more analytical approach to quantify the optimal value of ratios of pheromone components. We will illustrate this by two examples. One is a bark beetle species, *Ips duplicatus*, which utilizes two male-produced monoterpene alcohol pheromone components attracting both sexes (aggregation pheromone) (Bakke, 1975; Byers et al., 1990; Schlyter et al., 1992). The other is a pyralid moth, *Chilo partellus*, which uses two female-produced major components, a monounsaturated aliphatic alcohol and aldehyde, attracting males only (sex pheromone) (Nesbitt et al., 1979; Beevor et al., 1990; Unnithan and Saxena, 1990).

We could not find any readily available method, except simple interpolation, to pinpoint the exact peak location and no objective method for defining the width of the response window. To this end, we developed a simple model, using the Gaussian or normal curve fitted by nonlinear regression. Our model gives the location and width of the optimum for field attraction in four different populations of *Ips duplicatus* and a comparison to male pheromone production. For the moth *Chilo partellus*, we quantified the decline in response window width with the sequence of male flight-tunnel behaviors and compared it to the variation of female production. Our method is then applied to a range of literature data on lepidopteran, coleopteran, and hymenopteran systems for proportions, including a hybrid population with two optima, and one example of response to amounts in a dipteran pheromone system.

METHODS AND MATERIALS

Rationale for the Model. We expected a maximum frequency of signaling to occur at a point (an optimum) on the independent axis. Thus, we seek a function, $g(x)$, to describe the signalling frequency, y, depending on x,

$$y = g(x) \tag{1}$$

Such a function shall measure both the location (position) of the peak on the x axis and the width of the peak. The signaling frequency profile includes both response and production. The response profile is typically measured in field experiments as number of trapped specimens for a continuous range of baits or in the laboratory as the frequency of responding individuals to a range of stimuli. In the same way we may measure production profile as frequency distributions (histogram) of individually analyzed calling insects. It is expected that a signal system should possess a peak of response or production, as well as two tails. For the sake of simplicity, we seek a symmetrical distribution function that is unimodal as well. In addition, a function describing such a profile must be convex near the peak and have two longer or shorter tails. For these reasons, neither the asymmetrical Poisson, F, or χ^2 distributions nor power functions like $g(x) = x^2$ without tails are suitable. The

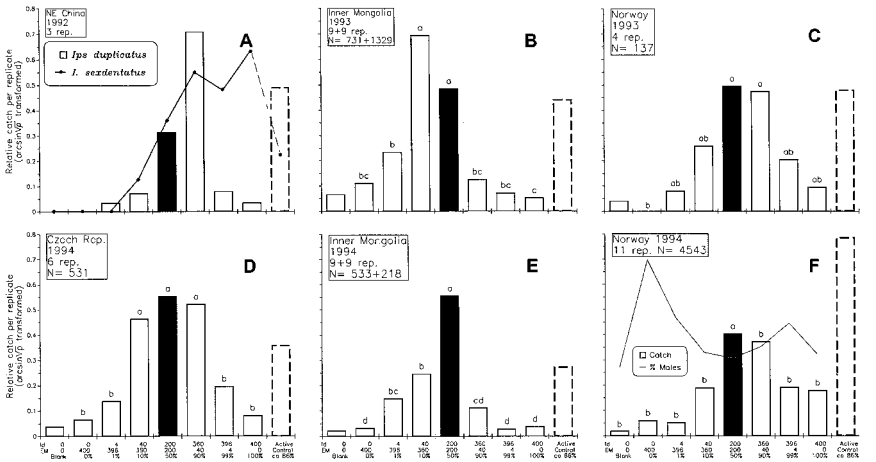


FIG. 1. Relative catches of *Ips duplicatus* in different experiments. Baits with 0–100% ipsdienol were released from wick dispensers containing solutions with varying ratios, but the same total amount. The relative amounts of each compound is given for each bait below the x axis. Active control is neat Id and EM in separate vials releasing about 86% Id at a dose of about 200 male equivalents (1 mg/day) (Schlyter et al., 1992). Bars with the same letter are not different by Duncan's multiple-range test ($\alpha = 0.05$). N is the total number beetles caught per experiment.

normal distribution and the t distribution have the desired properties. However, such functions are intrinsically nonlinear [cannot be linearized by a mathematical transformation (Norusis, 1994, p. 233; Sokal and Rohlf, 1995, p. 677)]. The Gaussian or normal curve was chosen for two reasons: (1) it was apparent from one data set (Figure 1A) that a response profile could be superficially close to such a curve; and (2) the normal curve and its parameters are well known to biologists as many characteristics in living organisms show such a distribution pattern and it makes statistical sense using it to describe a frequency distribution.

Equations for the Model. The formula for the well-known Gaussian or normal curve, with the two parameters for a population, μ, the mean or central moment of the population and σ, the theoretical population standard deviation (second moment) is;

$$g(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-(x-\mu)^2/2\sigma^2} \tag{2}$$

In an empirical sample, the estimated mean, $\bar{x}$, replaces μ, and similarly the standard deviation, SD, replaces σ. By analogy, we may interpret μ as the location of the optimum, or maximum response, of a response profile with a perfect bell shape.

The sample in this case is only the points defining the shape of the profile and the sample size, N, is 1. However, the sample size per se does not enter the equation (see formula above). (In a normal empirical sample, the sample size enters only when the uncertainty of the location of $\bar{x}$ must be estimated by the standard error of the mean ($\mathrm{SEM} = \mathrm{SD}/\sqrt{n}$) or by a confidence interval based on SEM and N, using the N dependent t distribution.)

For a production or response profile, we have used μ_p and σ_p, or μ_r and σ_r, for the two parameters estimated here by fitting the Gaussian curve. The subscripts p and r refers to production and response, repectively. The width of a response window we define in the simplest possible way as ranging from $\mu + 1\sigma_{p\,\mathrm{or}\,r}$ to $\mu - 1\sigma_{p\,\mathrm{or}\,r} = 2\sigma_{p\,\mathrm{or}\,r}$, which is equivalent to ca. 70% of a normal distribution. In addition, a scaling factor, f, was included to achieve a good fit in all cases, e.g. when the maxima were far from $x = 0.5$. Thus, the function fitted here included a factor to adjust the shape of the distribution for these more skewed and peaked data sets (with maxima close to $x = 0$ or 1), so that for describing a response or production profile the equation is:

$$g(x) = f\left[\frac{1}{\sigma\sqrt{2\pi}} e^{-(x-\mu)^2/2\sigma^2}\right] \tag{3}$$

The factor f corresponds to a combination of the more commonly used measures of the third and fourth moments of a distribution; skewness and kurtosis (peakedness).

Parameter Estimation for the Model. Profiles were fitted by the unconstrained nonlinear regression procedure (NLR) of SPSS/PC+5.01 for DOS and SPSS for Windows 6.01 and 8.0 and SPSS for Macintosh 6.1.1. Direct comparison of program output showed that numerical results from the three platforms agreed exactly. Such NLR procedures are available in all other major statistical packages. Empirical data was fitted only to proportions of components, except for one literature set on amounts. In principle, any variable of the signal could be used, like emission of, or response to, component proportions or amounts. In most cases, proportions (p) were arcsin $\sqrt{p}$ transformed, and this was especially important for those cases with maxima close to $x = 0$ or 1. To retransform the estimate of $2\sigma_{p\,\mathrm{or}\,r}$ from the arcsin transformed scale, one has to use the relation, $2\sigma = (\mu + \sigma) - (\mu - \sigma)$, which on the retransformed (linear) scale becomes

$$2\sigma = \sin(\mu + \sigma)^2 - \sin(\mu - \sigma)^2 \tag{4}$$

As for any nonlinear regression procedure, the NLR procedure used here is iterative, and by using a model with improper starting values one might arrive at a local solution with a poor global fit. For this reason, it is mandatory to make plots of predicted values from the statistical fitting together with the experimental data. When data are less well behaved, either by having few points on the x axis in the region where the true peak is located or by having their maxima at extreme

x values (close to 0 or 1), the predicted curve may peak at a higher value than the empirical maximum response. This is not an error of fitting, but simply reflects that the true maximum resides between or outside the independent data points. Such fitted functions, seemingly artificial in relation to the data, may then generate new testable hypotheses. In the plots displayed here, we have scaled the raw data by dividing the response variable by the maximum empirical value. This makes fitted models comparable across data sets, but is not necessary for applying the model on a single set. Details of parameter estimates obtained are given in Tables 1–3.

Bark Beetle (Ips duplicatus) *Data.* The double-spined spruce engraver of Eurasia, *Ips duplicatus* (Sahlb.), occurs in the Paleartic from central Scandinavia, across eastern Europe to Siberia and NE China, and in the mountains of central Europe (Postner, 1974; Lekander et al., 1977; Pfeffer, 1995). It has recently become of concern as a forest pest in the lowlands of central Europe (Mrkva, 1994; Knízek and Zahradník, 1996). *I. duplicatus* usually occurs on trees that are under attack by *I. typographus*, where it occupies the top position of the tree trunk (Schlyter and Anderbrant, 1993). *I. duplicatus* has not been considered as a major pest (Postner, 1974), but recent outbreaks in Czech Republic (Knízek and Zahradník, 1996) and in Inner Mongolia (Zhang et al., 1995) as well as its status as a vector of phytopathogenic fungi (Krokene and Solheim, 1996), might alter its economic status. *I. duplicatus* has been known to respond to racemic ipsdienol (±)-2-methyl-6-methylene-2,7-octadien-4-ol (Bakke, 1975; Schlyter et al., 1987), but its pheromone includes also (*E*)-myrcenol, (2*E*)-2-methyl-6-methylene-2,7-octadien-1-ol (Byers et al., 1990). West palearctic (Norway/Sweden) *I. duplicatus* contains a blend of about 90:10 of ipsdienol–E-myrcenol (Id/EM) and a synthetic blend of these de novo produced components (Ivarsson et al., 1993) is highly competitive to natural pheromone emitted by unpaired males in spruce logs (Schlyter et al., 1992).

In spite of several qualitative tests (Bakke, 1975; Schlyter et al., 1987, 1992; Byers et al., 1990) and quantitative variations of the Id and EM combination (Schlyter et al., 1992), no test has varied the ratio of the two components, to find the bait with the optimum ratio for attraction in the field.

Field Response of Both Ips *Sexes*

Wick-Dispensers. For *Ips* trapping, we used a wick dispenser made of cotton yarn (Birgersson and Lejfalk, 1997) to deliver the two compounds. When releasing components of similar volatility, this dispenser delivers a range of proportion released without any accompanying change of total amount of released components. Compounds were dissolved in 4 ml octane as 1:100 (alcohols–octane) solutions in dark glass bottles, evaporating from a thin-walled Teflon tube-enclosed cotton wick yarn end (tube: 2.5 mm OD, 1.7 mm ID) at approximately 1 mg/day. Solutions

TABLE 1. PARAMETER ESTIMATES AND OTHER STATISTICS OF NONLINEAR REGRESSION (NLR) FITTING OF GAUSSIAN-CURVE TO TRAPPING RESPONSE AND PRODUCTION PROFILES OF *Ips duplicatus* FIELD POPULATIONS

Data set (dependent regression form)	*df*		r^2	Parameter estimates from NLR				
	Regression	Residual		Location ($\mu_{p\,or\,r}$) ±SE[a]	Width/2 ($1\sigma_{p\,or\,r}$) ±SE[a]	Form factor (f) ±SE[a]	Location ($\mu_{p\,or\,r}$)[b]	Width ($2\sigma_{p\,or\,r}$)[b]
♂♂ + ♀♀ Field Response								
NE China, 1992	3	4	0.98	1.071	0.176	0.736	0.77	0.29
R-arcsin $\sqrt{p}$, f[c]				±0.012	±0.016	±0.064		
Norway, 1993	3	4	0.93	0.911	0.410	1.179	0.62	.71
R-arcsin $\sqrt{p}$, f				±0.042	±0.040	±0.107		
Norway, 1994 Raw data	2	75	0.44	0.966	0.416		0.68	0.69
R-arcsin $\sqrt{p}$				±0.038	±0.038			
Norway, 1994	2	5	0.86	0.966	0.416		0.68	0.69
R-arcsin $\sqrt{p}$				±0.053	±0.053			
Norway, 1994	3	4	0.98	0.942	0.452	1.240	0.65	0.75
R-arcsin $\sqrt{p}$, f				±0.023	±0.024	±0.055		
Inner Mongolia, 1993	3	4	0.94	0.509	0.255	0.822	0.24	0.41
R-arcsin $\sqrt{p}$, f				±0.023	±0.030	±0.072		
Inner Mongolia, 1994	3	4	0.98	0.703	0.319	0.813	0.42	0.59
R-arcsin $\sqrt{p}$, f				±0.026	±0.020	±0.051		
Czechia, 1994	3	4	0.84	0.819	0.453	0.729	0.53	0.78
R-arcsin $\sqrt{p}$, f				±0.062	±0.024	±0.089		
♂ Emission of pheromone								
Norway	3	5	0.966	0.906	0.063	0.158	0.91[e]	0.13[e]
E-p, f[d]				±0.005	±0.005	±0.010		
Norway	3	5	0.973	1.318	0.150	0.354	0.94	0.14
E-arcsin $\sqrt{p}$, f				±0.027	±0.022	±0.054		

[a] Asymptotic SE from NLR (Norusis, 1994).
[b] Value retransformed from arcsin $\sqrt{p}$.
[c] R = response frequency (y axis value, dependent variable in regression), p = proportion of pheromone components (x axis value, independent variable in regression), f = form factor in model equations 3 and 5.
[d] E = emission frequency of pheromone producing males.
[e] Value based on regression of the untransformed p variable, hence not retransformed, but displayed in this column to facilitate comparisons.

TABLE 2. PARAMETER ESTIMATES AND OTHER STATISTICS OF NLR FITTING OF GAUSSIAN CURVE TO MALE RESPONSE AND FEMALE PRODUCTION PROFILES OF EARLIER PUBLISHED DATA FOR LEPIDOPTERA

Data set (dependent regression form)	*df*		r^2	Parameter estimates from NLR				
	Regression	Residual		Location ($\mu_{p\,or\,r}$) ±SE[a]	Width/2 ($1\sigma_{p\,or\,r}$) ±SE[a]	Form factor (f) ±SE[a]	Location ($\mu_{p\,or\,r}$)[b]	Width ($2\sigma_{p\,or\,r}$)[b]
♂♂ *Pectinophora gossypiella* Response Linn and Roelofs (1985)								
R-arcsin $\sqrt{p}$, f[c]	3	14	0.94	0.916 ± 0.011	0.176 ± 0.011	0.464 ± 0.025	0.63	0.33
♀ *Pectinophora gossypiella* Content of gland Collins and Cardé (1985)								
C-arcsin $\sqrt{p}$, f[d]	3	16	0.97	0.738 ± 0.001	0.027 ± 0.001	0.692 ± 0.003	0.45	0.054
♂♂ *A. velutinana* Response Roelofs et al. (1975)								
R-arcsin $\sqrt{p}$, f	3	7	0.92	0.305 ± 0.011	0.097 ± 0.011	0.223 ± 0.022	0.090	0.110
♀ *A. velutinana* Content of gland Miller and Roelofs (1980)								
C-arcsin $\sqrt{p}$, f	3	8	0.97	0.301 ± 0.002	0.030 ± 0.002	0.071 ± 0.004	0.088	0.034
♂♂ *S. litura* field Response Tamaki (1977)								
R-arcsin $\sqrt{p}$, f	3	5	0.79	1.243 ± 0.060	0.231 ± 0.062	0.551 ± 0.139	0.90	0.27
♂♂ *A. segetum* field Response Löfstedt (1985)								
R-p start value 0.5 and 0.1 (μ and σ)	2	3	−2.5	0.440 ± 0.02	0.07 ± 0.01		0.44[e]	0.14[e]
R-p start value 0.5 and 0.5	2	3	0.87	0.568 ± 0.043	0.413 ± 0.037		0.57[e]	0.82[e]
R-arcsin $\sqrt{p}$, f	3	2	0.95	0.809 ± 0.042	0.414 ± 0.051	1.001 ± 0.095	0.52	0.74

[a] Asymptotic SE from NLR (Norusis, 1994).
[b] Value retransformed from arcsin $\sqrt{p}$.
[c] R = response frequency (y axis value, dependent variable in regression), p = proportion of pheromone components (x axis value, independent variable in regression), f = form factor in model equations 3 and 5.
[d] C = gland content frequency of pheromone component proportions in females.
[e] Value based on regression of the untransformed p variable, hence not retransformed, but displayed in this column to facilitate comparisons.

TABLE 3. PARAMETER ESTIMATES AND OTHER STATISTICS OF NLR FITTING OF GAUSSIAN CURVE TO MALE WIND-TUNNEL RESPONSE AND FEMALE PRODUCTION PROFILES OF *Chilo partellus* IN THE LABORATORY

Data set (dependent regression form)	*df*		r^2	Parameter estimates from NLR				
	Regression	Residual		Location ($\mu_{p\,or\,r}$) ±SE[a]	Width/2 ($1\sigma_{p\,or\,r}$) ±SE[a]	Form factor (f) ±SE[a]	Location ($\mu_{p\,or\,r}$)[b]	Width ($2\sigma_{p\,or\,r}$)[b]
♂♂ Wind-tunnel Response								
Wing fanning	3	4	0.37	0.9 ±3	2 ±5	5 ±10	0.6	0.6
R-arcsin $\sqrt{p}$, f[c]								
Take off	3	4	0.66	0.32 ±0.03	0.49 ±0.10	1.2 ±0.22	0.1	0.5
R-arcsin $\sqrt{p}$, f								
Orientation	3	4	0.95	0.359	0.219	0.534	0.15	0.30
R-arcsin $\sqrt{p}$, f				±0.010	±0.015	±0.030		
50 cm flight	3	4	0.85	0.414	0.209	0.474	0.16	0.30
R-arcsin $\sqrt{p}$, f				±0.023	±0.032	±0.061		
100 cm flight	3	4	0.79	0.402	0.203	0.457	0.15	0.28
R-arcsin $\sqrt{p}$, f				±0.026	±0.035	±0.061		
150 cm flight	3	4	0.81	0.393	0.195	0.438	0.15	0.27
R-arcsin $\sqrt{p}$, f				±0.022	±0.029	±0.056		
200 cm flight (arrival at source)	3	4	0.88	0.387	0.165	0.385	0.14	0.23
R-arcsin $\sqrt{p}$, f				±0.017	±0.020	±0.040		
Copulation attempt with source	3	4	0.77	0.385	0.140	0.303	0.14	0.19
R-arcsin $\sqrt{p}$, f				±0.025	±0.029	±0.053		
♀ Content of pheromone gland								
C-arcsin $\sqrt{p}$, f[d]	3	3	0.99	0.367	0.106	0.274	0.13	0.14
				±0.007	±0.006	±0.014		

[a] Asymptotic SE from NLR (Norusis, 1994).
[b] Value retransformed from arcsin $\sqrt{p}$.
[c] R = response frequency (y-axis value, dependent variable in regression), $p =$ proportion of pheromone components (x axis value, independent variable in regression), f = form factor in model equations 3 and 5.
[d] C = gland content frequency of pheromone component proportions in females.

were prepared by making a concentrated stock solution of each compound and adding it in the required amounts to make up a 1% solution with a total of 40 mg of the two compounds combined in each vial (see *x* axis in Figure 1). The two components were ipsdienol, provided by Borregaard (Sarpsborg, Norway), and (*E*)-myrcenol, provided in 1992 by W. Francke (Organic Chemistry, University of Hamburg, Germany) and in 1993–1994 purchased from Phero Tech Inc. (Delta, British Columbia, Canada). Both compounds are monoterpene alcohols and have evaporation rates in the same range (Schlyter et al., 1992). A positive control consisted of a standard synthetic bait using polyethylene vials (Schlyter et al., 1992) releasing at total of ca. 1 mg/day of about 86% Id or about 200 male equivalents.

Study Areas and Traps. In Norway, trapping was done with a set of nine black nonsticky, standard Norwegian drainpipe traps (N79) as in an earlier test (Schlyter et al., 1992). The test sites were clear-cuts in a Norway spruce [*Picea abies* (L.) Karst.] forest from recent winter logging near Ås in SE Norway (Schlyter et al., 1992) in May–June in 1993 and, 1994. Traps were set up in a line and randomized using the same scheme in all sites.

In the Peoples Republic of China, black nonsticky, flight barrier (slot) traps (Theyson, Germany) were used in NE China 1992 and in Inner Mongolia 1993 and, 1994. In NE China, beetle response was tested in June 1993 in a recent clear-cut in a natural forest of *Larix gmelinii* L. with no prior record of *I. duplicatus*. The site used was near Xin-Lin (51°48′N, 125°38′E), ca. 150 km W of the Amur river in the Great Xingan Mountains. Nine traps were set up in a line ca. 30 m from the stand margin with 25 m between neighboring traps. Inner Mongolia beetles were sampled in 1993 and 1994 in a reserve of ca. 2000 ha of natural spruce forest [*Picea mongolica* (Wu, H.-Q.) Xu, formerly *P. koraiensis* Nakai or *P. meyeri* or *P. meyeri* var. *mongolica* Wu, as discussed by Xu (1994) and Liu (1994)] at Baiyinaobao (43°30′–36′N, 117°06′–16′E), Keshiketeng Qi (formerly Hexigten Qi) Inner Mongolia, China, which is situated in the grassland at the east edge of the Hunshandake desert, with a thick layer of sandy soil. Since the 1950s these stands, at the southwest margin of the natural distribution of *Picea mongolica* Xu, have been severely infested by sawflies and *I. duplicatus* (Liu, 1994; Zhang et al., 1995). The larger bark beetle competitor, *I. typographus*, has not been recorded here. In 1993 and 1994, two sets of nine traps were set up in small glades at the margin of the surrounding stand of dead trees killed by *I. duplicatus* the previous year. Traps were arranged in a curve in order to ensure a similar distance (ca. 15 m) to the insect source (the dead trees) for each trap. The distance between traps was ca. 8 m.

In the Czech Republic, black nonsticky, multifunnel traps (Lindgren trap, Phero Tech, Vancouver, British Columbia) were used in NE Moravia in Chuchelná near Opava (49°59′N, 18°8′E) in May 1994. The test site was a 1-year-old clear-cut in a monoculture of artificially planted Norway spruce forest (80 years old) at approximately 300 m above sea leavel. Since 1992, the largest European outbreak

of *I. duplicatus* has been recorded in this area (Knízek and Zahradník, 1996). Although most trees were attacked by *I. typographus*, many apparently healthy trees were killed by *I. duplicatus* alone.

Statistics. Number of replicates (trap rotations following a Latin-square randomization scheme) (Byers, 1991) and total catch for each study area and year are given in Figure 1. Statistical analysis of field trapping data from each replicate (trap rotation) was done by ANOVA, followed by Duncan's multiple-range test with SPSS/PC+ V5.01, on arcsin $\sqrt{p}$ transformed data to approach normality and homoscedasticity as judged by Barlett's and Cochran's C tests.

Ips *Male Aggregation Pheromone Production.* Collection of volatiles from individual unpaired wild males, freshly collected from Skotterud, Norway, established in host logs were made in the laboratory in Göteborg applying the method of Birgersson and Bergström (1989) as modified by Ivarsson (Ivarsson et al., 1993; Ivarsson and Birgersson, 1995). Logs of Norway spruce, *P. abies*, infested with *I. duplicatus* were collected outside Skotterud, Hedmark County, Norway. The beetles were reared out of the logs at 23°C and relative humidity 75% and stored in cooled containers (4°C) with moist paper. Prior to experiments, male beetles were allowed to fly in transparent boxes at 25°C, 85% relative humidity and 35,000 lx for 6 hr. Each beetle was put on a fresh *P. abies* bolt in a shallow predrilled hole (2.5 mm ID) and covered with a gelatin capsule (#00) fitting in the grooves, made with a cork borer (4 mm dram.), around the hole. As the beetles began to enter the bolt, the capsules were removed and the bolt placed in a climate chamber (75% relative humidity 23°C). During the first 20 hr, no collection of volatiles was made as the beetles excavated their nuptial chambers (Schlyter et al., 1992). After this time, Teflon tube columns (20 × 3 mm), filled with 35 mg Porapak Q (60–80 mesh, Supelco Inc, Belfonte, Pennsylvania) were placed outside each individual entrance hole (Birgersson and Bergström, 1989) and air at 20 ml/min was pumped through each column. After 3 hr of collecting, the columns were removed and eluted with 500 μl diethyl ether (p.a.; Fluka Chemie AG, Buchs, Switzerland) with 500 ng of quantification standard (heptyl acetate) added. The aeration extracts were concentrated to 100 μl prior to analysis. After aeration, the corresponding individual male hindgut extracts were done by the standard method (Birgersson et al., 1984; Schlyter et al., 1992; Ivarsson and Birgersson, 1995).

Analyses of the aeration extracts were performed by GC-MS on a Hewlett-Packard 5890II GC–Finnigan TSQ-700 quadrupole MS at 70 eV. The GC was fitted with a fused silica capillary column (45 m × 0.25 mm) coated with DB-Wax (PEG, $df = 0.25$ μm; J&W, Folsom, California). Helium was used as carrier gas at 25 cm/sec, and the temperature program was constant at 50°C for 5 min, 10°C/min to 230°C, held for 10 min. Injector and transfer line temperatures were 210°C and 230°C, respectively. The amounts of collected pheromone components [ipsdienol and (*E*)-myrcenol], were quantified against the added standard, and calculated based on a standard curve, covering 0.5–5000 ng. All extracts included here for

calculation of component proportions were above limit of quantification (LOQ) for both compounds.

Moth Chilo partellus *Data. Chilo* Zincken is a large genus in the Pyralidae family. The larvae of all *Chilo* spp. are stalk borers, several being serious pests on graminaceous crops (Moyhuddin, 1990). The sex pheromone of *C. partellus* has been studied as a tool for population monitoring and control methods. Nesbitt et al. (1979) established that the aldehyde, (*Z*)-11-hexadecenal (*Z*11–16:Ald) and the corresponding alcohol (*Z*)-11-hexadecen-1-ol (*Z*11–16:OH) were the major female sex pheromone gland constituents of virgin females of *C. partellus*, originating from India. Both major components are found in airborne volatiles from calling females (Nesbitt et al., 1979), suggesting a behavioral role for both components. However, when tested at several sites in Africa, the aldehyde alone or blends of the aldehyde and the alcohol were later found to be less effective in attracting male moths to traps (Beevor et al., 1990; Unnithan and Saxena, 1990, 1991), as well as in India (Seshu Reddy, personal communication). To test if both the major components previously identified were necessary, and, if so, to optimize the pheromone blend of *C. partellus*, we needed to test both the total dose of the two-component synthetic blend and the ratio between the two components. This was done by comparing the male pheromone response in a wind-tunnel bioassay to different pheromone blends. This approach also required a quantification of the individual female variation in the sex pheromone content, to compare the relationship between female production and male response.

Male Chilo *Flight Tunnel Response*

Insects. Chilo partellus moths were obtained from a laboratory culture established from field-collected material from Kenya and reared on a semisynthetic diet based on white beans. The culture was maintained in a climatic chamber (24°C, 80% relative humidity, 12-hr reversed scotophase), and was renewed regularly with field-collected material. The insects were sexed as pupae and kept separately under the same light and temperature regime. Males were collected every day and kept in humidified plastic boxes until use.

Pheromone Formulations. Chemicals, purchased from The Research Institute for Plant Protection, Wageningen, The Netherlands, were at least 98% pure with respect to geometrical and positional isomers. Stock solutions of each compound (1–50 μg/μl) were made up in hexane (Chemicon, redistilled p.a. quality). Blends of pheromone components to be tested were made by mixing various amounts of the stock solution and adding hexane to the desired concentration. All mixtures were checked before use, on a Hewlett Packard 5890 GC-FID equipped with a 30-m $\times$ 0.25-mm DB-1-coated fused silica capillary column (J & W, Folsom, California) to ensure purity.

Extracts of calling virgin females were made as described by Svensson (1996b). The female extracts and synthetic blends to be tested in the wind tunnel were applied to 1-cm^2 filter papers (Wratten 80) and the solvent was left to evaporate. This was done immediately before use. Filter papers with mixtures applied were used for only 15 min in the wind tunnel and then exchanged for new ones to ensure a relatively stable release rate.

Wind Tunnel Behavioral Tests. Wind-tunnel tests were performed in a $0.9 \times 0.9 \times 2.5$ m Plexiglas tunnel (Löfstedt et al., 1985) as described by Svensson (1996a). The environmental conditions were: 21–22°C, 50–60% relative humidity, 0.3 m/sec wind speed, light intensity 2 lux. The wind-tunnel tests took place 7–9 hr into the scotophase. The male insects were tested the first night after their eclosion and were conditioned at least 1 hr before testing. The physical wind-tunnel conditions were checked prior to every test. Vital single males were chosen from boxes containing emerged males and placed in glass tubes. The tube was sealed in both ends with a fine mesh until placed in the wind tunnel. The mesh was removed from one end and the tube held in the pheromone plume 200 cm downwind of the odor source. Males were given 1 min to respond. Males not responding during that time were discarded and recorded as nonresponders.

Three calling females, placed in a 12-cm-long $\times$ 28-mm-diam. glass tube, open in both ends, were used as a biological standard (the best possible stimuli) for every other treatment. All other treatments were run with the tester blinded with respect to the treatment tested. A mixture of 100 ng *Z*11–16:Ald and 30 ng *Z*11–16:OH applied on filter paper was used as the synthetic control in all tests unless otherwise described. By including both the best biological standard and a highly repeatable synthetic control, both the ultimate goal (attracting a virgin female) and the testing conditions (control of abiotic conditions) could be compared between different tests. All equipment, including all glassware that was used inside the wind tunnel, was heated over night between tests; metal stands for 12 hr 180°C, and glass tubes for 12 hr at 360°C, to prevent contamination. Eight behavioral steps were recorded (Table 3) and response expressed as percentage or proportion of tested males performing a behavior. Wind-tunnel data were analyzed by the method of adjusted significance levels of proportions (Ryan, 1960) for post hoc comparisons between treatments.

Dose–Response to Two-Component Synthetic Blend and Alcohol/Aldehyde. First, a dose–response study of the total amount at a fixed proportion was done. A two-component blend of *Z*11–16:Ald + *Z*11–16:OH (100 + 30 units of each, i.e., 23% alcohol of the total blend) applied on filter paper was made with the following doses: 1, 3, 10, 30, 300, 1000, and 3000 ng *Z*11–16:Ald, respectively. Each combination was tested on at least three different occasions, with calling females as controls. Secondly, different proportions of the two main components were tested at an optimal level of total amount. We added 2–40 ng *Z*11–16:OH to a fixed amount

of 100 ng *Z*11–16:Ald in an attempt to optimize the synthetic two-component blend. Seven different ratios of *Z*11–16:Ald and *Z*11–16:OH (from 100 + 2 to 100 + 40 units, or from 2 to 29% alcohol of total, Figure 3) were tested.

Female Chilo *Sex Pheromone Production*

Virgin females, in their second scotophase, were taken for extraction of pheromone components 9 hr into the scotophase and dissected. Single sex pheromone glands were extracted in 10 μl redistilled hexane (p.a. quality, Chemicon), with an internal standard of 10 ng (*Z*)-8-tridecenyl acetate, for 60 min. The extract was transferred to a 50-μl glass capillary, which was sealed immediately under an open flame. The capillaries were kept in a freezer until analysis. The analyses of individual female pheromone gland extracts were performed on a Hewlett Packard 5890 GC-FID. Gas chromatography parameters: 25 m $\times$ 0.25 mm DB-Wax column; temperature program: 70°C for 10 min, 70 to 230°C at 5°C/min, 230°C for 20 min. Hydrogen was used a carrier gas at 30 cm/sec.

RESULTS

Empirical Data

Field Response of Four Ips *Populations.* The first test in NE China in 1992 showed a marked preference by *I. duplicatus* for a high proportion of ipsdienol (% Id), close to 90% (90:10 Id/EM, Figure 1A). Overall catches were low due to a low population and allowed only three replicates. However, the validity of this small data set is shown by the unambiguous positive dose–response by the congener *I. sexdentatus* to an increase of Id up to and including 100% (Figure 1A). In contrast, *I. duplicatus* showed an optimum response, as the catches were lower at 99% Id and 100% Id baits than at the 90% Id, and this fitted well with the necessity of (*E*)-myrcenol as a synergist, as found in earlier tests in Norway (Byers et al., 1990; Schlyter et al., 1992). From the bell-shaped response profile it was obvious that the optimum was not located precisely at the treatment with the highest catch. The 90% Id bait had highest catch, but catches of the surrounding treatments were not symmetrically distributed around the 90% Id maximum (Figure 1A). Similar optima, situated between treatment values were observed later (Figure 1B–F).

In spite of few replicates due to cold weather, the test in Norway in 1993 showed a good response to 50% and 90% Id, as expected based on earlier results from this area (Schlyter et al., 1992) (Figure 1C). Data from Inner Mongolia in 1993 were quite surprising and almost a mirror image of those from NE China in 1992 and Norway in 1993. The best bait here was the 10% Id (10:90 Id/EM) followed by 50% Id (Figure 1B). These mirror-like results shed some doubt on

the reliability of bait preparation/labeling for China in 1993. Bait dispensers from China were not available for GC analysis, but three dispensers with pheromone solution left from Norway could be analyzed. GC results from these three baits showed no error in the Norway baits, proving the correctness of the solutions prepared, whereas the possibility of labeling errors for baits sent to China could not be disregarded.

The 1994 results from Inner Mongolia, Norway, and Czech Republic confirmed results of previous years (Figure 1D,1E). Inner Mongolia had the best catches for 50% Id followed by 10% Id, while Norway and Czech Republic had highest response to baits with $\geq$50% Id. The distribution of catch in Norway 1994 with ample replication, was very close to the results of the previous year (Figure 1C,F). The data from Inner Mongolia in 1994 were somewhat less extreme than data of the previous year, but both years had ample replication (Figure 1E). The results from Czech Republic had good catches with somewhat fewer replicates, and they were similar to the Norway data (Figure 1D). Sex ratios showed generally that the baits with the strongest attractivity had the lowest ratio of males. In other words, males—the pheromone-producing sex in *Ips*—had a wider response window than females, as shown for data from Norway in 1994 (Figure 1F).

Male Ips *Aggregation Pheromone Production.* In entrainments of emitted volatiles from field collected Norwegian males, there was a proportion (mean $\pm$ SD) of 86 $\pm$ 15% Id ($N = 30$). The same mean ratio of pheromone components was quantified from hindgut extracts: 85 $\pm$ 9% Id ($N = 27$). The frequency profile of the components emitted from males had a peak around 0.90 Id/(Id + EM), with a small left-tailed skewness (Figure 2). The absolute amounts quantified were higher in entrainments than in hindguts. For ipsdienol the amount in entrainments was 1800 $\pm$ 2100 ng with a range of 0–9600 ng ($N = 36$), while hindguts had only 300 $\pm$ 340 ng with a range of 0–1580 ng ($N = 32$).

Earlier data, based on batch analyses of males, agrees favorably with the present data, both from entrainments (85% Id, laboratory strain) and from hindgut extracts (82 and 86% Id, wild and laboratory strain) (Schlyter et al., 1992, 13–50 males per batch).

Chilo partellus *Dose–Response to Two-Component Synthetic Blend.* For all eight behavioral steps in the flight tunnel, the 100- or 300-ng doses gave the highest response. However, by post-hoc multiple range test there was no significant difference between baits with 30–1000 ng loading of the aldehyde for any of the eight steps (Figure 3A). The response pattern, e.g., doses that evoked the highest responses, became narrower and bell-shaped in the later behavioral steps (Figure 3A).

Chilo partellus *Dose–Response to Proportions of Alcohol–Aldehyde.* Seven different ratios of *Z*11–16:Ald and *Z*11–16:OH were tested for behavioral activity (Figure 3B). We found that an optimum synthetic blend of *Z*11–16:Ald and *Z*11–16:OH released a behavioral response as good as the corresponding amount

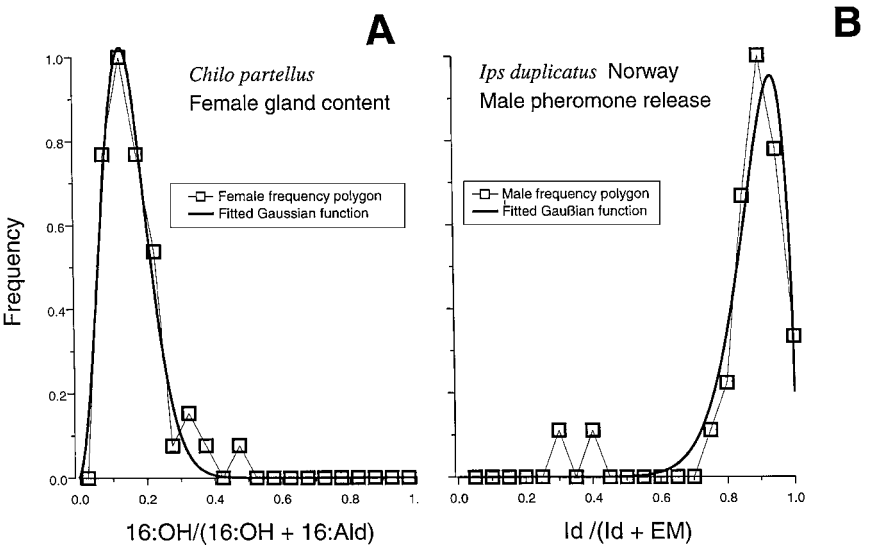


FIG. 2. Frequency distributions of production of pheromone component proportions (-□-) with overlaid estimated Gaussian curve (▬). Frequencies (*y* axes) were rescaled so that the bin with the highest frequency = 1. (**A**) Female pheromone production blends frequency profile of *Chilo partellus*, $N = 44$ females from a laboratory culture. See Table 3 for parameter estimates. (**B**) Male pheromone emission in *Ips duplicatus* from Norway, $N = 30$ wild collected males. For details of parameter estimates see Table 1.

of female gland extract. The male response to calling females was consistently around 90% in all tests ($N = 140$). However, calling females gave a slightly better response rate than synthetic baits at the later behavioral steps (Svensson et al., unpublished data). Both pheromone sources resulted in ca. 80% response at the 200-cm (source contact) behavioral step (Figure 3B). Response to the different ratios was apparently different, especially for the later behavioral steps (Figure 3B). The 17% OH treatment was the optimum, showing the highest response in all seven behavioral steps. However, by post hoc multiple-range test, usually only the 2% OH treatment was consistently different from the optimum (17% OH).

Female Chilo *Sex Pheromone Production.* Extracts of $N = 46$ *Chilo partellus* females showed that most female glands contained between 5% and 25% OH of the two main components, with a mean of ±SD 16.5% ± 8.9 (range 0–49%). However, there was a tail of the distribution of the production profile skewed towards higher values (Figure 2B). Some females had ≥30% alcohol component in their glands. By visual inspection, the male response profile (Figure 3B) seemed to have a somewhat wider window than that of the female production profile (Figure 2B). Gland extracts contained, on average, 74.2 ± 43.7 ng (11–202 ng) of the

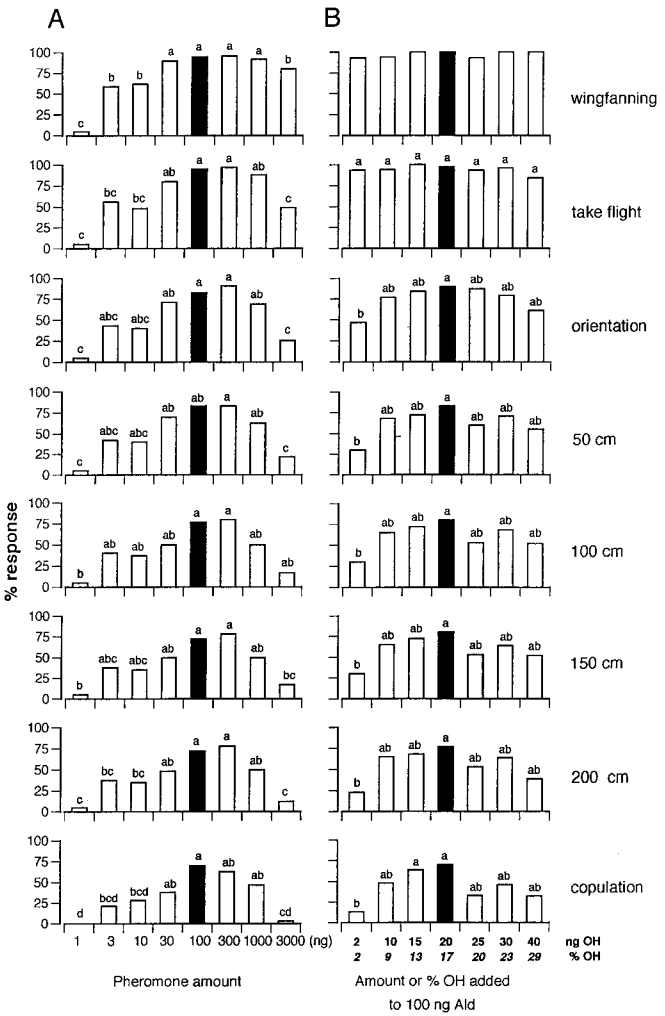


FIG. 3. Comparison of response frequency during different behavioral steps of male *Chilo partellus* in a wind tunnel to variation in total dose and proportions of components. Each combination was tested on at least three different occasions, with three calling females as controls. Bars with different letters are significantly different at $P < 0.05$, test of adjusted significance levels of proportions (Ryan, 1960). (A) Variation of total amount of the two-component mixture of Z11-16:Ald + Z11-16:OH (100:30) applied on filter paper, $N = 22$, 19, 24, 29, 31, 28, 28, and 22 for 1, 3, 10, 30, 300, 1000, and 3000 ng Z11–16:Ald, respectively. (B) Variation in proportion; different amounts of Z11–16:OH added to 100 ng Z11–16:Ald (100 + 2 to 100 + 40), comparable to 3 FE, and applied on filter paper, $N = 30$, 31, 25, 30, 15, 28, and 31 for 2, 10, 15, 20, 25, 30, and 40 ng OH, respectively.

major *Z*11–16:Ald component and 15.6 ± 13.7 ng (0–63 ng) of the minor *Z*11–16:OH component. The variation in amount of the aldehyde (CV % = 59), the main component, was thus somewhat lower than in amount of the alcohol (CV % = 88).

Model Parameter Estimation

Regression analyses for proportion (p) of pheromone components were generally more successful when arcsin $\sqrt{p}$ transformed $\bar{y}$ values were used than when the raw or linear y values were used (Tables 1 and 2). The arcsin $\sqrt{p}$ transformation of the independent axis was particularly valuable when empirical data was close to the extremes (0 or 1). The data on frequency of response or production (y axis) were rescaled so that the highest frequency was set at 1 for each data set, to facilitate comparisons between data sets. Plots of overlaid predicted values with the experimental data showed a subjectively pleasing fit in most cases (Figures 2, 3, and 6). Better estimates were obtained when using the mean of the response for each treatment ($\bar{y}$ values), rather than the raw data (e.g., Norway, 1994 data in Table 1).

Examples of plots with functions estimated from data that used the transformed x axis values but plotted on both the linear and transformed axis are given in Figure 4. On an arcsin $\sqrt{p}$ transformed independent axis (Figure 4A) both the data points and the profile obtained for response of *Ips duplicatus* in Norway, 1993 is more compliant with a Gaussian curve than when they are plotted on the linear (untransformed) x axis (Figure 4B). The fitted functions from other data sets for this species show the same change in shape (Figures 4C, D).

Iterations usually showed convergence in less than 100 iterations when using suitable starting values on good empirical material. In data sets with few values on the x axis or with a jagged profile, the convergence either took longer (>100 iterations) or sometimes arrived at local solutions giving globally impossible results. In some cases this was remedied by choosing starting values closer to those suggested by the raw data (e.g., *Agrotis segetum* males in Table 2). With the better empirical data sets, starting values were not at all important. This is normal for iterations of nonlinear regression systems in general (Norusis, 1994).

Ips duplicatus *Population Response and Male Production Models.* Fit to mean trap data estimates of Gaussian curves by NLR generally showed good correlation ($r^2 > 0.8$, Table 1); an example is the curve for Norway 1993 data shown in Figure 4A. The data from Norway 1993 and 1994 gave functions that looked similar (Figure 4C) and had fitted parameter estimates close to each other, although the better replicated data from 1994 had a somewhat better r^2 (0.98) than the 1993 data ($r^2 = 0.93$, Table 1). Especially for NE China 1992 data, the fitted Gaussian curve was narrower and the peak was much higher than the data points, but these x-axis points were based on only three replicates. Similarly, a somewhat higher absolute response value may be seen for Inner Mongolia 1993

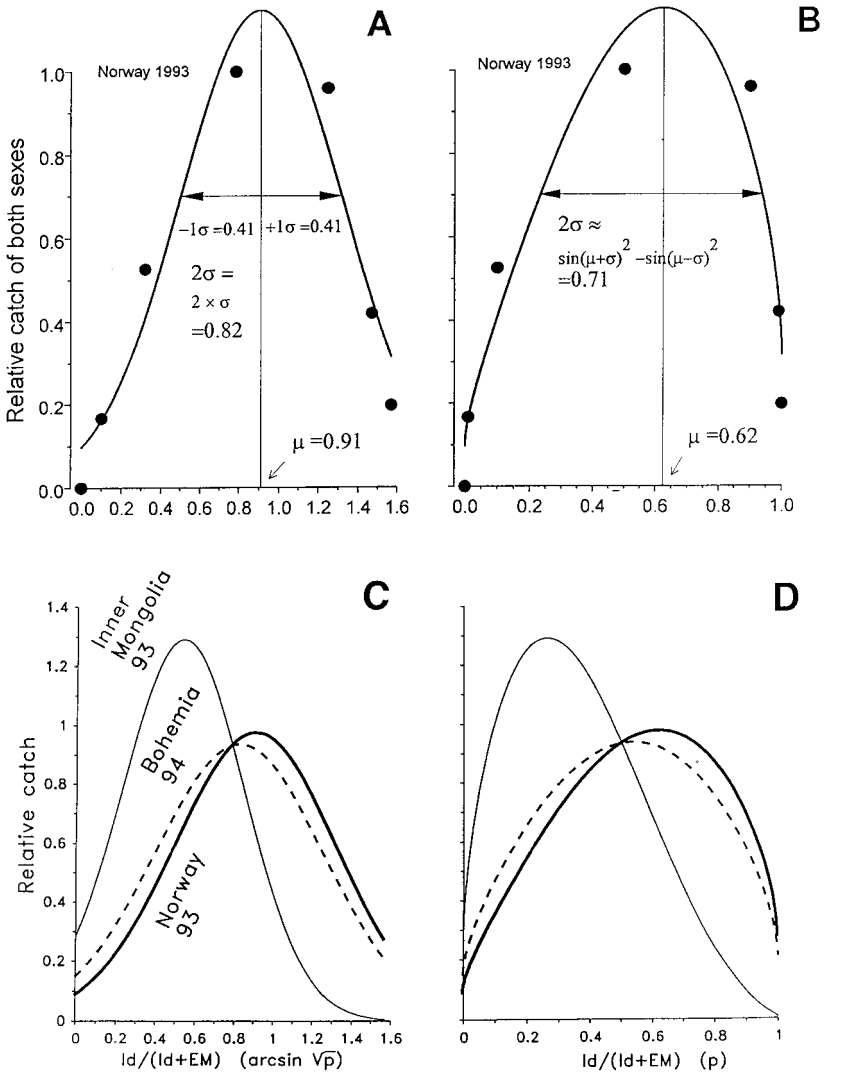


FIG. 4. Illustration of estimation on both transformed and untransformed independent axes by nonlinear regression (NLR) of Gaussian curve to relative catch profiles of *Ips duplicatus*. (**A**) The data set from Figure 1C with the fitted Gaussian curve; the x axis scaled as arcsin $\sqrt{p}$. (**B**) Same data and NLR curve as in A, but plotted on the x-axis scaled as p and with the formula for obtaining the window width on the untransformed scale. (**C**) Fitted Gaussian curves for selected data sets from Figure 1 with the x scale as arcsin $\sqrt{p}$. (**D**) As in C, but plotted on the x scale as p. Catch data (y axis) were rescaled so that the treatment with the highest catch = 1 for each data set. See Table 1 for parameter estimates.

data (Figure 4B). However, as the peaks of the fitted curves are between the data points, this is not an error in curve fitting. Instead, this means that the true maximum response is located between the fixed treatment values (x axis points for p values).

A summary of re-transformed estimates is shown below (number of trap rotations, N, in parenthesis):

NE China	1992	$\mu = 0.77$	$2\sigma = 0.29$	(3)
Inner Mongolia	1993	$\mu = 0.24$	$2\sigma = 0.42$	(9 + 9)
Inner Mongolia	1994	$\mu = 0.42$	$2\sigma = 0.59$	(9 + 9)
Norway	1993	$\mu = 0.62$	$2\sigma = 0.71$	(4)
Norway	1994	$\mu = 0.65$	$2\sigma = 0.75$	(11)
Czechia	1994	$\mu = 0.53$	$2\sigma = 0.79$	(6)

(For details of estimates see Table 1)

All data sets showed a peak of response (μ_r) located above 50% Id, except for the two sets from Inner Mongolia. The width of the response window ($2\sigma_r$) did not vary consistently with the location of the optimum. The number of replicates did not seem to influence window width, as the NE China 1992 sample with the fewest replicates ($N = 3$) had the lowest $2\sigma_r$, while the two Inner Mongolia samples with the largest number ($N = 18$) had intermediate values.

As an alternative way of separating the response from the different populations, cluster analysis was performed on the whole data set (Tóth et al., 1992), but with variable results. When including the active control, i.e., the earlier used 200 male equivalents bait (Schlyter et al., 1992), there was better resolution. However, in the cluster analysis, one may use five different methods for measurement of distances between clusters and four ways to combine cases into clusters, yielding $4 \times 5 = 20$ different plots. Of these, the cosine measurement together with average linkage combination of clusters methods gave a clustering result similar to the results obtained by our model.

Norway male beetles emitting pheromone (Figure 2) had a somewhat higher estimated peak location ($\mu_p = 0.94$ Id proportion) compared to the arithmetic mean of the sample, $\bar{x} = 86\%$ Id. This is due to the two values in the extreme left-hand tail of the male frequency polygon with a high EM content, influencing the $\bar{x}$, but not the Gaussian model production curve fitted by NLR (Figure 2). These data points were not included, as the model (equation 3) assumes and fits only a single, unimodal peak. To represent these two points would have required a model for two peaks or optima, as in equation 5. The estimated window width for production was $2\sigma_p = 0.14$, which was much smaller than that of Norway beetles responding (Figure 5), these having a window width of $2\sigma_r \approx 0.7$ both in 1993 and 1994 for their fitted response profiles (for details see Table 1).

Chilo partellus *Male Behavioral Steps and Female Production Models.* Male response width, when estimated by NLR, showed a marked decrease with the sequence of behavioral steps. The decreasing width of the response profile is plain to see when plotting the NLR estimated Gaussian curves for the sequential behavioral steps (Figure 5A). The overall fit of the regression was good (see Table 3 for details of parameter estimates), but the peak of the model response curve did not reach as high as 1.0, due to adjustment to the low values for the 20% OH treatment (Figure 3B and 5A). Quantification of the width of the response window with the progression of behavioral steps is shown in Figure 5B. The location of the peak did not change much after the two first behavioral steps (Figure 6B). After the first behavioral step, the continuous decline in response window width ($2\sigma_r$) with subsequent steps was highly significant ($r_S = -0.96$, $P = 0.0005$, $N = 7$). In contrast, the location of the peak response (μ_r), did not vary with behavioral step [$r_S = -0.14$, $P = 0.76$ (N.S), $N = 7$].

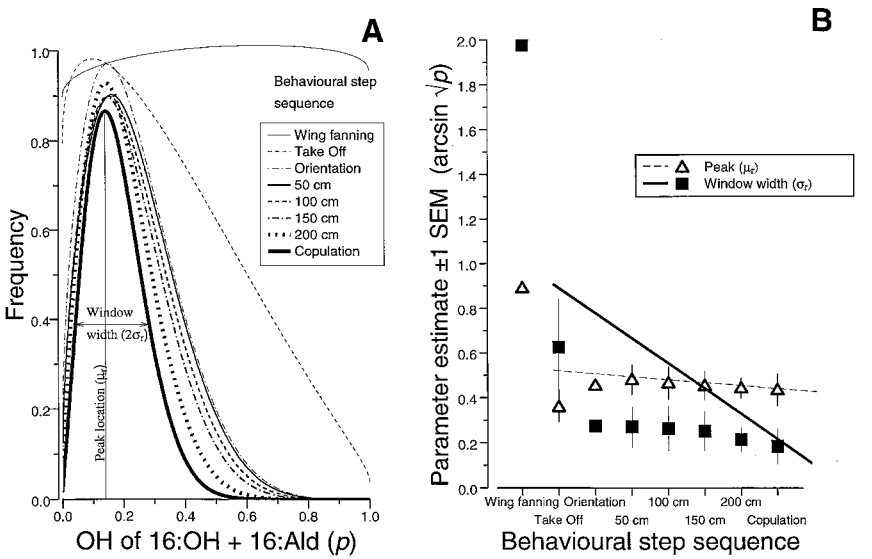


FIG. 5. Decrease in response width (σ) with behavioral steps sequence by male moths in flight tunnel. (**A**) Gaussian curve fitting by NLR to relative response frequencies of *Chilo partellus.* For raw data see Figure 3B and for details of parameter estimates see Table 3. (**B**) Decline in parameter values of μ_r (location of response peak) and σ_r (width of response profile) with the sequence of behavioral steps recorded. —) Regression for decline in response width, $P < 0.001$, $N = 7$, ----) Regression for change in peak location, $N = 7$, $p > 0.1$ NS. For both parameters the first point was omitted from analysis as their confidence intervals included zero (see Table 3).

The peaks of male response and female production were similar in their location ($\mu_p \approx 0.4$ on arcsin scale, Table 3). In contrast, the width of the female production window was somewhat smaller than that of the male response window, even at the last behavioral step. For the female gland content, the width was $2\sigma_p = 0.14$ compared to $2\sigma_r \approx 0.2$ for males in their two latest steps (Table 3).

DISCUSSION

Literature Data. The method for estimation of location and width of the response window developed here can be easily extended to other pheromone systems. Examples are given in Figure 6. Best fits are obtained with data that have a symmetrical profile and many values on the independent axis, such as those for *Pectinophora gossypiella* (Lepidoptera: Gelechiidae) (Linn and Roelofs, 1985) in Figure 6A. Highly asymmetrical profiles with few data points, such as that for *Spodoptera litura* (Lepidoptera: Noctuidae) (Tamaki, 1977), also can be fitted reasonably well (Figure 6B,C).

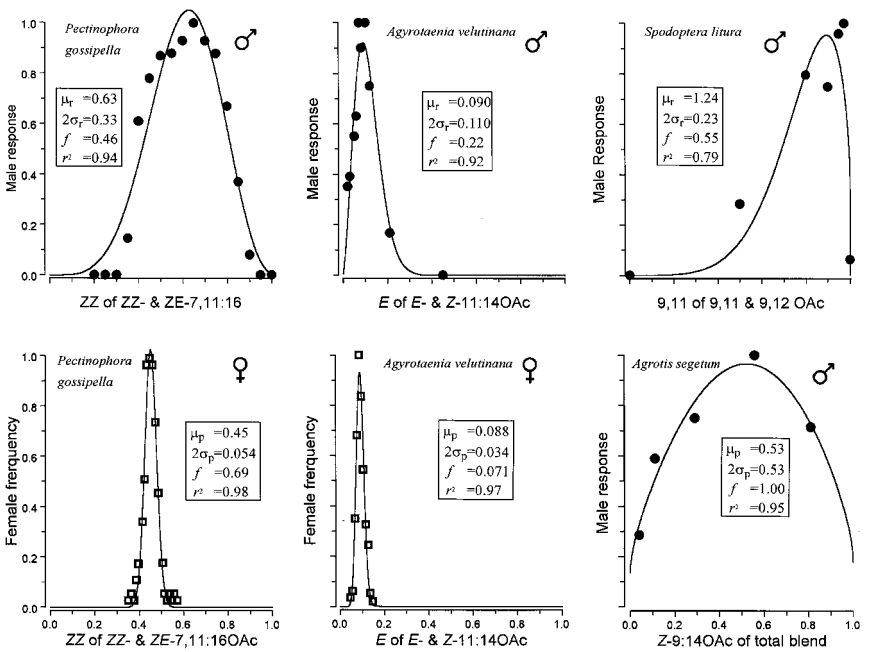


FIG. 6. Analysis of selected literature data on lepidopteran female sex pheromones; examples of Gaussian curve fitting by NLR to relative responses in males and production of females. For details of parameter estimates and references see Table 2.

Löfstedt (1990) plotted together the male response and female production of pheromone component ratios of *P. gossypiella* and *Argyrotaenia velutinana* and concluded that the male window of response was "relatively wider." Qualitatively, this conclusion seems reasonable when comparing Figure 6A vs. 6D and Figure 6B vs. 6E. Quantitatively, the $2\sigma_p$ for female production is 0.054 and 0.033, while the corresponding male window is more than twice as wide, having $2\sigma_r = 0.33$ and 0.11, for *P. gossypiella* and *Argyrotaenia velutinana*, respectively (Table 3).

Clearly, pheromone responses in other insect orders can also be analyzed as can responses to variation in amount of stimuli. Quantification of the response to variation in extreme enantiomer ratios in a sawfly is shown in Figure 7A. The variation in amount of pheromone in a fruit fly can be easily quantified by our method (Figure 7B).

The model may also be extended to analysis of data sets with a mixture of response profiles, where more than one peak of activity exists. A nice example of a sample with two optima for response and production is the *Ips pini* population in Princeton, British Columbia, Canada reported by Miller et al. (1996). This population seems to have two types of members belonging to one of two different signaling types, each producing and responding to ipsdienol of specific chirality, one with a peak near racemic (around 50/50) and one at high (+)-enantiomeric composition. A model for such a two-peak system can be expressed as the sum of two equations:

$$y = g_1(x) + g_2(x) = f_1\left[\frac{1}{\sigma_1\sqrt{2\pi}}e^{-(x-\mu_1)^2/2\sigma_1^2}\right] + f_2\left[\frac{1}{\sigma_2\sqrt{2\pi}}e^{-(x-\mu_2)^2/2\sigma_2^2}\right] \tag{5}$$

The two subfunctions, $g_1(x)$ and $g_2(x)$, are of the same form as equation (3) and their parameters are estimated by NLR in the same way. For the two-peaked model of this *I. pini* population sample, we assumed the width parameters to be the same, $\sigma_{p_1 \text{ or } r_1} = \sigma_{p_2 \text{ or } r_2}$, for the two response profiles in the population, while f, μ_p, and μ_r were allowed to vary. The fit to the raw frequencies of male production and population(s) response is good (Figure 8). In principle, the one-dimensional fitting along an x axis done here may be extended to a true two-dimensional response window, sensu Roelofs (1978), by fitting data on response to variation in both amount and proportion. Our present data sets do not allow this, as dose and proportion have not been independently varied over the whole parameter space.

Empirical Data. The wick dispensers for *Ips duplicatus* proved satisfactory over time, as they lasted for about 10 replicates (rotations) and with uniform release, as all baits went dry at approximately the same time. The optimum catch of the wick dispenser in Norway was similar to that of open PE vials used earlier (here active control) giving ≈200 ME (Schlyter et al., 1992). The traps used in the four different populations differed in their design and partly in their trapping principle.

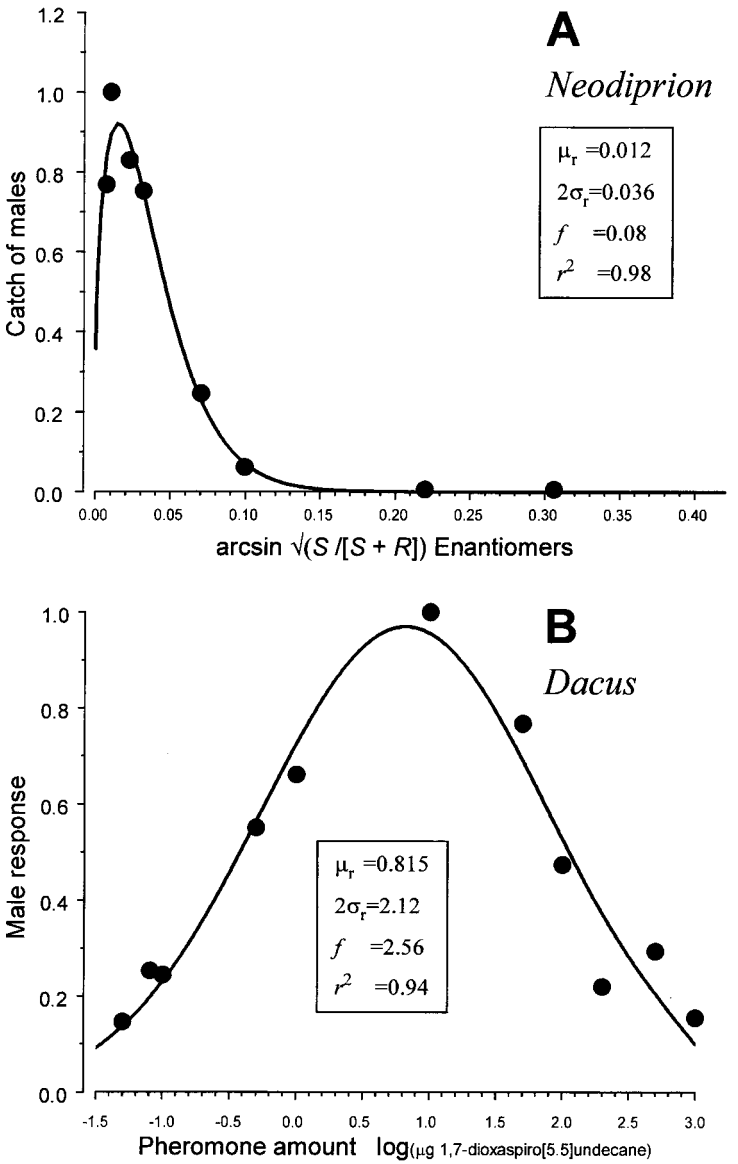


FIG. 7. Analysis of variation in attraction to extremely small proportion differences and to amounts of pheromone in two different insect orders. (**A**) Literature data of pine sawfly *Neodiprion sertifer* female sex pheromone; variation in proportion of diprionol enantiomers (Löfqvist, 1986). (**B**) Literature data of olive fruit fly *Dacus oleae*; variation in amount pheromone (Haniotakis and Pittara, 1994).

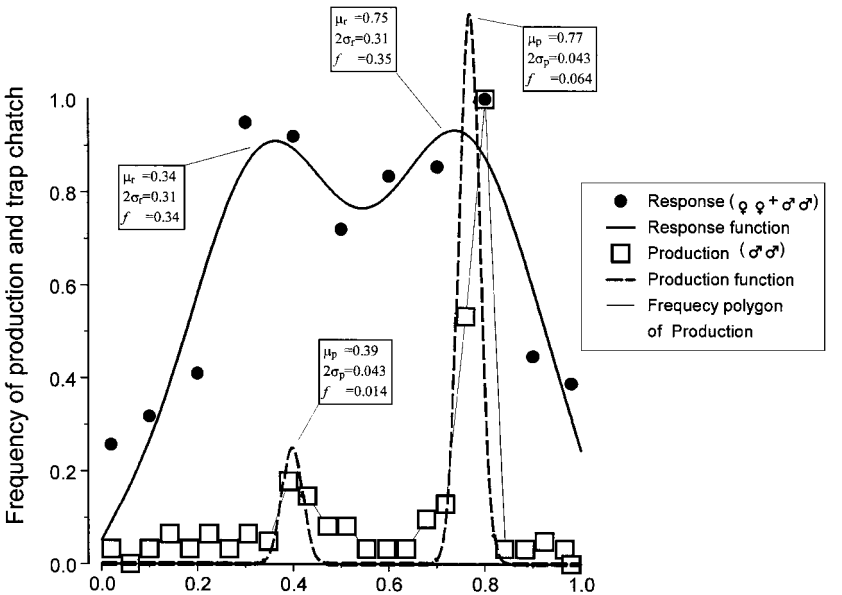


FIG. 8. Literature data of bark beetle *Ips pini* (Princeton, British Columbia, Canada) male aggregation pheromone; variation in proportion of (*R*)-(+)-ipsdienol enantiomer (Miller et al., 1996). Male enantiomer composition frequency profile (-□-) with double peaked Gaussian curve overlaid (**----**) and relative catches of both sexes at baits with varied enantiomer composition (●) with overlaid double peaked Gaussian curve (**—**). The type of equation fitted is equation 5, see Discussion.

While all provide a dark silhouette as a visual cue, the N79 model used in Norway in 1993 and 1994 requires beetles to both land and enter holes to get trapped. The Lindgren multifunnel trap (Czech Republic 1994) and the Theyson slot trap (China 1992–1994) are partly barrier traps in their function and require only that beetles orient close enough to the odor source to be intercepted and collected by a funnel or extended slot. A fraction of beetles probably still lands on the trap surfaces and later fall down to be trapped, but this has not been studied in detail. The similar sex ratios recorded in the whole material and the similarities between catches in Norway and Bohemia (Czech Republic) make it unlikely that trap design would have affected results. The most striking differences were in fact found between two localities in China using the same traps during the three years of study.

The results from the two years of sampling in Norway are almost identical in peak location and response width (window size). They also showed a very small cluster distance in the hierarchical cluster analysis. The two samples from Inner Mongolia differed somewhat in their location of optima but less in window width.

Correspondingly, the two Inner Mongolia data sets combined at larger cluster distance than the two Norway sets. The data sets that stand out from the others are the catches from Inner Mongolia in 1993 and 1994. Based on raw data showing the highest response for 50% and 10% Id baits, cluster analysis, and fitting of our model, this east Palearctic population seems to be unique. It should be noted that the geographic location of this population is 9° or about 1000 km west of the NE China locality of 1992. Thus, the Inner Mongolia population is not the population most distant from Europe, but it is biologically distinctive since it is isolated from other populations of *Ips duplicatus* and *Picea* (Zhang et al., 1995). Its host *Picea mongolica* Xu has been considered as a probably endemic taxon separate from both *P. koraiensis* and *P. meyeri* (Xu, 1981, 1994; Liu, 1994). In addition, both the bark beetle and the spruce tree live in a highly isolated environment in respect to the taiga, surrounded by grassland and on sandy soil. No *Ips typographus* could be detected by trapping with Pheropraxc pheromone dispenser in this area (Zhang, unpublished data). Therefore, the evolutionary direction of *I. duplicatus* population in Baiyinaobao, Inner Mongolia, together with its host tree, might have been genetically different from other populations, as in NE China and in Europe.

The analysis, or even accurate description, of differences in response to an array of several stimuli for different populations is not simple. Strictly speaking, this is a multivariate problem and cluster analysis is one possible method (Tóth et al., 1992). However, cluster analysis is a purely descriptive tool, and by using the many possible combinations of clustering techniques and distance measurements it is possible to get almost any dendrogram. We believe our method of fitting a Gaussian curve by NLR is more objective and quantitative, although it is still mainly descriptive. However, by use of the asymptotic SE (Table 1), it is possible to construct confidence intervals for the estimated parameters, as for any other standard univariate statistic. With our model, the several stimuli variables are reduced to one fitted function and the inherently more complex multivariate analysis types need not be evoked.

The variation of the proportion of the two major sex pheromone components of *C. partellus* was made in a fashion where the amount also was changed. The change in amount loaded on filter paper, however, was a minor one: the lowest dose was 100 μg and the highest 140 μg when ratio was changed (Figure 3B). The test of different total amount showed almost no difference between filter paper loads of 100 and 300 μg (Figure 3A). The two separate experiments gave the same result; unimodal pattern for both amount and ratio. This pattern is also seen in the other systems (Löfstedt, 1990).

Nesbitt et al. (1979) identified two major components in both female gland extracts and in female effluvia from *C. partellus*. However, they could only confirm a positive attraction effect for one of the components (*Z*11–16:Ald). Additions of the corresponding alcohol (*Z*11–16:OH) reduced the attraction. Our results

confirm the presence of these two major components in the gland extracts but, in contrast, indicate that both components are needed to achieve a high male response. The pheromone of *C. partellus* includes two major components; *Z*11–16:Ald and the corresponding alcohol *Z*11–16:OH, indicating a shared biosynthetic pathway.

The two pheromone components have also been identified in other *Chilo* species. *Z*11–16:OH is the major component of the sex pheromone of *C. infuscatellus* identified by Wu et al. (1984). A mixture of *Z*11–16:Ald and *Z*13–18:Ald was first identified as the female sex pheromone of the rice stem borer moth, *Chilo suppressalis* (Walker) (Nesbitt et al., 1975; Ohta et al., 1976). It was later shown that a synthetic two-component blend was less attractive in the field than live virgin females, although both components were shown to be essential for male attraction. A positional isomer of *Z*11–16:Ald, i.e., *Z*9–16:Ald, was later identified as a third pheromone component which drastically enhanced attractiveness to male moths in the field when combined with the two previously identified components (Tatsuki et al., 1983). However, in *C. partellus* we have no evidence that any of the minor gland constituents of the female have a positive effect on male pheromone attraction (Svensson et al., unpublished data).

Difference in Signaling Width of Production and Response

In most mating systems, the reproductive success of a male can be measured by the number of females he inseminates. In addition, the often strikingly male-biased operational sex ratio (OSR) in most insect species further rewards male reproductive strategies that lead to detection of as many females as possible (Emlen and Oring, 1977; Ims, 1988). In female-produced pheromone systems, the male detection propensity may be interpreted as the male response window. A male that is broadly tuned to female signals will probably have a greater chance of finding mates than a more finely tuned male, depending on female population variation. However, there may be a trade-off between the width of the male response window and his overall sensitivity (Svensson, 1996a), as well as a stabilizing or directional selection on male response if responding to sympatric species or different ecological races (Phelan, 1992). The differences in signaling width (2σ) between female pheromone production and male response in *C. partellus* seems to follow these predictions. The male response window is, however, not always much wider than the female production, as is the case in *Pectinophora gossypiella* (Figure 8). This may be explained by factors such as differences in the pheromone chemistry (*P. gossypiella* uses an easily controlled ratio between two isomers with the same vapor pressure) as well as differences in the OSR.

The aggregation pheromone systems in both *I. pini* (Figure 8) and *I. duplicatus* (Figures 1 and 2) show similar differences between production and response. The composition of pheromone components produced by males of both species is narrowly canalized ($2\sigma = 0.10$ and 0.14), whereas the response window by both

sexes in both *Ips* spp. is much wider ($2\sigma = 0.5$ and 0.3–0.8) (Table 1 and Figure 8). Schlyter and Birgersson (1989) quantified variation in amounts in samples of lepidopteran sex pheromones and in scolytid bark beetle aggregation pheromones. They found the coefficient of variation to be significantly higher in scolytid samples ($N = 9$) for the amounts of pheromone components than in moths ($N = 15$). This finding seems to contrast with the data on proportions of components quantified here. However, Schlyter and Birgersson (1989) did not find any difference between variation in proportions of components ($n_{\text{moths}} = 13$, $n_{\text{bark beetles}} = 6$) in samples from the two taxa. Thus, irrespective of the sex and taxa of the communicating insect, the responder seems be less precise than the sender. Interestingly, the absolute values estimated from both moths and bark beetles by our method were $2\sigma \leq 0.15$ for production but 0.3–0.8 for response. This fact may reflect both a common basic structure of constraints in these communication systems as well as the generality of our model.

A direct comparison of the descriptive and analytical power of our method is not possible as few authors have quantified widths of signaling windows, but our successful quantification of 26 profiles in 13 populations from 9 species indicates a rather wide usefulness.

Acknowledgments—We thank our colleagues in Lund and Alnarp for fruitful discussions of an early draft and Dr. A. El-Zayed for suggestions on a later draft, Mr. Erling Jirle for bark beetle breeding, and Ms. Eva Annerfeldt for field bait preparation. Dr. Marie Bengtsson supplied moth test solutions. We are grateful to Mr. Guang-Tian Liu (Baiyinaobao) and Mr. Dong Chu (Shenyang) for assistance in the field. Field work in China was partly supported by the Ministry of Forestry, Peoples Republic of China. We appreciate the financial support of the SAREC and NFR projects.

REFERENCES

BAKKE, A. 1975. Aggregation pheromone in the bark beetle, *Ips duplicatus* (Sahlberg). *Norw. J. Entomol*. 22:67–69.

BEEVOR, P. S., DAVID, H., and JONES, O. T. 1990. Female sex pheromones of *Chilo* spp. (Lepidoptera: Pyralidae) and their development in pest control applications. *Insect Sci. Appl.* 11:787–794.

BIRGERSSON, G., and BERGSTRÖM, G. 1989. Volatiles released from individual spruce bark beetle entrance holes: Quantitative variations during the first week. *J. Chem. Ecol.* 15:2465–2483.

BIRGERSSON, G., and LEJFALK, C. 1997. Wick-baits—a novel delivery system for testing potential semiochemicals. Abstract, 14th Annual Meeting of the International Society of Chemical Ecology Vancouver, July 12–16, P-31.

BIRGERSSON, G., SCHLYTER, F., LÖFQVIST, J., and BERGSTRÖM, G. 1984. Quantitative variation of pheromone components in the spruce bark beetle *Ips typographus* from different attack phases. *J. Chem. Ecol.* 10:1029–1055.

BYERS, J. A. 1991. BASIC algorithms for random sampling and treatment randomization. *Comp. Biol. Med.* 21:69–77.

BYERS, J. A., SCHLYTER, F., BIRGERSSON, G., and FRANCKE, W. 1990. *E*-myrcenol in *I. duplicatus*: An aggregation pheromone component new for bark beetles. *Experientia* 46:1209–1211.

COLLINS, R. D., and CARDÉ, R. T. 1985. Variation in and heritability of aspects of pheromone in pink boll worm moth, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 11:1583–1590.

EMLEN, S. T., and ORING, L. W. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science* 197:215–223.

HANIOTAKIS, G. E., and PITTARA, I. S. 1994. Response of *Bactrocera (Dacus) oleae* Males (Diptera: Tephritidae) to pheromones as affected by concentration, insect age, time of day, and previous exposure. *Environ. Entomol.* 23:726–731.

IMS, R. A. 1988. The potential for sexual selection in males: Effect of sex ratio and spatiotemporal distribution of receptive females. *Evol. Ecol.* 2:338–352.

IVARSSON, P., and BIRGERSSON, G. 1995. Regulation and biosynthesis of pheromone components in the double spined bark beetle *Ips duplicatus* (Coleoptera: Scolytidae). *J. Insect Physiol.* 41:843–849.

IVARSSON, P., SCHLYTER, F., and BIRGERSSON, G. 1993. Demonstration of *de novo* pheromone biosynthesis in *Ips duplicatus* (Coleoptera: Scolytidae): Inhibition of ipsdienol and *E*-myrcenol production by Compactin. *Insect Biochem. Mol. Biol.* 23:655–662.

KNÍZEK, M., and ZAHRADNÍK, P. 1996. Mass outbreak of *Ips duplicatus* Sahlberg, (Coleoptera, Scolytidae). Proceedings of XXth International Congress of Entomology, Firenze, Italy, August 25–31, p. 527.

KROKENE, P., and SOLHEIM, H. 1996. Fungal associates of five bark beetle species colonizing Norway spruce. *Can. J. For. Res.* 26:2115–2122.

LEAL, W. S., HASEGAWA, M., SAWADA, M., ONO, M., and UEDA, Y. 1994. Identification and field evaluation of *Anomala octiescostata* (Coleoptera: Scarabaeidae) sex pheromone. *J. Chem. Ecol.* 20:1643–1655.

LEKANDER, B., BEJER-PEDERSEN, B., KANGAS, E., and BAKKE, A. 1977. The distribution of bark beetles in the Nordic countries. *Acta Entomol. Fenn.* 32:1–100.

LINN, C., JR., and ROELOFS, W. 1985. Response specificity of male pink boll worm moths to different blends and dosages of sex pheromone. *J. Chem. Ecol.* 11:1583–1590.

LINN, C., JR., and ROELOFS, W. 1994. Pheromone communication in moths, pp. 263–300, *in* D. M. Lambert and H. Spencer (eds.). Speciation and the Recognition Concept: Theory and Application, John Hopkins University Press, Baltimore, Maryland.

LINN, C., JR., CAMPBELL, M., and ROELOFS, W. 1991. The effects of different blend ratios and temperature of the Oriental fruit moth sex pheromone. *Physiol. Entomol.* 16:211–222.

LIU, T. 1994. Studies on the recovery and development of *Picea mongolica* forest on Hunshandake desert. Inner Mongolia Forestry University, MSc thesis. 52 pp. (in Chinese; English abstract).

LÖFQVIST, J. 1986. Species specificity in response to pheromone substances in diprionid sawflies, pp. 123–129, *in* T. L. Payne, M. C. Birch, and C. E. J. Kennedy (eds.). Mechanisms in Insect Olfaction, Oxford University Press, Oxford.

LÖFSTEDT, C. 1985. Behavioral responses of male turnip moths *Agrotis segetum* to sex pheromone in a flight tunnel and in the field. *J. Chem. Ecol.* 11:1209–1221.

LÖFSTEDT, C. 1990. Population variation in moth pheromone communication systems and its genetic control. *Entomol. Exp. Appl.* 54:199–218.

LÖFSTEDT, C. 1993. Moths pheromone genetics and evolution. *Phil. Trans. R. Soc. Lond. B* 340:167–177.

MILLER, J. R., and ROELOFS, W. L. 1980. Individual variation in sex pheromone component ratios in two populations of the redbanded leafroller moth *Argyrotaenia velutinana*. *Environ. Entomol.* 9:359–363.

MILLER, D. R., BORDEN, J. H., and SLESSOR, K. N. 1996. Enantiospecific pheromone production and response profiles for populations of pine engraver, *Ips pini* (Say) (Coleoptera: Colytidae), in British Columbia. *J. Chem. Ecol.* 22:2157–2172.

MOYHUDDIN, A. I. 1990. Biological control of *Chilo* spp. in maize, Sorghum, and millet. *Insect Sci. Appl.* 11:721–732.

MRKVA, R. 1994. Die geographische Verbreitung und Schädlichkeit des nordischen Fichtenborkenkäfers (*Ips duplicatus* Sahlberg) in der Tschechischen Republik, pp. 76–88, *in* Anonymous (ed.). Sborník referátu z Celostátní konference Kurovcová kalamita: príciny, rozsah, ochrana Faculta lesnická a drevarská, Brno (in Czech; German abstract).

NESBITT, B. F., BEEVOR, P. S., HALL, D. R., LESTER, R., and DYCK, V. A. 1975. Identification of the female sex pheromones of the moth, *Chilo suppressalis. J. Insect Physiol.* 21:1883–1886.

NESBITT, B. F., BEEVOR, P. S., HALL, D. R., LESTER, R., DAVIES, J. C., and SESHU REDDY, K. V. 1979. Components of the sex pheromone of the female spotted stalk borer, *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae): Identification and preliminary field trials. *J. Chem. Ecol.* 5:153–163.

NORUSIS, M. J. 1994. SPSS Advanced Statistics 6.1. SPSS Inc., Chicago.

OHTA, K., TATSUKI, S., UCHIUMI, K., KURIHARA, M., and FUKAMI, J. 1976. Structures of sex pheromones of rice stem borer. *Agric. Biol. Chem.* 40:1887–1899.

PFEFFER, A. 1995. Zentral- und westpaläarktische Borken- und Kernkäfer (Coleoptera: Scolytidae, Platypodidae). Pro Entomologia, c/o Naturhistorische Museum Basel, Basel.

PHELAN, P. L. 1992. Evolution of sex pheromones and the role of asymmetric tracking, pp. 265–314, *in* B. D. Roitberg and M. B. Isman (eds.). Insect Chemical Ecology: An Evolutionary Approach, Chapman & Hall, New York.

POSTNER, M. 1974. Ips duplicatus, p. 455, *in* W. Schwenke (ed.). Die Forstschädlinge Europas 2, Paul-Parey, Hamburg.

ROELOFS, W. L. 1978. Threshold hypothesis for pheromone perception. *J. Chem. Ecol.* 4:685–700.

ROELOFS, W. L., HILL, A., and CARDÉ, R. T. 1975. Sex pheromone components of the redbanded leafroller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 1:83–89.

RYAN, J. A. 1960. Significance tests for multiple comparison of proportions, variances and other statistics. *Psychol. Bull.* 57:318–328.

SCHLYTER, F., and ANDERBRANT, O. 1993. Competition and niche separation between two bark beetles: Existence and mechanisms. *Oikos* 68:437–447.

SCHLYTER, F., and BIRGERSSON, G. 1989. Individual variation in bark beetle and moth pheromones—a comparison and an evolutionary background. *Holarc. Ecol.* 12:457–465.

SCHLYTER, F., LÖFQVIST, J., and BYERS, J. A. 1987. Behavioral sequence in the attraction of the bark beetle *Ips typographus* to pheromone sources. *Physiol. Entomol.* 12:185–196.

SCHLYTER, F., BIRGERSSON, G., BYERS, J. A., and BAKKE, A. 1992. The aggregation pheromone of *Ips duplicatus* and its role in competitive interactions with *I. typographus. Chemoecology* 3:103–112.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry, The Principles and Practice of Statistics in Biological Research, 3rd ed. W.H. Freeman and Company, New York, p. 677.

SVENSSON, M. 1996a. Pheromone-mediated mating system in a moth species. PhD thesis no. 116. Department of Ecology, Animal Ecology, Lund University, 128 pp.

SVENSSON, M. 1996b. Sexual selection in moths: The role of chemical communication. *Biol. Rev.* 71:113–135.

TAMAKI, Y. 1977. Complexity, diversity, and specificity of behavioral-modifying chemicals in Lepidoptera and Diptera, pp. 253–285, *in* H. H. Shorey and J. J. McKelvey, Jr. (eds.). Chemical Control of Insect Behavior, John Wiley & Sons, New York.

TATSUKI, S., KURIHARA, M., USUI, K., OHGUCHI, Y., UCHIUMI, K., FUKAMI, J., ARAI, K., YABUKI, S., and TANAKA, F. 1983. Sex pheromone of the rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae): The third component, *Z*-9-hexadecenal. *Appl. Entomol. Zool.* 18:443–446.

TEALE, S. A., HAGER, J. H., and WEBSTER, F. X. 1994. Pheromone-based assortative mating in a bark beetle. *Anim. Behav.* 48:569–578.

TÓTH, M., LÖFSTEDT, C., BLAIR, B. W., CABELLO, T., FARAG, A. I., HANSSON, B. S., KOVALEV, B. G., MAINI, S., NESTEROV, E. A., PAJOR, I., SAZONOV, P., SHAMSHEV, I. V., SUBCHEV, M., and SZÖCS, G. 1992. Attraction of male turnip moths *Agrotis segetum* (Lepidoptera: Noctuidae) to sex pheromone components and their mixtures at 11 sites in Europe, Asia, and Africa. *J. Chem. Ecol.* 18:1337–1347.

UNNITHAN, G. C., and SAXENA, K. N. 1990. Population monitoring of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) using pheromone traps. *Insect Sci. Appl.* 11:795–800.

UNNITHAN, G. C., and SAXENA, K. N. 1991. Pheromonal trapping of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) moths in relation to male population density and competition with females. *Appl. Entomol. Zool.* 26:17–28.

WU, D.-M., YAN, Y.-H., WANG, B.-H., and HUANG, H.-L. 1984. The chemical structure and field attractant effect of the sex pheromone of sugar-cane stem borer *Chilo infuscatellus* (Snellen). *Acta Entomol. Sin.* 27:367–368.

XU, W.-D. 1981. Community characteristics of the spruce forest in Baiyinaobao, Inner Mongolia. *J. Northeast For. Inst.* 2:61–68 (in Chinese; English abstract).

XU, W.-D. 1994. Study on the taxonomy of the spruce in Baiyinaobao, Inner Mongolia. *Res. Plants* 14:59–67 (in Chinese; English abstract).

ZHANG, Q.-H., SCHLYTER, F., and LIU, G.-T. 1995. Distribution, mortality, and sex-ratio of overwintering *Ips duplicatus* (Coleoptera: Scolytidae) in the soil of a *Picea koraiensis* reserve in Inner Mongolia, China, with a model of diffusion, pp. 109–122, *in* F. P. Hain, S. S. Salom, W. F. Ravlin, T. L. Payne and K. F. Raffa (eds.). Behavior, Population Dynamics, and Control of Forest Insects. Ohio State University OARDC, Wooster, Ohio.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

DENSITY-DEPENDENT CHEMICAL INTERFERENCE—AN EXTENSION OF THE BIOLOGICAL RESPONSE MODEL

A. SINKKONEN*

University of Turku
Satakunta Environmental Research Center
and Section of Ecology, Department of Biology
Konttorinkatu 1, 28900 Pori, Finland

(Received June 12, 2000; accepted March 11, 2001)

Abstract—The response of plants to many phytochemicals changes from stimulatory to inhibitory as the concentration of the phytochemical increases. In this paper, a previous biological response model is extended to yield estimates of plant responses to changes in phytochemical concentrations in the case of density-dependent phytotoxicity. This requires a knowledge of plant densities, phytochemical concentrations in soil, and the relationship between the two. According to this model extension, inhibition is a probable outcome in density-dependent chemical interference, but phytotoxic effects may become stimulatory as plant density increases. In addition, low phytochemical concentrations in soil may cause an increase in the slope of the biomass–density relationship compared to the slope of control treatments. Experimental data from the literature support this model extension, and in several cases density-dependent chemical interference can be estimated mathematically.

Key Words—Chemical interference, phytotoxicity, competition, model, density, biological response, plant–plant interactions.

INTRODUCTION

To date, only a few methods have been described to distinguish the effects of chemical interference from those of resource competition (Lockerman and Putnam, 1981; Weidenhamer et al., 1989; Nilsson, 1994; Pellissier, 1995; Weidenhamer, 1996; Wu et al., 2000). The methods described are suitable only in certain situations. Growth experiments that utilize the density-dependent nature of phytotoxic effects can be used when the source of phytotoxins is independent of the activity of target plants

* e-mail: aki.sinkkonen@utu.fi

0098-0331/01/0700-1513$19.50/0

and phytotoxins are homogenously distributed in the environment (Weidenhamer et al., 1989; Thijs et al., 1994; Weidenhamer, 1996; Wu et al., 2000). Examples of such situations are monocultures under phytotoxic exposure or vegetation growing beneath allelopathic trees.

Weidenhamer et al. (1989) expressed the density-dependent nature of phytotoxic interactions as a graphic model (Figure 5 in Weidenhamer et al., 1989). In this model, the relationship between log mean plant weight and log plant density is linear if direct chemical interference does not exist. If chemical interference exists, the log-weight–log-density slope shifts downwards as plant density decreases or phytotoxin concentration increases in the substratum. In other words, the fewer individuals that share the toxins, the more their total response is affected. If the concentration is sufficiently high, growth may be completely inhibited at low plant densities. Weidenhamer et al. (1989) presented several data sets in support of their model, with varying target species, effective chemicals, harvest times, plant densities, and rates of phytotoxins.

Carballeira et al. (1988) modeled plant responses to varying allelochemical concentrations graphically. They proposed that the response is stimulatory at low concentrations, reaches a maximum at a certain concentration, becomes inhibitory as the concentration increases, and ends in total inhibition. An et al. (1993) presented the phenomenon as a mathematical model, based on enzyme kinetics. In this model, the biological response (P) to an allelochemical is the difference between biological responses to the stimulatory (S) and inhibitory (I) attributes of the allelochemical:

$$P_x = 100 + S_x - I_x \tag{1}$$

$$= 100 + \frac{S_m X^q}{(K_S)^q + X^q} - \frac{I_m X^q}{(K_I)^q + X^q} \tag{2}$$

in which 100 is the control (%), P_x is the biological response at concentration X, S_x is the stimulatory attribute at concentration X, S_m is the stimulatory response at saturating concentration, and K_S is the concentration at which $S = S_m/2$. The term q is a constant that controls the shape of the curve and is connected to the number of active sites per enzyme molecule for the substrate. I_x, I_m, and K_I are the respective parameters of the inhibitory attribute (An et al., 1993).

In this article, the biological response model of An et al. (1993) is combined with the density-dependent model of Weidenhamer et al. (1989). The resulting extension of the biological response model is applied to data presented by Hoffman and Lavy (1978), Andersen (1981), Weidenhamer et al. (1989), and Thijs et al. (1994). Finally, some practical and theoretical implications of the model extension are discussed.

METHODS AND MATERIALS

Description of Model Extension. It is hypothesized that the models of both Weidenhamer et al. (1989) and An et al. (1993) are applicable. The model of An et al. (1993) presents the biological response of plants to varying phytochemical concentrations. The density-dependent model by Weidenhamer et al. (1989) is based on the dilution of these phytochemical concentrations. Obviously, such dilution occurs when all plants try to take up the same phytochemicals at any plant density. When N plants compete for the same phytochemicals, any one of them cannot take up more than one Nth of those phytochemicals. If plants differ in size or activity, some of them may absorb more than others, but the mean exposure to phytochemicals does not change significantly when the model of Weidenhamer et al. (1989) is applicable (see Weidenhamer et al., 1989; Thijs et al., 1994; Wu et al., 2000). Thus, the phytochemical concentration X_i per plant at density i is a fraction (or multiple) of a known concentration X_0 per plant at a known number of individuals (N_0). As a result, there is a linear correlation

$$X_i = \frac{N_0 X_0}{N_i} \tag{3}$$

between the concentration at density 0 and the concentration at density i. X in equation 2 can therefore be replaced with X_i in equation 3:

$$P_{itot} = C_{itot} + \frac{S_m(N_0X_0/N_i)^q}{(K_S)^q + (N_0X_0/N_i)^q} - \frac{I_m(N_0X_0/N_i)^q}{(K_I)^q + (N_0X_0/N_i)^q} \tag{4}$$

Equation 4 computes the total response (P_{itot}) of treated plants compared to a control treatment (C_{itot}) at density i. Note that equation 2 is expressed in percentages, but equation 4 is not. Note also that the model of Weidenhamer et al. (1989) considers the responses of individual treated plants at a range of densities where total biomasses are constant ($= C_{0tot}$) in control treatments. Therefore, P_{itot} is divided by the plant number (N_i) at density i, giving the final model (Figure 1):

$$P_i = C_i + \frac{\frac{S_m(N_0X_0/N_i)^q}{(K_S)^q + (N_0X_0/N_i)^q} - \frac{I_m(N_0X_0/N_i)^q}{(K_I)^q + (N_0X_0/N_i)^q}}{N_i} \tag{5}$$

C_i is the response of an average control plant at density i. If total plant biomasses are C_{0tot} in control treatments, C_i is a fraction (or multiple) of C_{0tot}:

$$C_i = C_{0tot}/N_i \tag{6}$$

Note that if $S_x > I_x$, $P_i > C_i$, and if $N_i \to \infty$, $(P_i - C_i) \to 0$. Note also that if $X_{iSOIL} < X_{jSOIL}$, then $N_{P_i=C_i} < N_{P_j=C_j}$, where the subscript *SOIL* refers to phytochemical concentration in soil and $i > 0$. Equations 3 and 6 must be true only at

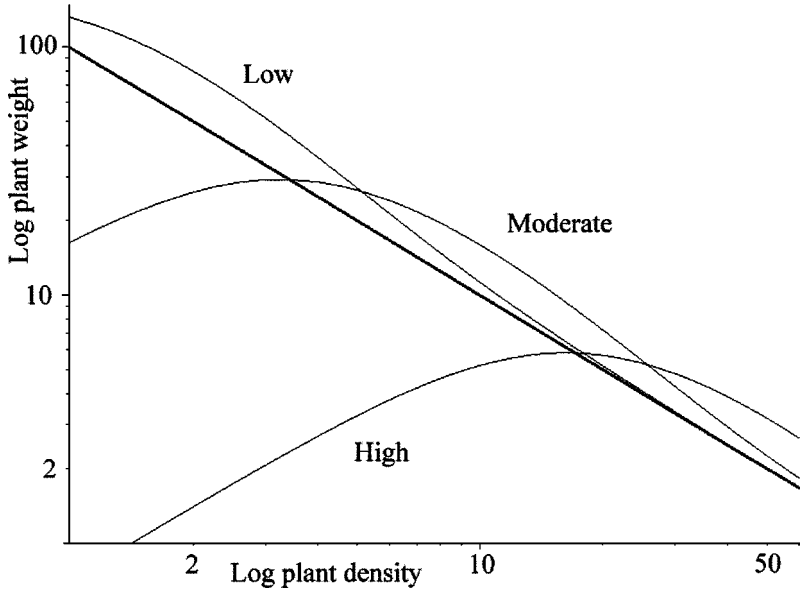


FIG. 1. Response of individual plants to plant density in arbitrary units at three phytotoxin concentrations. Straight line = nontreated controls. Default parameter values are $C_i = 100/N_i$, $N_0 = 1$, $q = 2$, $S_m = 100$, $I_m = 200$, $K_S = 5$ and $K_I = 30$. X_0 (low) = 20; X_0 (moderate) = 100; X_0 (high) = 500.

the densities and concentrations used. If $C_{itot} \neq C_{0tot}$, equation 6 may be redefined to suit the current situation.

Application of Model Extension. To examine the validity of the model, parameters were derived to suit data by Hoffman and Lavy (1978), Andersen (1981), Weidenhamer et al. (1989), and Thijs et al. (1994). Lagrangian interpolation and the Levenberg-Marquardt nonlinear regression were used to determine models for control treatments. The NLIN Procedure in SAS System for Windows Release 8 (SAS Institute Inc., Cary, North Carolina) was used to iterate all model parameters that minimized the difference between actual data values and the corresponding estimates of the used model. Finally, Pearson correlation coefficients were used to verify the accuracy of each model.

RESULTS

The effects of metribuzin residues on the yield of soybean (*Glycine max* L.) (Andersen, 1981) and the response of oat (*Avena sativa* L.) to atrazine (Hoffman and Lavy, 1978) have been parameterized (Figures 2 and 3). The lowest oat density

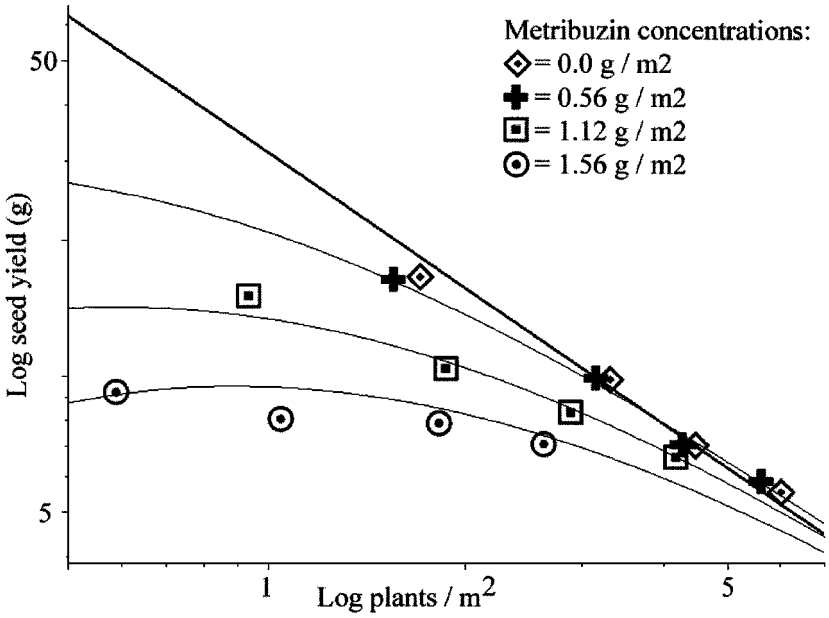


FIG. 2. Relationship of log plant density and log mean seed yield of individual soybean plants in the presence of residues of metribuzin. $C_i = 31.38/N_i . q = 1.11$, $S_m = 7.17$, $I_m = 40.35$, $K_S = 0.039$, $K_I = 0.73$, $N_0 = 1. r = 0.98$, $P < 0.001$. Values have been calculated from Andersen (1981).

(20 plants/pot) was excluded from analysis due to the exceptionally low dry weight of the control treatment (see Discussion).

Figure 4 shows the parameters modeled for soybean biomass at varying atrazine concentrations in a target-neighbor design by Thijs et al. (1994), where soybeans grew as neighbor plants and phytochemicals were added to the soil every other day. Competitive and chemical interference by the target plant, maize (*Zea mays* L.), decreased the suitability of the model extension at the lowest neighbor plant density, because the impact of the target plant increased as the number of neighbor plants decreased (see Niemeyer, 1988; Thijs et al., 1994). The lowest soybean density was, therefore, excluded from the analysis after many unsuccessful attempts ($P > 0.05$).

Figure 5 shows the parameters derived for shoot dry weights of bahiagrass (*Paspalum notatum* Fluegga) grown for five weeks in soil that had been treated with gallic acid and hydroquinone (Weidenhamer et al., 1989). It was not possible to calculate single parameters (see Discussion). Instead, the biomasses at different phytochemical concentrations of the substratum followed separate parameter values.

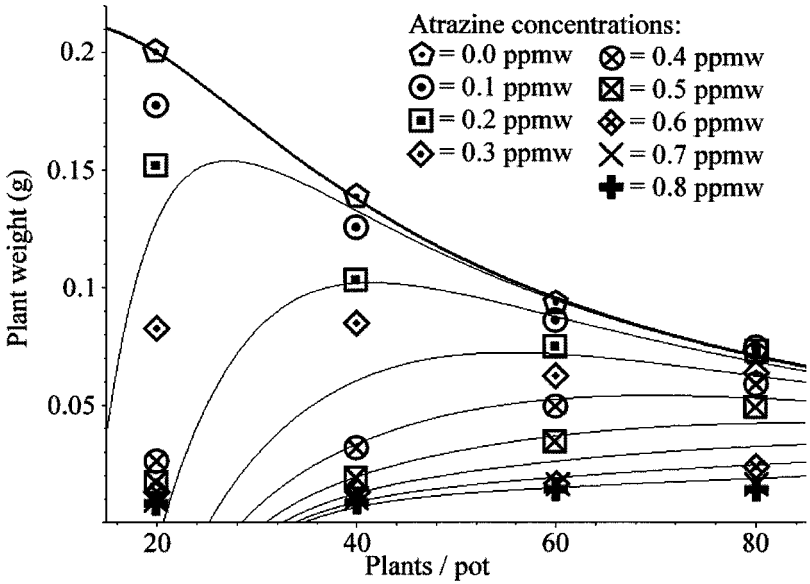


FIG. 3. Relationship of plant density and mean weight of individual oat plants grown in soil treated with atrazine. $C_i = 0.214 - 0.207^* e^{-196.16^* N_i^{-1.43}}$, $N_0 = 1, q = 3.21, S_m = 8.74$, $I_m = 14.2, K_S = 0.0065, K_I = 0.0067. r = 0.97, P < 0.001$. Values have been calculated from Hoffman and Lavy (1978).

DISCUSSION

Benefits of the Present Model. This extension of the model of An et al. (1993) is useful when estimating whether the response of plants to direct chemical interference can be distinguished from pure competition (Figures 2–5). In addition, this model extension attaches (both graphically and mathematically) growth-promoting interactions to the model of density-dependent phytotoxicity by Weidenhamer et al. (1989). They were aware of the possibility of growth- promoting chemical interference but did not include it in their graphic model. In density-dependent chemical interference, stimulatory effects do not necessarily differ greatly from the slope of control treatments at any plant density. The possibility of chemical interference should, therefore, be taken into consideration in cases where it may have been excluded previously.

This extension of the model of An et al. (1993) also has other benefits compared to the graphic model of Weidenhamer et al. (1989). The model extension does not require logarithmic conversion of the data. The best fit of the control treatments can be calculated with or without any conversion, and the result can be

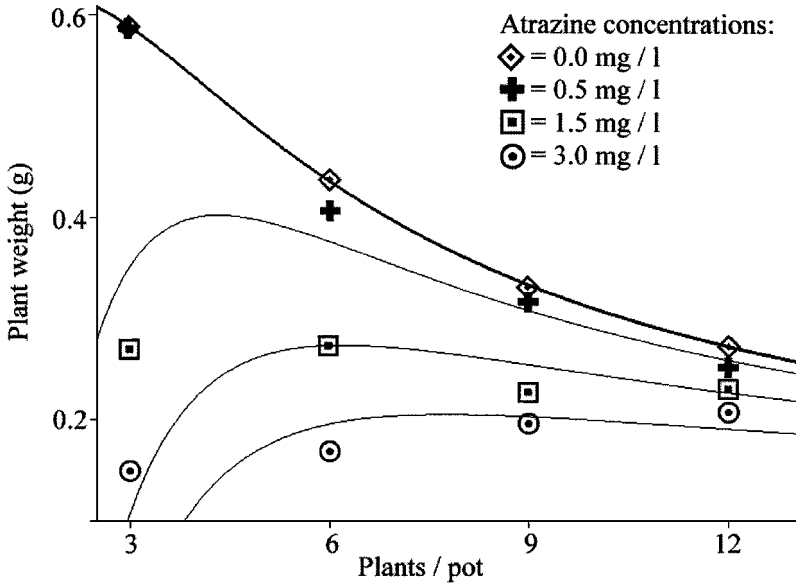


FIG. 4. Relationship of soybean density and individual soybean dry mass when corn was planted as target species and soil solution was treated with atrazine. $C_i = 0.627 - 0.525^* e^{-11.70^* N_i^{-1.37}}$. $N_0 = 1$, $q = 1.052$, $S_m = 4.545$, $I_m = 6.877$, $K_s = 0.143$, $K_I = 0.194$, $r = 0.98$, $P < 0.001$. Data from Thijs et al. (1994).

used as C_i in equation 5. If plants share most active chemicals at low densities, this model extension may not be limited to the densities of equal total plant biomasses in control treatments.

Limitations of the Present Model. Very high phytochemical concentrations and very low plant densities may not fit into this model extension. Plants may be so small or distances so great that phytochemicals cannot be diluted. Those conditions may create phytochemical-poor patches around plant roots that can be utilized with minor phytotoxic effects. A possible example of such conditions is the oat–atrazine bioassay by Hoffman and Lavy (1978). The dry matter of the control plants at the lowest density was two thirds that of the dry matter of control plants at other densities, and none of their treatments ended up in total growth inhibition (Figure 3). In addition, in that experiment, the seed number was approximated and no replacements of nongerminated seeds were made, which may negatively affect the accuracy of the parameters modeled.

The sensitivity of plants to phytochemicals may vary among treatments due to density-dependent stress (see Reigosa et al., 1999). The significance of such stress probably changes when the total dry matter of the treated plants has

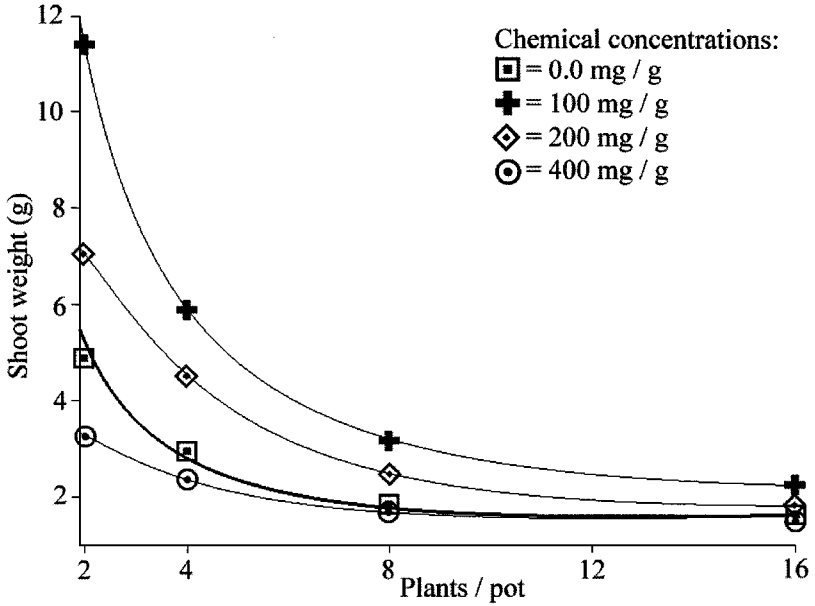


FIG. 5. Shoot dry weight of bahiagrass individuals grown in soil treated with gallic acid and hydroquinone. $C_i = 10.21/N_i + N_i/16.16$, $N_0 = 1$; ✚ : $q = 0.60$, $S_m = 32.91$, $I_m = 51.04$; $K_S = 1.16$ and $K_I = 42.80$; ◈ : $q = 1.51$, $S_m = 18.00$, $I_m = 33.79$; $K_S = 2.98$ and $K_I = 15.00$; ⊙ : $q = 1.15$, $S_m = 23.50$, $I_m = 39.55$; $K_S = 21.68$ and $K_I = 30.37$. $r = 1.00$, $P < 0.001$. Data from Weidenhamer et al. (1989).

reached its maximum. In accordance with that assumption, the lowest density could not be parameterized in the atrazine bioassay by Hoffman and Lavy (1978) (Figure 3), and the biomasses of the two lowest surviving plant densities possibly raised parametrized slopes in the metribuzin bioassay by Andersen (1981) (Figure 2).

The degradation of phytochemicals may also depend on the plant density or phytochemical concentration in the soil. For example, the quality of phytochemicals varied among treatments in the bahiagrass experiment by Weidenhamer et al. (1989). Hence, it was not surprising that common parameters could not be iterated for all three used phytochemical concentrations in that study. Instead, every concentration fit excellently in concentration-specific model parameters, as predicted by the present model (Figure 5). On the other hand, if the proportion of degraded phytochemicals was the same at all densities and concentrations used, and if the biological response of plants to phytochemicals was relatively linear at all occurring concentrations (see An et al., 1993), it should be possible to find common parameters for all treatments. The atrazine bioassay by Andersen (1981) may be

an example of such a situation, since single parameters were found even though atrazine probably degraded during the growing season (Figure 2).

When bioassays are conducted to study density-dependent chemical interference, it is essential to be aware of the other limitations of this model extension. Stimulatory effects may be slight due to intensive resource competition and other stress factors. More than one trend in the availability and activity of phytochemicals may be involved (see Gerig and Blum, 1991). In addition, the organisms studied may be autotoxic (Hall, 1989; Choesin and Boerner, 1991; Hedge and Miller, 1992). In general, as a consequence of any of these reasons, the results of a bioassay may not show parametrizable nonlinear weight–density slopes. In such cases, it may still be possible to determine graphically whether or not chemical interference is significant in a bioassay (see Weidenhamer et al., 1989) (Figure 1).

Implications of the Present Model. The case studies presented in this paper satisfy many of the assumptions of this model extension. When phytochemicals were added regularly, uniform parameters could be determined (Figure 4); when phytotoxins degraded nonlinearly, uniform parameters could not be found (Figure 5). Furthermore, changes in resource availability per plant affected the accuracy of the parameters modeled (Figure 3). However, bioassays designed to test this model extension are useful if we want to know whether or not the parameterization of a data set is actually due to the phytochemicals themselves. A time series of phytochemical concentrations in the soil may be a useful way to test the validity of this model in density-dependent growth experiments. If the assumptions of the model extension are true, uniform parameterization of randomly degrading phytochemical concentrations in the soil (with respect to original concentrations at different plant densities) will not be possible, but similarly degrading phytochemical concentrations may or may not be parameterized with single parameters depending on the linearity of the slope of plant responses to phytochemical concentrations (see An et al., 1993).

Based on the examples presented in this paper, the biological response model by An et al. (1993) is suited for modeling plant responses to density-dependent chemical interference. However, it may be possible to modify the model equations in some cases. First, equation 3 may be modified if an environmental factor, such as absorption of phytochemicals by soil particles or degradation of phytochemicals by soil microorganisms, alters the equation in a predictable way. Second, it may be possible to add a density-dependent stress factor to the model. This stress factor would change the power of stimulatory and inhibitory attributes at different plant densities. However, every modification must be based on empirical data showing a distinctive pattern that can be modeled.

One of the major problems in allelopathic studies has been the impossibility of estimating the role of rhizosphere microorganisms in allelopathic phenomena (Williamson and Weidenhamer, 1990; Inderjit and Dakshini 1995; Pellissier and Souto, 1999; Blum et al., 2000). In density-dependent chemical interference, the

phenomena observed may be affected by the quality, quantity, and patchiness of rhizosphere microorganisms, together with plant density, plant size, and phytochemical concentration. It is impossible to know whether this occurred in the examples presented in this paper. An appropriate method for testing the influence of soil microorganisms might be to measure the concentrations of potential phytochemicals in the soil as a time series in the presence and absence of rhizosphere bacteria and to compare this time series to parameterized plant responses in both situations.

Weidenhamer et al. (1989) speculated on the possible ecological implications of the density dependence of phytotoxic effects on the target species. Since stimulatory effects may exist regularly in the field, and since the balance between stimulation and inhibition depends on both plant density and the phytochemical concentration in the soil, the consequences of the effects of phytochemicals may perhaps be more diverse than has previously been assumed. The target species may, for instance, tend to evolve towards stimulation at higher phytochemical concentrations. In that case, however, the stimulatory effects will probably be weaker at low concentrations. Thus, the opposite of improved tolerance is also possible if plant densities are permanently high or phytochemical concentrations low. It may, therefore, be difficult to identify evolutionary adaptations to phytotoxicity without a detailed knowledge of the characteristics of every population and every chemical involved.

Acknowledgments—The author is grateful to Pia Mutikainen, Erkki Haukioja, Mikko Ojanen, Marie-Charlotte Nilsson, Kyösti Lempa, and two anonymous reviewers for commenting on earlier versions of the manuscript; Pekka Kaitaniemi for help with SAS; Ellen Valle for checking the language; and Emil Aaltonen Foundation for economic support.

REFERENCES

AN, M., JOHNSON, I. R., and LOVETT, J. V. 1993. Mathematical modeling of allelopathy: Biological response to allelochemicals and its interpretation. *J. Chem. Ecol*. 19:2379–2388.

ANDERSEN, R. N. 1981. Increasing herbicide tolerance of soybeans (*Glycine max*) by increasing seeding rates. *Weed Sci*. 29:336–338.

BLUM, U., STAMAN, K. L., FLINT, L. J., and SHAFER, S. R. 2000. Induction and/or selection of phenolic acid-utilizing bulk-soil and rhizosphere bacteria and their influence on phenolic acid phytotoxicity. *J. Chem. Ecol*. 26:2059–2078.

CARBALLEIRA, A., CARRAL, E., and REIGOSA, M. J. 1988. Asymmetric small-scale distribution and allelopathy: Interaction between *Rumex obtusifolius* L. and meadow species. *J. Chem. Ecol*. 14:1775–1786.

CHOESIN, D. N., and BOERNER, R. E. J. 1991. Allyl isothiocyanate release and the allelopathic potential of *Brassica napus* (Brassicaceae). *Am. J. Bot*. 78:1083–1090.

GERIG, T. M., and BLUM, U. 1991. Effects of mixtures of four phenolic acids on leaf area expansion of cucumber seedlings grown in Portsmouth B1 soil materials. *J. Chem. Ecol*. 17:29–40.

HALL, M. H. 1989. Alfalfa autotoxic fraction characterization and initial separation. *Crop Sci*. 29:425–428.

HEGDE, R. S., and MILLER, D. A. 1992. Concentration dependency and stage of crop growth in alfalfa autotoxicity. *Agronomy J*. 84:940–946.

HOFFMAN, D. W., and LAVY, T. L. 1978. Plant competition for atrazine. *Weed Sci*. 26:94–99.

INDERJIT, and DAKSHINI, K. M. M. 1995. On laboratory bioassays in allelopathy. *Bot. Rev.* 61:28–44.

LOCKERMAN, R. H., and PUTNAM, A. R. 1981. Mechanisms for differential interference among cucumber (*Cucumis sativus* L.) accessions. *Bot. Gaz*. 142:427–430.

NIEMEYER, H. M. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the Gramineae. *Phytochemistry* 27:3349–3358.

NILSSON, M.-C. 1994. Separation of allelopathy and resource competition by the boreal dwarf shrub *Empetrum hermaphroditum* Hagerup. *Oecologia* 98:1–7.

PELLISSIER, F. 1995. Allelopathy from ecosystem to cell in spruce [*Picea abies* (L.) Karst.] forests, pp. 65–79, *in* S. S. Narwal and P. Tauro (eds.). Allelopathy: Field Observations and Methodology. Scientific Publishers, Jodhpur, India.

PELLISSIER, F., and SOUTO, X. C. 1999. Allelopathy in northern temperate and boreal semi-natural woodland. *Crit. Rev. Plant Sci*. 18:637–652.

REIGOSA M. J., SÁNCHEZ-MOREIRAS A., and GONZÁLES L. 1999. Ecophysiological approach in allelopathy. *Crit. Rev. Plant Sci*. 18:577–608.

THIJS, H., SHANN, J. R., and WEIDENHAMER, J. D. 1994. The effect of phytotoxins on competitive outcome in a model system. *Ecology* 75:1959–1964.

WEIDENHAMER, J. D. 1996. Distinguishing resource competition and chemical interference: Overcoming the methodological impasse. *Agron. J*. 88:866–875.

WEIDENHAMER, J. D., HARTNETT, D. C., and ROMEO, J. T. 1989. Density-dependent phytotoxicity: Distinguishing resource competition and allelopathic interference in plants. *J. Appl. Ecol*. 26:613–624.

WILLIAMSON, G. B., and WEIDENHAMER, J. D. 1990. Bacterial degradation of juglone: Evidence against allelopathy? *J. Chem. Ecol*. 16:1739–1742.

WU, H., PRATLEY, J., LEMERLE, D., and HAIG, T. 2000. Laboratory screening for allelopathic potential of wheat (*Triticum aestivum*) accessions against annual ryegrass (*Lolium rigidum*). *Aust. J. Agric. Res*. 51:259–266.

Journal of Chemical Ecology, Vol. 27, No. 7, 2001

ASSOCIATION OF CARBON DISULFIDE WITH PLANTS IN THE FAMILY FABACEAE

JASON PILUK,[1] PETER G. HARTEL,[1,*] BRUCE L. HAINES,[2] and DAVID E. GIANNASI[2]

Departments of [1]Crop and Soil Sciences and [2]Botany
University of Georgia
Athens, Georgia 30602-7272

(Received September 20, 2000; accepted March 11, 2001)

Abstract—We examined 40 taxa from nine genera within the subfamily Mimosoideae; 29 (73%) from six genera produced carbon disulfide (CS_2). In addition, 19 of 40 taxa (48%) produced carbonyl sulfide (OCS). Of nine mimosoid taxa that produced CS_2, all possessed a djenkolic acid and a cysteine lyase. Of three mimosoid taxa that did not produce CS_2, two lacked a cysteine lyase and one lacked both a lyase and a djenkolic acid. Of 16 taxa from 14 genera from the other two subfamilies of the Fabaceae, the Caesalpinioideae and Papilionoideae, none produced CS_2. The results suggest that CS_2 production is common in the Mimosoideae and uncommon in the Caesalpinioideae and Papilionoideae and that plants in the Mimosoideae that do produce CS_2 must possess both a djenkolic acid and a cysteine lyase for this production to occur.

Key Words—Carbonyl sulfide, cysteine lyase, djenkolic acid, organic sulfide.

INTRODUCTION

The family Fabaceae consists of three subfamilies, the Caesalpinioideae, Mimosoideae, and Papilionoideae. When the roots of some plants in the Fabaceae are injured or wetted, they produce carbon disulfide (CS_2), a volatile, toxic, foul-smelling liquid (Haines et al., 1987, 1989; Hartel and Reeder, 1993; Feng and Hartel, 1996). Within the Mimosoideae, the presence of CS_2 has been reported in *Acacia pulchella* R. Br. (Whitfield et al., 1981), *Enterlobium cyclocarpum* (Jacq.) Griseb., *Leucaena multicapitula* Schery, *Mimosa pigra* L.,

* To whom correspondence should be addressed. e-mail:pghartel@arches.uga.edu

0098-0331/01/0700-1525$19.50/0

Pithecellobium arboreum (L.) Urban [=*Cojoba aborea* (L.) Britton & Rose], *Pithecellobium catenatum* Donn. Sm., *Pithecellobium pedicellare* Ajillo, *Stryphnodendron excelsum* Harms (Haines et al., 1987, 1989), *Mimosa pudica* (L.) (Hartel and Reeder, 1993), and all known *Leucaena* spp. (Feng and Hartel, 1996). Within the Caesalpinioideae and Papilionoideae, the presence of CS_2 has been reported in *Gliricidia sepium* (Jacq.) Kunth ex. Walp. (Haines et al., 1987), *Medicago sativa* L., *Phaseolus* sp., and *Pisum* sp. (Westberg and Lamb, 1984). Thus, it appears that the presence of CS_2 is potentially common in the Fabaceae.

Our studies with the roots of the mimosoid species *M. pudica* suggest that CS_2 is produced through the hydrolysis of a djenkolic acid by a cysteine lyase (Piluk et al., 1998). Djenkolic acid [3,3′-methylenedithiobis(2-aminopropionic acid)] is a nonprotein, sulfur amino acid consisting of two cysteine molecules bound at the sulfhydryl groups by a C atom. With regards to djenkolic acid, many other mimosoid species contain djenkolic acid or its analogs, including *Acacia acapulcensis* Kunth (Evans et al., 1993), *Acacia farnesiana* (L.) Willd. (Gmelin et al., 1962), *Acacia georginae* F.M. Bailey (Ito and Fowden, 1972), *Acacia lemmonnii* Rose (Gmelin, 1959), *Acacia saficina* Lindley (Seneviratne and Fowden, 1968), *Acacia willardiana* Rose (Gmelin, 1959), *Albizia lophantha* (Willd.) Benth. [=*Paraserianthes lophantha* (Willd.) I. Nielsen subsp. *lophantha*] (Gmelin et al., 1957), *Desmanthus brachylobus* Benth. (von Krauss and Reinbothe, 1970), *Desmanthus chacoensis* Burkart (von Krauss and Reinbothe, 1970), *Desmanthus depressus* Hook & Berneby ex. Willd. (von Krauss and Reinbothe, 1970), *Desmanthus virgatus* (L.) Willd. (von Krauss and Reinbothe, 1970), *Dichrostachys glomerata* (Forsskal) (Gmelin et al., 1962), *Neptunia gracilis* Benth. (von Krauss and Reinbothe, 1970), *Piptadenia macrocarpa* Benth. (von Krauss and Reinbothe, 1970), and *Pithecellobium lobatum* Benth. (von Krauss and Reinbothe, 1970). With regards to a cysteine lyase, seeds (Gmelin et al., 1957) and roots (Schwimmer and Kjaer, 1962) of *Albizia lophantha* and the roots of *A. julibrissin* Durazz. (Mazelis and Fowden, 1973) and *Acacia farnesiana* (Mazelis and Creveling, 1975) possess *S*-alkyl cysteine *a-b* lyase (EC 4.4.1.6). It may be that production of CS_2 through the hydrolysis of a djenkolic acid by a cysteine lyase is the general mechanism for CS_2 production in the Fabaceae.

The objectives of this study were twofold: (1) to determine how common CS_2 production was in plant species within the Fabaceae, and (2) to determine from a select subset of CS_2-producing plants if this production was always associated with the presence of a djenkolic acid and a cysteine lyase. To do this, plants of 40 species from 9 genera within the subfamily Mimosoideae and 16 species from 14 genera in the subfamilies Caesalpinioideae and Papilionoideae were selected for testing. Because microorganisms can produce CS_2 (Kelly and Smith, 1990), all plants were grown gnotobiotically.

METHODS AND MATERIALS

For the taxa surveyed, seeds were obtained from Banco Latinoamericano de Semillas Forestales, Centro Agronomico Tropical de Investigacion y Ensenanza (CATIE); J. L. Hudson, Redwood City, California; GoldKist Farm and Garden, Athens, Georgia; Oxford Forestry Institute, Oxford, United Kingdom; Peter B. Dow Seed Company, Gisborne, New Zealand; Southern Regional Plant Introduction Station, Griffin, Georgia; Vaughan's Seed Company, Downers Grove, Illinois, and the seed collection at the University of Georgia. In addition, researchers collected seeds of various species for us from Costa Rica, Georgia (USA), Guatemala, India, and Senegal.

To increase germination rates, seeds possessing a thick seed coat (>0.5 mm) were scarified around their edges with fine sandpaper (220 grit) until the endosperm or cotyledon was just visible; areas adjacent to the hilum were not scarified. Seeds with thin and thick seed coats were surface-sterilized with 0.7 *M* NaOCl for 15 and 30 min, respectively and germinated on water agar (15 g agar per liter of distilled water) until the radicles emerged. Individual seedlings were aseptically transferred to gnotobiotic plant assemblies (Hartel et al., 1993) supplying both macro- (McClure and Israel, 1979) and micronutrients (Ahmed and Evans, 1960). Plants were grown under metal halide lights with an average light intensity of 550 μmol photons/m^2/sec. Average day/night temperatures on a 16L:8D cycle were 26 and 24°C, respectively.

Potential maximum production of CS_2 was quantified by using a sulfur-specific gas chromatograph calibrated against a CS_2 permeation standard as described by Piluk et al. (1998). The low limit of detection for CS_2 was 0.1 ng/ml. Some plant species also produced carbonyl sulfide (OCS), hydrogen sulfide (H_2S), methane thiol (CH_3SH), ethane thiol (CH_3CH_2SH), or a mixture of these gases. When detected, these compounds were also quantified against the appropriate permeation standard. The low limits of detection for OCS, H_2S, CH_3SH, and CH_3CH_2SH were 0.2, 0.2, 0.2 and, 0.5 ng/ml, respectively.

The presence of a cysteine lyase among a select group of CS_2-producing plants was determined as described by Piluk et al. (1998). The presence of a djenkolic acid among the same group of plants was determined using high-performance liquid chromatography with modifications of the methods of Bidlingmeyer et al. (1984) and Randle et al. (1995) as described in Piluk et al. (1998). *Mimosa pudica* was the positive control, and *Glycine max* (L.) Merr. and *Pisum sativum* L. Alaska were the nonmimosoid, non-CS_2-producing controls.

RESULTS

Of 40 taxa from nine genera within the subfamily Mimosoideae, 29 taxa (73%) from seven genera produced CS_2 from their roots (Table 1). In addition,

TABLE 1. RELEASE OF CS_2 AND OCS FROM ROOTS OF GNOTOBIOTICALLY GROWN PLANTS IN SUBFAMILY MIMOSOIDEAE (FAMILY FABACEAE)

Species	Individuals		Days after planting	Incubation time (min)	Root production (ng/mg dry weight, mean ± SE)		Seed source[a]
	Tested (*N*)	Positive (*N*)			OSC	CS_2	
Acacia acuminata Benth.	4	0	30	8	ND[b]	ND	1
A. aneura F. Muell. ex. Benth.	3	0	25	8	ND	ND	1
A. auriculiformis A. Cunn ex. Benth.	4	4	50	10	ND	0.01 ± 0.01	1
A. baileyana F. Muell.	3	1	35	>10	ND	0.11	1
A. baileyana cv. purperea	5	4	30	8	0.01	0.15 ± 0.05	1
A. brachybotrya Benth.	5	5	50	9	0.01	0.07 ± 0.03	1
A. caffra (Thunb.) Willd.[e]	6	6	35	8	0.19 ± 0.05	0.84 ± 0.58	1
A. cyclocarpum (J.C. Wendl.) Willd.	8	8	50	9	0.07 ± 0.03	0.04 ± 0.02	1
A. dealbata Link.[c]	4	4	35	8	0.10 ± 0.09	0.36 ± 0.36	1
A. elata A. Cunn. ex. Benth.	5	4	45	9	0.12 ± 0.06	0.18 ± 0.09	1
A. farnesiana (L.) Willd.[c]	5	5	20	10	1.90 ± 3.20	0.43 ± 0.36	1
A. floribunda (Vent.) Willd.	4	4	45	10	ND	0.04 ± 0.02	1
A. x *giraffae* Willd.[c]	4	4	25	10	ND	0.84 ± 0.84	1
A. glaucocarpa Maiden and Blakely	2	2	40	8	0.26 ± 0.03	0.44 ± 0.06	1
A. greggii A. Gray	5	0	25	8	ND	ND	1
A. macradenia Benth.	5	5	45	9	0.25 ± 0.17	0.66 ± 0.22	1
A. melanoxylon R. Br.[d]	5	3	25	9	ND	0.20 ± 0.13	1
A. mucronate H.L. Wendl.	5	3	45	10	0.02	0.03 ± 0.01	1
A. nilotica (L.) Willd. ex. Del.[c–e]	5	5	15	10	0.01	0.15 ± 0.04	9
A. nilotica var. *tomentosa* (Benth.) Brenan	3	3	25	>15	ND	< 0.01	8
A. suaveolens (Smith) Willd.	5	1	45	>10	ND	0.01	1
A. tortilis (Forsskal) Hayne	4	2	15	>10	ND	0.10	8

A. triptera Benth.	4	0	45	8	ND	ND	1
A. uncinate Lindley	9	8	35	8	0.07 ± 0.04	1.01 ± 0.85	1
Albizia caribeae (Urban) Britton and Rose	3	0	30	10	ND	ND	7
A. lophantha (Willd.) Benth.	5	5	30	10	0.21 ± 0.06	0.26 ± 0.11	2
A. julibrissin Durazz.	4	0	30	10	ND	ND	6
Desmanthus illinoensis (Michaux) MacMillan ex. Robinson & Fern.[c]	5	5	20	20	0.51 ± 0.37	1.08 ± 0.33	1
D. velutinus Scheele	4	4	30	10	0.53 ± 0.16	0.54 ± 0.18	3
D. virgatus (L.) Willd.	3	3	30	9	6.26 ± 4.85	4.10 ± 2.40	3
D. virgatus var. depressus (Humb. and Bonpl. ex Willd.) B. Turner	4	1	30	>10	ND	0.32	3
Enterlobium cyclocarpum (Jacq.) Griseb.	3	0	30	10	ND	ND	4
Mimosa diplotricha C. Wright ex. Sauvalle	4	0	30	10	ND	ND	3
Neptunia dimorphantha Domin	4	4	25	9	0.91 ± 0.45	0.15 ± 0.06	1
Pithecellobium elegans Ducke[c]	5	5	30	10	1.00 ± 0.41	1.85 ± 0.50	5
P. unguise-cati (L.) Benth.	4	3	30	9	0.01	0.38 ± 0.14	5
Prosopis alba Griseb.	3	0	30	10	ND	ND	1
P. chilensis (Molina) Stuntz	3	0	30	10	ND	ND	1
P. pubescens Benth.	4	0	30	10	ND	ND	1
Schrankia microphylla (Dryander) J.F. Macbr.	5	4	35	10	ND	0.15 ± 0.12	6

[a] 1, J.L. Hudson, Redwood City, Calif.; 2, Peter B. Dow Seed Company, Gisborne, New Zealand; 3, Southern Regional Plant Introduction Station, Griffin, Ga.; 4, Banco Latinoamericano de Semillas Forestales, Centro Agronomico Tropical de Investigacion y Ensenanza (CATIE); collected from: 5, Costa Rica; 6, Georgia (USA); 7, Guatemala; 8, Senegal; or 9, India.
[b] ND, none detected.
[c] Traces of hydrogen sulfide detected.
[d] Traces of methane thiol detected.
[e] Traces of ethane thiol detected.

18 of 40 (45%) taxa produced OCS from roots. No taxon produced OCS without also producing CS_2. Of three *Albizia* species, only *A. lophantha* produced CS_2 and OCS from roots. Of 24 *Acacia* taxa, 20 (84%) produced CS_2 from their roots and 12 (50%) also produced OCS. Roots of *Desmanthus virgatus* produced the highest amounts of both CS_2 and OCS. Only two species, *Desmanthus illinoensis* (Michaux) MacMillan ex. Robinson & Fern. and *D. velutinus* Scheele, produced CS_2 and OCS from shoot tissues (includes cotyledons), and these were detected in only trace amounts. Of the 29 mimosoid taxa that produced CS_2, individuals of 17 taxa (59%) produced CS_2 every time. In the remaining 12 taxa, CS_2 production was variable and at least one or more individual plants did not produce CS_2.

Hydrogen sulfide was also produced from the roots of *Acacia* x *giraffae* Willd. (2.4 ± 1.2 ng/mg dry root) and *Pithecellobium elegans* Ducke (3.1 ± 2.3 ng/mg dry root), and occasionally in the roots of *Acacia dealbata* (Link.), *Acacia farnesiana, Acacia nilotica* (L.) Willd. ex. Del., and *Desmanthus illinoensis*. The roots of some mimosoid taxa also produced trace amounts of methane thiol (*Acacia melanoxylon* R. Br. and *Acacia nilotica*) and ethane thiol (*Acacia caffra* (Thunb.) Willd. and *Acacia nilotica*). With the exception of *Acacia* x *giraffae* and *Pithecellobium elegans*, not every individual plant was positive for these trace gases.

Of 15 species from 14 genera in the subfamilies Caesalpinioideae and Papilionoideae, none produced CS_2 (data not shown). These were *Caesalpinia ferrea* C. Martius ex. Tul., *Cercidium microphyllum* (Torrey) Rose & I.M. Johnson, *Clianthus formosus* (G. Don) Ford & Vick, *Crotalaria longirostrata* Hook. & Arn., *Dalea purpurea* Vent., *Desmodium gangeticum* (L.) D.C., *Desmodium incanum* D.C., *Gledistia triacanthos* L.f. *inermis* (L.) C. Schneider, *Gliricidia sepium, Glycine max, Parkinsonia aculeata* L., *Pisum sativum* Alaska, *Schizolobium parahyba* (Vell. Conc.) S.F. Blake, *Sesbania formosa* (F. Muell.) N. Burb., and *Vigna acontifolia* (Jacq.) Marechal. Incidental, trace amounts of methane thiol were detected in the roots of some individuals of *S. parahyba*, and ethane thiol was detected in some individuals of *G. max* and *S. formosa*. As with some plants from the Mimosoideae, not every individual plant was positive for these trace gases.

Of nine mimosoid taxa that produced CS_2 from roots, all showed the presence of a cysteine lyase and a djenkolic acid (Table 2). Of three mimosoid species that did not produce CS_2, two lacked a cysteine lyase and one lacked both a lyase and a djenkolic acid. Neither of the two nonmimosoid, non-CS_2-producing species examined, *Glycine max* and *Pisum sativum* Alaska, possessed a cysteine lyase or a djenkolic acid.

DISCUSSION

This is the first report of CS_2 production from the roots of *Albizia, Desmanthus, Neptunia,* and *Schrankia* species, and additional reports of CS_2

TABLE 2. CS_2 PRODUCTION, PRESENCE OF DJENKOLIC ACID, AND CYSTEINE LYASE ACTIVITY DETECTED (+) OR NOT DETECTED (−) IN ROOTS OF PLANTS WITHIN SUBFAMILY MIMOSOIDEAE[a]

Subfamily (species)	CS_2	Djenkolic acid	Lyase activity
Mimosoideae			
Acacia elata	+	+	+
Acacia x *giraffae*	+	+	+
Acacia greggii	−	+	−
Albizia julibrissin	−	−	−
Albizia lophantha	+	+	+
Desmanthus virgatus	+	+	+
Leucaena cuspidata	+	+	+
Leuceana retusa	+	+	+
Mimosa pudica	+	+	+
Neptunia dimorphantha	+	+	+
Pithecellobium elegans	+	+	+
Prosopis alba	−	+	−
Papilionoideae			
Glycine max	−	−	−
Pisum sativum Alaska	−	−	−

[a] *Mimosa pudica* was the positive control, and *Glycine max* and *Pisum sativum* Alaska were the non-mimosoid, negative controls. Authority names are given in the text and Table 1.

production from two *Pithecellobium* and 20 *Acacia* species. Therefore, of ca. 65 genera in the subfamily Mimosoideae, eight of the 10 tested genera (for a total of 50 species) produce CS_2. As with some *Acacia*, *Pentaclethra*, and *Pithecellobium* species (Haines et al., 1987), some species within a genus did not produce CS_2 (e.g., *Albizia*). One species, *E. cyclocarpum*, previously described as a CS_2 producer (Haines et al., 1987), did not produce CS_2 when grown under gnotobiotic conditions.

Of 15 gnotobiotically grown taxa from the Caesalpinioideae and Papilionoideae, none produced CS_2. Although earlier reports described CS_2 production from *G. sepium* and *Pisum* sp. (Westberg and Lamb, 1984; Haines et al., 1987), we did not observe CS_2 production when these species were tested. Although this may be because of genetic variability, a more likely possibility is that the plants in these earlier studies were grown in nonsterile soil and the soil microorganisms produced the CS_2 (Kelly and Smith, 1990).

In addition to CS_2, many plants from the subfamily Mimosoideae also produced OCS. Carbonyl sulfide is toxic, although its toxicity is only half that of CS_2 (Peyton et al., 1978). With the exception of several *Leucaena* species (Feng and Hartel, 1996) and two *Desmanthus* species, all OCS production was in the roots.

The quantitative amounts of CS_2, OCS, and trace sulfides produced from gnotobiotic plants were consistent for some species and variable for others. Reasons

for this variability are unclear but may be because of genetic variability, the labile nature of many S-containing compounds (in which the intermediate products of an enzyme reaction can often lead to more than one end product; Cavallini et al., 1980), or the highly reactive nature of S-containing gases with various materials (Kuster and Goldan, 1987). In this study, it is unlikely that environmental conditions were responsible for the variability because conditions for sulfide production were optimal (Feng and Hartel, 1996).

Within the family Fabaceae, our results suggest that CS_2 production is common in the Mimosoideae and uncommon in the Caesalpinioideae and Papilionoideae. Within the Mimosoideae, the results show that both a djenkolic acid and a cysteine lyase were required for CS_2 production. This was shown for eight of the 11 previously untested species (i.e., *Acacia elata* A. Cunn. ex. Benth., *Acacia* x *giraffae*, *Albizia lophantha*, *Desmanthus virgatus*, *Leucaena cuspidata* Standley, *Leucaena retusa* Benth., *Neptunia dimorphantha* Domin, and *Pithecellobium elegans*). Of the remaining three mimosoid species, where a cysteine lyase or a djenkolic acid or both were absent, no CS_2 production occurred (i.e., *Acacia greggii* A. Gray, *Albizia julibrissin*, and *Prosopis alba* Griseb.).

The ecological role of CS_2 in plants in the Fabaceae is probably that of a deterrent to pathogens. Carbon disulfide is bacteriostatic (Bremner and Bundy, 1974; Hartel and Haines, 1992), fungicidal (Lewis and Papavizas, 1971; Banwart and Bremner, 1975; Filip and Roth, 1977), nematicidal (Guba, 1932), and insecticidal (Punj and Girish, 1969). Carbon disulfide produced by *M. pudica* decreases fungal colonization on the rhizoplane (Feng et al., 1998). It is also possible that plant-produced CS_2 affects soil ecological processes. Carbon disulfide is a potent inhibitor of nitrification (Bremner and Bundy, 1974; Ashworth et al., 1975; Hyman et al., 1990), and if a plant could produce sufficient CS_2 to inhibit nitrification, then this could be a N-conserving mechanism. The vast majority of soils have a net negative charge, thus, NH_4^+ does not leach through these soils as readily as NO_3^-. It would not be surprising if a djenkolic acid was indeed the parent compound for all plant-mediated CS_2 production in the Mimisoideae. Mimosoid legumes are well-known for containing nonprotein amino acids, and there is considerable evidence for their functional multiplicity in physical stress, insect deterrence, and phytotoxicity (e.g., Romeo, 1998).

Acknowledgments—We thank Mark Eiteman, Orla Finlay–Moore, and Robert Potter for their technical assistance. This research was supported by Hatch funds to the Georgia Experimental Station and a Core Biological Instrumentation and Facilities grant from the National Science Foundation to the University of Georgia Research Foundation.

REFERENCES

AHMED, S., and EVANS, H. J. 1960. Cobalt: A micronutrient element for the growth of soybean plants under symbiotic conditions. *Soil Sci*. 90:205–210.

ASHWORTH, J., BRIGGS, G. G., and EVANS, A. A. 1975. Field injection of carbon disulphide to inhibit nitrification of ammonia fertilizer. *Chem. Ind*. 6:749–750.

BANWART, W. L., and BREMNER, J. M. 1975. Formation of volatile sulfur compounds by microbial decomposition of sulfur-containing amino acids in soils. *Soil Biol. Biochem*. 7:359–364.

BIDLINGMEYER, B. A., COHEN, S. A., and TARVIN, T. L. 1984. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr*. 336:93–104.

BREMNER, J. M., and BUNDY, L. G. 1974. Inhibition of nitrification in soil by volatile sulfur compounds. *Soil Biol. Biochem*. 6:161–165.

CAVALLINI, D., FREDERICI, G., DUPRÈ, S., CANNELLA, C., and SCANDURRA, R. 1980. Ambiguities in the enzymology of sulfur-containing compounds, pp. 510–523, *in* D. Cavallini, G. E. Gaull, and V. Zappia (eds.). Natural Sulfur Compounds. Plenum Press, New York.

EVANS, C. S., SHAH, A. J., ADLARD, M. W., and LOURDES RICO ARCE, M. 1993. Nonprotein amino acids in seeds of neotropical species of *Acacia*. *Phytochemistry* 32:123–126.

FENG, Z., and HARTEL, P. G. 1996. Factors affecting production of COS and CS_2 in *Leucaena* and *Mimosa* species. *Plant Soil* 178:215–222.

FENG, Z., HARTEL, P. G., RONCADORI, R. W., and SUNG, S. J. S. 1998. Inhibition of fungal colonization on the rhizoplane of the CS_2-producing plant, *Mimosa pudica* L., pp. 115–126, *in* J. E. Box, Jr. (ed.). Root Demographics and their Efficiencies in Sustainable Agriculture, Grasslands, and Forest Ecosystems. Proceedings of the 5th Symposium of the International Society of Root Research. Kluwer Academic Publishers, Boston.

FILIP, G. M., and ROTH, L. F. 1977. Stump injections with soil fumigants to eradicate *Armillariella mellea* from young-growth Ponderosa pine killed by root rot. *Can. J. For. Res*. 7:226–231.

GMELIN, R. 1959. Die freien Aminosäuren der Samen von *Acacia willardiana* (Mimosaceae). Isolierung von Willardiin, einer neuen pflanzlichen Aminosäure, vermutlich L-Uracil-[β-(α-Amino-Propionsäure)]-(3). *Hoppe-Seyler's Z. Physiol. Chem*. 316:164–169.

GMELIN, R. 1962. Dichrostachinsäure, eine neue schwefelhaltige Aminosäure aus den Samen von *Dichrostachys glomerata* (Forsk.) Hutch und Dalz. (Mimosaceae). *Hoppe-Seyler's Z. Physiol. Chem*. 327:186–194.

GMELIN, V. R., HASENMAIER, G., and STRAUSS, G. 1957. Über das Vorkommen von Djenkosaüre und einer C-S Lyase in den Samen von *Albizia lophantha* Benth. (Mimosaseae). *Z. Naturforsch. B: J. Chem. Sci*. 12:687–697.

GMELIN, V. R., KJAER, R. A., and LARSEN, P. O. 1962. *N*-acetyl-L-djenkolic acid, a novel amino acid isolated from *Acacia farnesiana* Willd. *Phytochemistry* 1:233–236.

GUBA, E. F. 1932. Carbon disulfide emulsion for the control of the root-knot nematode. Massachusetts Agricultural Experimental Station, Bull. 292. Massachusetts State College, Amherst, Massachusetts.

HAINES, B., BLACK, M., FAIL, J. Jr., MCHARGUE, L., and HOWELL, G. 1987. Potential sulphur gas emissions from a tropical rainforest and a Southern Appalachian deciduous forest, pp. 599–610, *in* T. C. Hutchinson and K. M. Meema (eds.). Effects of Atmospheric Pollutants on Forests, Wetlands and Agricultural Ecosystems. NATO ASI Series, Vol. G16. Springer-Verlag, Berlin.

HAINES, B., BLACK, M., and BAYER, C. 1989. Sulfur emissions from roots of the rain forest tree *Stryphnodendron excelsum*. Ecosystem, community, and physiological implications, pp. 58–69, *in* E. S. Saltzman and W. J. Cooper (eds.). Biogenic Sulfur in the Environment. ACS Symposium Series No. 393. American Chemical Society, Washington, D.C.

HARTEL, P. G., and HAINES, B. L. 1992. Effects of potential plant CS_2 emissions on bacterial growth in the rhizosphere. *Soil Biol. Biochem*. 24:219–224.

HARTEL, P. G., and REEDER, R. E. 1993. Effects of drought and root injury on plant-generated CS_2 emissions in soil. *Plant Soil* 148:271–276.

HARTEL, P. G., VAUGHN, T. M., and WILLIAMSON, J. W. 1993. Rhizosphere competitiveness of genetically altered *Pseudomonas solanacearum* in a novel gnotobiotic plant assembly. *Soil Biol. Biochem*. 25:1575–1581.

HYMAN, M. R., KIM, C. Y., and ARP, D. J. 1990. Inhibition of ammonia monooxygenase in *Nitrosomonas europaea* by carbon disulfide. *J. Bacteriol*. 172:4775–4782.

ITO, K., and FOWDEN, L. 1972. New characterization of amino acids and g-glutamyl peptides from *Acacia georginae* seed. *Phytochemistry* 11:2541–2545.

KELLY, D. P., and SMITH, N. A. 1990. Organic sulfur compounds in the environment. Biogeochemistry, microbiology, and ecological aspects, pp. 345–385, *in* K. C. Marshall (ed.). Advances in Microbial Ecology, Vol. 11. Plenum Press, New York.

KUSTER, W. C., and GOLDAN, P. D. 1987. Quantitation of the losses of gaseous sulfur compounds to enclosure walls. *Environ. Sci. Technol*. 21:810–815.

LEWIS, J. A., and PAPAVIZAS, G. C. 1971. Effect of sulfur-containing volatile compounds and vapors from cabbage decomposition on *Aphanomyces euteiches*. *Phytopathology* 61:208–214.

MAZELIS, M., and CREVELING, R. K. 1975. Purification and properties of *S*-alkyl-L-cysteine lyase from seedlings of *Acacia farnesiana* Willd. *Biochem. J*. 147:485–491.

MAZELIS, M., and FOWDEN, L. 1973. Relationship of endogenous substrate to specificity of *S*-alkyl cysteine lyases of different species. *Phytochemistry* 12:1287–1289.

MCCLURE, P. R., and ISRAEL, D. W. 1979. Transport of nitrogen in xylem of soybean plants. *Plant Physiol*. 64:411–416.

PEYTON, T., STEELE, R., and MABEY, W. 1978. Carbon disulfide, carbonyl sulfide: Literature review and environmental assessment. EPA 600/9-78-009. Environmental Protection Agency, Washington, D.C.

PILUK, J., HARTEL, P.G., and HAINES, B. L. 1998. Production of carbon disulfide (CS_2) from L-djenkolic acid in the roots of *Mimosa pudica* L. *Plant Soil* 200:27–32.

PUNJ, G. K., and GIRISH, G. K. 1969. Relative toxicity of certain fumigants to *Trogoderma granarium* Everts (Coleoptera, Dermestidae). *J. Stored Prod. Res*. 4:339–342.

RANDLE, W. M., LANCASTER, J. E., SHAW, M. L., SUTTON, K. H., HAY, R. L., and BUSSARD, M. L. 1995. Quantifying onion flavor compounds responding to sulfur fertility—sulfur increases levels of alk(en)yl cysteine sulfoxides and biosynthetic intermediates. *J. Am. Soc. Hortic. Sci*. 120:1075–1081.

ROMEO, J. T. 1998. Functional multiplicity among nonprotein amino acids in mimosoid legumes: A case against redundancy. *Ecoscience* 5:287–294.

SCHWIMMER, S., and KJAER, A. 1962. Purification and specificity of the C-S-lyase of *Albizzia lophantha*. *Biochim. Biophys. Acta* 42:316–324.

SENEVIRATNE, A. S., and FOWDEN, L. 1968. The amino acids of the genus *Acacia*. *Phytochemistry* 7:1039–1045.

von KRAUSS, G. J., and REINBOTHE, H. 1970. Die Aminosäuren der Gattung *Albizia* Durazz. (Mimosaceae). *Biochem. Physiol. Pflanzen* 161:243–265.

WESTBERG, H., and LAMB, B. 1984. Estimation of biogenic sulfur emissions from the Continental U.S., pp. 41–53, *in* V.P. Aneja (ed.). Environmental Impact of Natural Emissions. Transactions of the Air Pollution Control Association Specialty Conference (TR-2). Air Pollution Control Association, Pittsburgh, Pennsylvania.

WHITFIELD, F. B., SHEA, S. R., GILLEN, K. J., and SHAW, K. J. 1981. Volatile components from the roots of *Acacia pulchella* R. Br. and their effect on *Phytophthora cinnamomi* Rands. *Aust. J. Bot*. 29:195–208.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

COMMUNICATION ECOLOGY OF WEBBING CLOTHES MOTH: 1. SEMIOCHEMICAL-MEDIATED LOCATION AND SUITABILITY OF LARVAL HABITAT

STEPHEN TAKÁCS, GERHARD GRIES,* and REGINE GRIES

Center for Environmental Biology
Department of Biological Sciences
Simon Fraser University
Burnaby, B.C., V5A 1S6, Canada

(Received November 2, 2000; accepted April 3, 2001)

Abstract—We tested two hypotheses: 1) that there is semiochemical-mediated attraction of male and female webbing clothes moth (WCM), *Tineola bisselliella* (Hum.) (Lepidoptera: Tineidae) to suitable larval habitat; and 2) that selection of optimal larval habitat has fitness consequences. In binary or ternary choice arena bioassay experiments that prevented WCM from contacting test stimuli, males and females were attracted to dried but untanned animal pelts (red squirrel, muskrat, beaver, coyote, red fox and bobcat) and preserved horseshoe crab but not to unprocessed sheep's wool, demonstrating semiochemical-based recognition of, and discrimination between, potential larval habitats. Selection of habitat has fitness consequences for ovipositing females, because significantly more male and female WCM completed development when the larval diet consisted of intact animal pelt (hide plus hair) rather than hide or hair alone. Equal attraction of male WCM to muskrat pelt volatiles in Porapak Q or solvent extracts of muskrat pelts indicated that volatile semiochemicals could be obtained by both methods.

Key Words—*Tineola bisselliella*, Lepidoptera, Tineidae, semiochemicals, larval-habitat location, host-habitat location, dietary requirements, fitness, larval development, untanned animal pelts, fur.

INTRODUCTION

Larvae of the webbing clothes moth (WCM), *Tineola bisselliella* (Hum.) (Lepidoptera: Tineidae), develop in temporary microhabitats not exposed to direct light, including sheltered nests (Woodroffe, 1953), animal lairs (Mallis, 1969;

*To whom correspondence should be addressed.

0098-0331/01/0800-1535$19.50/0

Hill, 1990), and dry carrion (Bornemissza, 1957). Like other scatophagous and necrophagous insects, adult WCM discriminate between and select a particular stage of temporary microhabitats (Bornemissza, 1957; Read, 1958; Stafford, 1971; Anderson and VanLaerhoven, 1996). For example, tineids such as WCM appear during the dry stage of carrion when only skin, hair, bones, and a little dry flesh remain (Bornemissza, 1957; Read, 1958; Stafford, 1971), whereas dipterans arrive quickly at and colonize fresh carrion (Rodriguez and Bass, 1983; Parker, 1970; Early and Goff, 1986; Anderson and VanLaerhoven, 1996; VanLaerhoven and Anderson, 1999).

In buildings, WCM larvae may feed year round on woollen textiles and other animal-based products, such as furs and carpets causing economic depreciation of goods disproportionate to the physical damage (Roth and Willis, 1952; Hinton, 1956; Mallis et al., 1959; Baker and Bry, 1982; Story, 1985). Depreciation caused by keratophagous insects including WCM approximates one billion dollars annually in the United States alone (Metcalf and Metcalf, 1994).

In optimal larval habitat and environmental conditions, female WCM may lay up to 250 eggs in 2 weeks. Larvae hatch in 3–7 days and develop through 5 instars (Titschak, 1922; Griswold, 1944). When feeding on raw animal pelts, they spin webbing tubes close to the hide to help maintain their water balance (Chauvin and Gueguen, 1978; Chauvin et al., 1981). Consuming first the base of the hair and surface of the hide, larvae ingest mainly soft keratin while avoiding sulphur-rich hard keratin in the distal shaft of the hair. This feeding habit leads to the debriding effect often seen in museum specimens. Under favorable conditions, larval and pupal development is complete after 3–5 weeks. In sub-optimal habitats like wool fabric, development may exceed 4 years because larvae may moult $\geq$40 times and repeatedly enter diapause (Titschak, 1922; Griswold 1944; Baker and Bry, 1982).

Previous studies suggested that tactile and contact chemoreceptive stimuli mediate selection of oviposition sites by females (Kan and Waku, 1985; Tranyier et al., 1994). Chemicals in eluents of acetone-washed raw sheep's wool and high concentrations of wool wax or vegetable oil deterred oviposition, whereas yeast or feces from larvae feeding on yeast-supplemented wool induced oviposition (Kan and Waku, 1985; Tranyier et al., 1994). Both of these studies were conducted in small Petri dishes (10 cm diam.), making it difficult to discern between tactile, contact chemoreceptive, and olfactory moieties of test stimuli.

We are engaged in a large study on sonic- and semiochemical-based communication in clothes moths. Herein we report long-range attraction *sensu* Kennedy[1] (1974) of male and female WCM to larval habitat, and differential development of larvae in habitats of different quality.

[1]An organism is attracted over long range if the distance to the stimulus exceeds a few body lengths of the organism.

METHODS AND MATERIALS

Experimental Insects

WCM adults were collected in the Natural History Museum of Simon Fraser University and placed in 1-l glass containers with mesh lids at 20–25°C, 40–70% R. H. and a 10L: 14D photoperiod. Larvae were provided with either freshly sheared untreated sheep's wool (courtesy of Anne Embra, Langley Spinners and Weavers Guild, Langley, B. C.) supplemented with brewer's yeast or with untanned animal pelts (Western Canada Fur Auctions, Vancouver, B. C.). These untanned pelts were from animals trapped and skinned during the 1996-1997 season. They were skinned, stretched, scraped of flesh and fat, air dried, and stored at ≤ -1°C before use. Every day, within 3 hr after dawn, adult WCM were collected and their sex determined through the presence of claspers and aedagus (male) or ovipositor (female).

Resource-Dependent Development of Larvae

Experiment 1 tested development of larvae on three resources: muskrat hair, muskrat hide with no hair, or muskrat hide with hair (intact pelt). Six each of these three resources (treatments) were kept separately for 18 months in 1-l glass containers with mesh lids at 25°C, 70% R. H. and a 10L:14D photoperiod. To obtain experimental insects, eggs were floated in 2% formaldehyde, rinsed three times with sterile water, and transferred with a brush to filter paper-lined Petri dishes. On hatching, 50 neonate larvae were transferred to each treatment container. Eclosed males and females were removed and recorded every day until no further moths were found for 14 consecutive days. Numbers of males and females per treatment were compared using the Kruskal Wallis test with Tukey type multiple comparisons ($\alpha = 0.05$) (Zar, 1984).

Capture of Habitat-Derived Semiochemicals

Dry, untanned pelts (150 cm^2) of eight vertebrate species, as well as formaldehyde-preserved dry horseshoe crab (Table 1), all potential habitats for WCM larvae, were placed in a cylindrical Pyrex^{TM} glass chamber. For one week, charcoal-filtered, humidified air was drawn at 2 l/min with a water-aspirator through the chamber and a glass column (14 cm $\times$ 13 mm O. D.) filled with Porapak Q (50–80 mesh, Waters Associates, Inc., Milford, MA). Volatiles were eluted from the Porapak Q with 5 ml of redistilled pentane. The eluent was concentrated to 2 ml by distillation in a 30 cm Dufton column, adjusting the volatile extract so that 2 μl equalled 5 pelt (or horseshoe crab)-min of volatile collection. Untanned pelts of muskrat (40 cm^2) were also extracted in dichloromethane (Cl_2CH_2) for 10 min. The supernatant was pipetted into vials, sealed, and refrigerated until used.

TABLE 1. SUMMARY OF EXPERIMENTS INVOLVING WEBBING CLOTHES MOTHS (*T. bisselliella*) AND STIMULI TESTED IN BINARY CHOICE ARENA BIOASSAY EXPS. 2–16

Exp.	No. of replicates	Sex	Mating status[a]	Test stimuli[b]
2	6	Female	Mated	Sheep's Wool
3	6	Female	Mated	Horseshoe Crab
4	10	Female	Mated	Red Squirrel
5	6	Female	Mated	Muskrat
6	6	Female	Mated	Beaver
7	6	Female	Mated	Coyote
8	6	Female	Mated	Red fox
9	6	Female	Mated	Bobcat
10	6	Female	Mated	Mink
11	6	Male	Unknown	Horseshoe Crab
12	6	Male	Unknown	Red Squirrel
13	6	Male	Unknown	Muskrat
14	6	Male	Unknown	Beaver
15	6	Male	Unknown	Coyote
16	6	Male	Unknown	Mink

[a] Mating status of males is unknown because 2 males had been confined with 1 female to ensure mating of females.

[b] *Sheep's Wool*, from domestic sheep sheared in spring 1995, stored for one year in polyethylene bag at room temperature; *Horseshoe crabs*, *Limulus polyphemus* L. (dry, formaldehyde-preserved); *Red squirrel*, *Tamiasciurus hudsonicus* (Erxleben), Peace River District, British Columbia, 20 dorsal and ventral samples cut from separate pelts; *Muskrat*, *Ondatra zibethica* (L.), Northwest Territories, 20 dorsal and ventral samples cut from separate pelts; *Beaver*, *Castor canadensis* Kuhl, Northwest Territories, 6 dorsal samples cut from 2 pelts; *Coyote*, *Canis latrans* Say, Northwest Territories, 6 ventral samples cut from 1 pelt; *Red fox*, *Vulpes fulva* (Desmarest), Peace River District, British Columbia, 6 ventral samples cut from 1 pelt: *Bobcat*, *Lynx rufus* (Schreber), Prince George District, British Columbia, 6 ventral samples cut from 1 pelt: *Mink*, *Mustela vison* Schreber, Vancouver Island, British Columbia, 12 dorsal and ventral samples cut from 3 pelts. All pelt samples originated from specimens trapped in winter 1996, and measured 150 cm^2. Control treatments consisted of cardboard or plastic models similar in size and shape to sheep's wool, horseshoe crab, or pelts.

Arena Bioassay Experiments

Tactic responses of WCM to untanned pelt samples or dry horseshoe crabs (Table 1) paired with cardboard or plastic model control stimuli in experiments 2–16 were assessed in a closed Plexiglas™ arena olfactometer (Figure 1). Bioassay experiments in moving air olfactometers proved unsuitable (see also Trematerra and Fontana, 1996). Stimuli were suspended 2 cm above the centre of Tanglefoot-coated disks kept 80 cm apart. Volatile extracts as test stimuli (Table 2, experiments 17–26) and solvent controls were pipetted on filter paper before being tested in two-choice (experiments 17–24) or three choice (experiments 25, 26) bioassays in the same arena olfactometer. Twenty-five male or 25 female moths per replicate were released from a Petri dish in the center of the arena after a 30-min

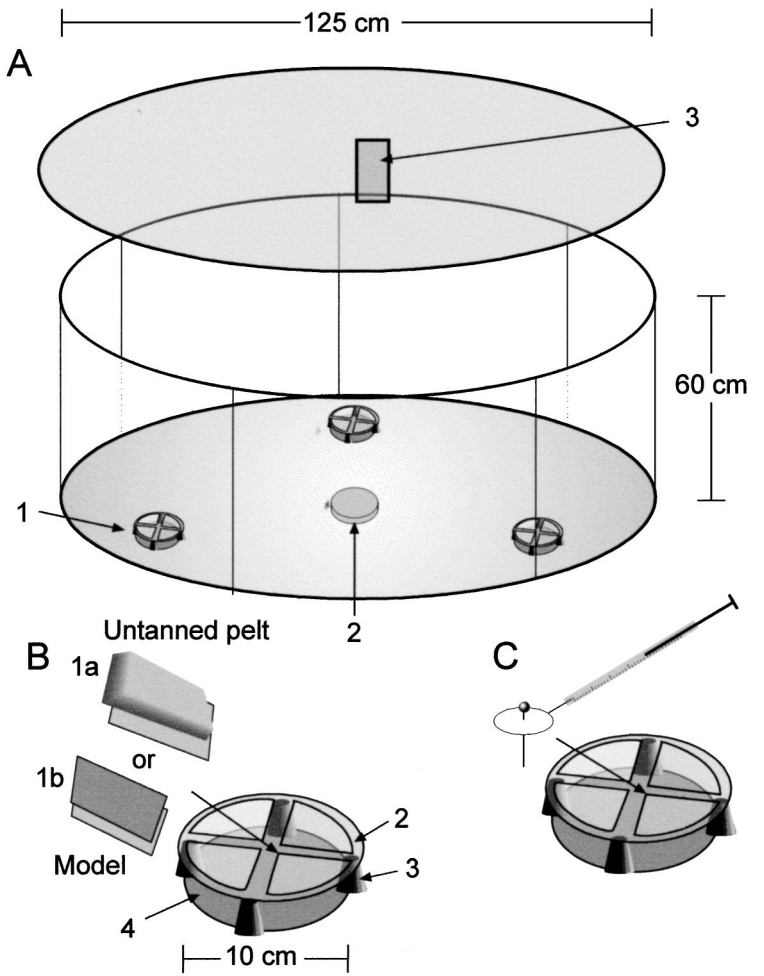


FIG. 1. Experimental design for ternary-choice arena bioassays. **A**: Plexiglas arena with capture device (1), Petri dish from which moths were released (2), and access port (3). For binary choice bioassays, one of the capture devices was replaced by the Petri dish (2) for release of moths. **B**: Capture device with animal pelt (150 cm^2) (1a, treatment) or cardboard model (1b, control) placed on a cardboard platform (2) suspended on rubber stoppers (3) (height 2.5 cm) above a Tanglefoot-coated cardboard disc (4) (10 cm diam.). **C**: Capture device with pin-supported filter paper impregnated with natural candidate semiochemicals in HPLC-grade solvent.

TABLE 2. SUMMARY OF EXPERIMENTS INVOLVING WEBBING CLOTHES MOTHS (*T. bisselliella*) AND STIMULI TESTED IN BINARY OR TERNARY CHOICE ARENA BIOASSAY EXPERIMENTS 17–26

Exp.	No. of replicates	Sex	Mating status	Experimental treatments[a]		
				Treatment 1		Treatment 2
17	10	Female	Mated	Volatiles of Horseshoe Crab		Pentane
18	10	Male	Unknown	Volatiles of Horseshoe Crab		Pentane
19	10	Female	Mated	Volatiles of Beaver Pelt		Pentane
20	10	Male	Unknown	Volatiles of Beaver Pelt		Pentane
21	10	Female	Mated	Volatiles of Red Squirrel Pelt		Pentane
22	10	Male	Unknown	Volatiles of Red Squirrel Pelt		Pentane
23	10	Female	Mated	Extract of Muskrat Pelt		Cl_2CH_2
24	10	Male	Unknown	Extract of Muskrat Pelt		Cl_2CH_2
				Treatment 1	Treatment 2	Treatment 3
25	10	Female	Mated	Extract of Muskrat Pelt	Volatiles of Muskrat Pelt	Both
26	10	Male	Unknown	Extract of Muskrat Pelt	Volatiles of Muskrat Pelt	Both

[a] In each replicate of experiments 17–24, treatment 1 received 75 horseshoe crab- or pelt-minutes of Porapak Q volatile extract; treatments 1 and 2 received the same amount of solvent ranging from 30–35 μl. In each replicate of experiments 25 and 26, treatment 1 received 10 μl (2%) of Cl_2CH_2 extract of muskrat pelt (150 cm^2) plus 30 μl of pentane, treatment 2 received 75 pelt minutes of Porapak Q volatile extract plus 10 μl of Cl_2CH_2.

acclimation period. After 12 hr, moths captured on the sticky disks were recorded as responders, whereas all other moths were collected from the arena and classed as non-responders. After completion of a replicate (a), every third replicate (b), and experiments (c), the interior surfaces of the arena olfactometer were wiped with a paper towel saturated with a Sparkleen^{TM}-solution (a, b, c), followed by hexane (b, c) and methanol (c); cleaned surfaces were allowed to dry and were ventilated for $\geq$ 7 hr (a, b) or $\geq$ 36 hr (c).

Experiments 2–16 (Table 1) tested the response of male and female WCM to potential larval habitats. Numbers of captured moths were analysed by the Wilcoxon's paired sample test for two choice experiments ($\alpha = 0.05$) and by the Kruskal Wallis test with Tukey type multiple comparisons for three choice experiments ($\alpha = 0.05$) (Zar, 1984).

RESULTS AND DISCUSSION

Most, fewer, and least adult WCM eclosed when larvae were reared on muskrat hide with hair, hide alone, and hair alone, respectively (Figure 2; experiment 1).

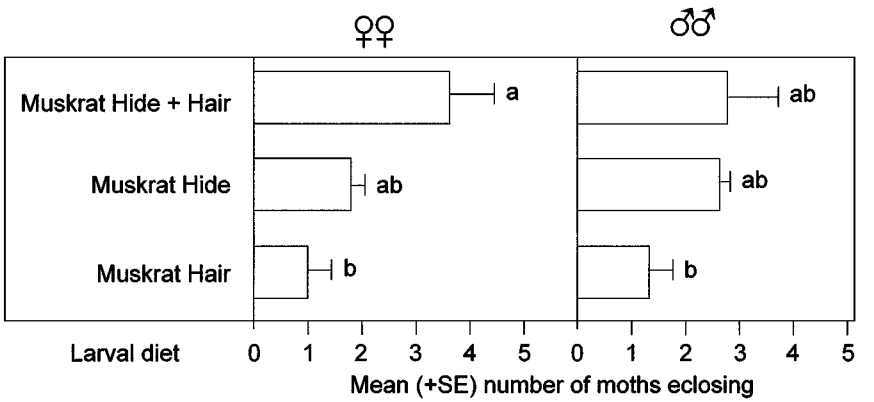


FIG. 2. Number of *T. bisselliella* completing development and emerging as adults when provided with different resources. Bars with different letters are significantly different [Kruskal Wallis test with Tukey type non-parametric multiple comparison ($P < 0.05$)].

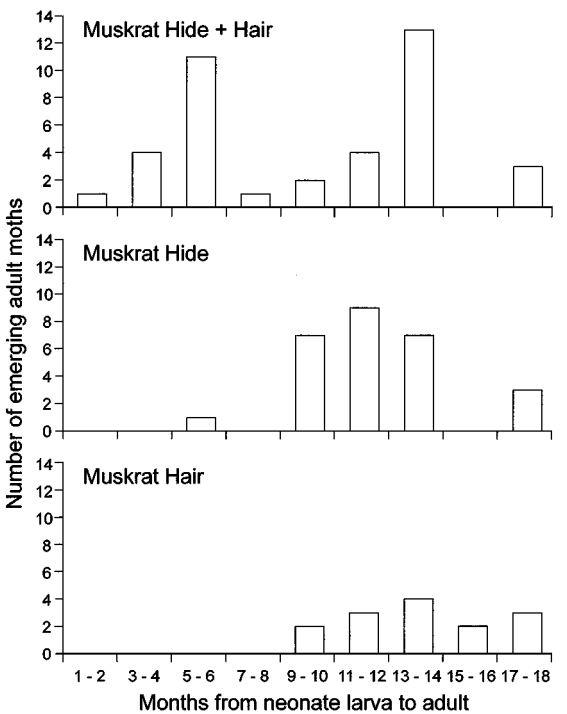


FIG. 3. Total number of *T. bisselliella* completing development and emerging as adults over time when provided with different resources.

Adult moths eclosed earlier and in greater numbers from muskrat hide with hair than from hide or hair alone, indicating that selection of suitable larval habitat has fitness consequences for ovipositing females (Figures 2, 3). Muskrat hide with hair met dietary requirements of developing larvae better than hair or hide alone. Although hair provides proteinous keratin for production of larval body mass (Cowell and McKay, 1937; Baker and Bry, 1982), hide-derived nutrients likely supplement the diet and facilitate development of larvae. B-complex vitamins and sterols essential for growth and development of insects are absent from clean animal hair (Fraenkel and Blewett, 1946) but present in vertebrate body fluids and cells (Mallis et al. 1959; Mallis et al., 1962). Untanned hide with glandular and cellular skin components contain these essential nutrients. WCM larvae ingesting these nutrients on the hide surface and soft keratin at the base of hair shafts cause the debriding effect seen on museum specimens.

The results of arena bioassay experiments provide evidence for long-range attraction[2] of both males and females to potential larval habitats. Female and male WCM were strongly attracted to preserved horseshoe crab (Figure 4; experiments 3 and 11) and pelt samples from red squirrel, muskrat, beaver, coyote, and mink (Figure 4; experiments 4–7, 10, 12–16). Females were also attracted to pelt samples from red fox and bobcat (Figure 4; experiments 8 and 9), but not to wool ($P > 0.05$) (Figure 4; experiment 2). This rejection of raw sheep's wool by female WCM even in the absence of alternative larval habitat is in agreement with Kan and Waku (1985) and Traynier et al. (1994), and supports the results of experiment 1 that hair alone is suboptimal for larval development. Wool or wool-fabric with proteinous keratin as the main constituent lack nicotinic acid, pantothenic acid, and sterols and are thus inadequate for larval development (Titschak, 1922; Griswold 1944; Fraenkel and Blewett, 1946; Mallis et al., 1959; Mallis et al., 1962; Baker and Bry, 1982).

Extracts of Porapak Q-captured volatiles from horseshoe crab, beaver, or red squirrel pelts were also highly attractive to both males and females (Figure 5; experiments 17–22), providing further evidence for olfactory recognition of larval habitat. Unlike females ($P > 0.05$), males preferred solvent extracts of muskrat pelt over solvent (Cl_2CH_2) alone (Figure 5; experiments 23 and 24), and Porapak Q-captured volatile extract plus solvent extract of muskrat pelt over either stimulus alone (Figure 5; experiments 25 and 26). Equal attraction of males to semiochemicals Porapak Q captured or solvent-extracted from muskrat pelt (Figure 5; experiments 25 and 26), suggested that essential attractants can be obtained by either method of volatile acquisition. Enhanced attraction of male WCM to Porapak Q plus solvent extracts from muskrat pelts (Figure 5; experiment 26) was likely

[2]The term long range attraction *sensu* Kennedy (1974) is applicable because webbing clothes moths (<0.8 cm in body length) were attracted to test stimuli positioned 2.5 cm above the sticky surface of the trap.

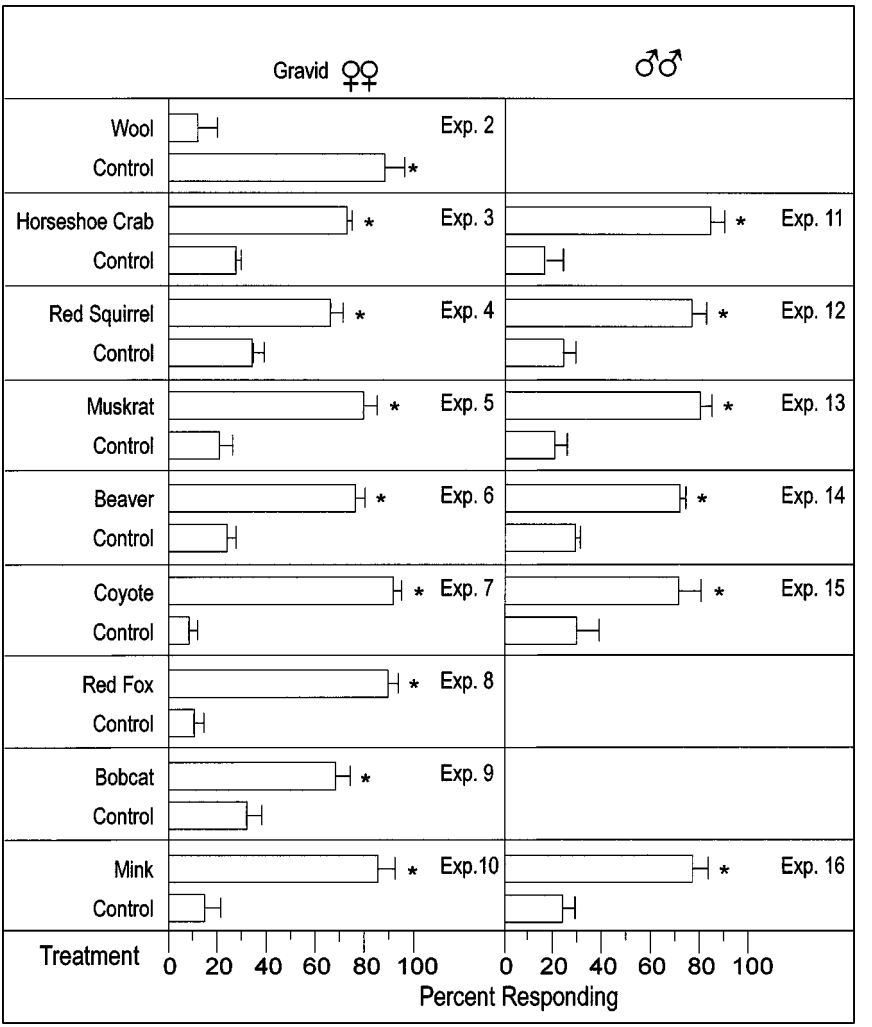


FIG. 4. Responses of adult *T. bisselliella* in binary choice arena bioassays to test stimuli (Table 1) in experiments 2–16. For each experiment, asterisks indicate a significant preference for a particular treatment [Wilcoxon paired-sample test ($P < 0.05$)]. Non-responders as follows: Exp. 2, 67 +/− 11%; Exp. 3, 31 +/− 3%; Exp. 4, 39 +/− 4%; Exp. 5, 39 +/− 7%; Exp. 6, 31 +/− 7%; Exp. 7, 28 +/− 5%; Exp. 8, 59 +/− 7%; Exp. 9, 55 +/− 9%; Exp. 10, 45 +/− 5%; Exp. 11, 27 +/− 10%; Exp. 12, 34 +/− 5%; Exp. 13, 50 +/− 9%; Exp. 14, 41 +/− 5%; Exp. 15, 50 +/− 13%; Exp. 16, 59 +/− 7%.

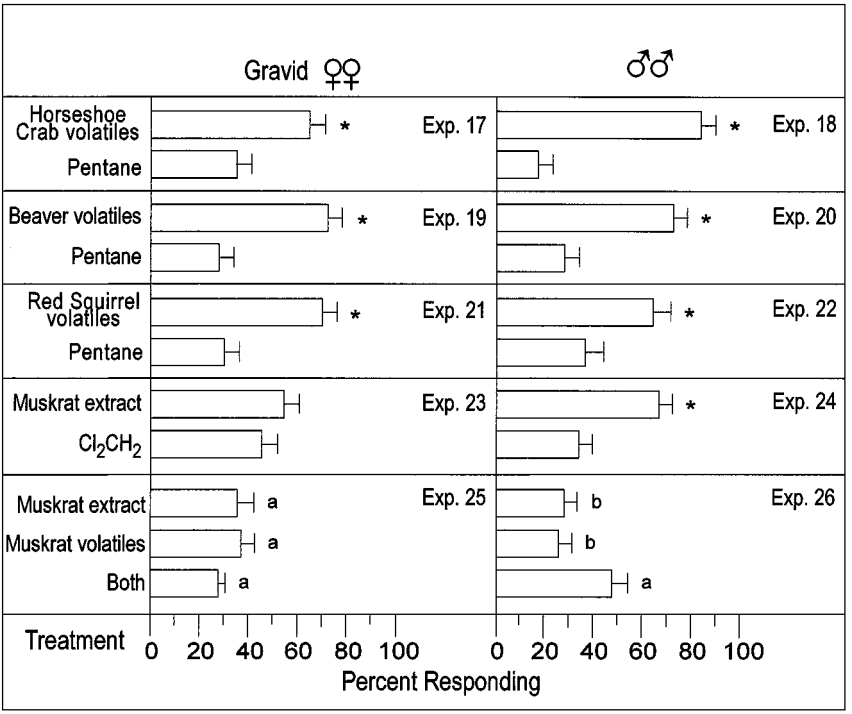


FIG. 5. Responses of adult *T. bisselliella* to test stimuli (Table 2) in binary choice arena bioassays (experiments 17–24) or ternary choice arena bioassays (experiments 25–26). For each of experiments 17–24, asterisks indicate a significant preference for a particular treatment [Wilcoxon paired-sample test ($P < 0.05$)]. For experiments 25 and 26, bars with different letters are significantly different [Kruskal Wallis test with Tukey type nonparametric multiple comparison ($P < 0.05$)]. Non-responders in experiments as follows: Exp. 17, 41 +/− 3%; Exp. 18, 27 +/− 8%; Exp. 19, 49 +/− 10%; Exp. 20, 52 +/− 6%; Exp. 21, 54 +/− 5%; Exp. 22, 39 +/− 6%; Exp. 23, 58 +/− 4%; Exp. 24, 27 +/− 5%; Exp. 25, 17 +/− 3%; Exp. 26, 12 +/− 3%.

due to increased quantities of semiochemicals, because GC-MS analyses failed to reveal any qualitative differences between these two sources of semiochemicals. Because Porapak Q-captured volatile extracts are void of fatty acids and are well suited for chromatographic analyses, they will be used to determine the identity of the semiochemicals that mediate attraction of male and female WCM to larval habitat.

Acknowledgments—We thank H. D. Pierce, Jr., for assistance in volatile collections, S. Halford, D. Baber and J. Moldenado for collecting and rearing *Tineola bisselliella*, and J. H. Borden and A. S. Harestad for review of the manuscript. This study was supported, in part, by an industrially oriented

research grant (IOR) from the Natural Sciences and Engineering Research Council of Canada (NSERC) with S.C. Johnson & Son, Inc. as the industrial partner, and by a Graduate Fellowship from Simon Fraser University to S. T.

REFERENCES

ANDERSON, G. S., and VANLAERHOVEN, S. L. 1996. Initial studies on insect succession on carrion in Southwestern British Columbia. *J. Foren. Sci*. 41:617–625.

BAKER, J. E., and BRY, R. E. 1982. Nutritional ecology of wool and fur-feeding insects, pp. 971–992, *in* F. Slasky and J. G. Rodriuez. (eds.). Nutritional Ecology of Insects, Mites, Spiders, and Related Invertebrates. Wiley: Toronto.

BORNEMISSZA, G. F. 1957. An analysis of arthropod succession in carrion and the effect of its decomposition on the soil fauna. *Aust. J. Zool.* 5:1–12.

CHAUVIN, G., and GUEGUEN, A. 1978. Developpement larvaire et bilan d'utilisation d'energie en fonction de l'hygrometrie *Tinea pellionella* L. (Lepidoptera: Tineidae). *Can. J. Zool*. 56:2176–2185.

CHAUVIN, G., GUEGUEN, A., and VANNIER, G. 1981. Croissance, bilan d'energie et production d'eau metabolique chez la larvae de *Tineola bisselliella. Can. J. Zool*. 59:297–304.

COWEL, M. F., and MCCAY, C. M. 1937. Nutritional studies of the webbing clothes moth, *Tineola bisselliella* Hum. *Physiol. Zool.* 10:368–372.

EARLY, M., and GOFF, M. L. 1986. Arthropod succession patterns in exposed carrion on the island of O'Ahu, Hawaiian Islands, USA. *J. Med. Entomol.* 23:520–531.

FRAENKEL, G., and BLEWETT, M. 1946. The dietetics of the clothes moth *Tineola bisselliella* Hum. *J. Exp. Biol*. 22:156–161.

GRISWOLD, G. H. 1944. Studies on the biology of the webbing clothes moth (*Tineola bisselliella* (Hum.)). *Mem. Cornell Agric. Exp. Sta.* 262, 59 pp.

HILL, D. S. 1990. Pests of Stored Products and Their Control. Belhaven Press: London.

HINTON, H. E. 1956. The larvae of species of Tineidae of economic importance. *Bull. Entomol. Res.* 47:251–346.

KAN E., and WAKU, Y. 1985. Analysis of oviposition preference in the webbing clothes moth, *Tineola bisselliella* Hum. (Lepidoptera: Tineidae). *Appl. Ent. Zool*. 20:322–330.

KENNEDY, J. S. 1974. Changes in the patterning of behavioural sequences, pp. 1–6, *in* L. B. Brown (ed.). Experimental Analysis of Behaviour. Springer-Verlag: New York.

MALLIS, A. 1969. Handbook of Pest Control: The Behavior, Life History, and Control of Household Pests. Mac Nair-Dorland Company: New York.

MALLIS, A., MILLER, A. C., and HILL, R. C. 1959. The attraction of stains to three species of fabric pests. *J. Econ. Entomol.* 52:382–384.

MALLIS, A., BURTON, B. T., and MILLER A. C. 1962. The attraction of salts and other nutrients to the larvae of fabric insects. *J. Econ. Entomol*. 55:351–355.

METCALF, R. L., and METCALF, R. A. 1994. Attractants, repellents, and genetic control in pest management, pp. 355–356, *in* R. L. Metcalf and W. H. Luckman (eds.). Introduction to Insect Pest Management. Wiley: New York.

PARKER, G. A. 1970. Sperm competition and its evolutionary effect on copula duration in the fly, *Scatophaga stercoraria*. *J. Insect Physiol*. 16:1301–1328.

READ, H. B. JR. 1958. A study of dog carcass communities in Tennessee, with special reference to the insects. *Am. Mid. Nat.* 59:213–245.

RODRIGUEZ, W. C., and BASS, W. M. 1983. Insect activity and its relationship to decay rates of human cadavers in east Tennessee. *J. Foren. Sci*. 28:423–432.

ROTH, L. M., and WILLIS, E. R. 1952. Observations on the behaviour of the webbing clothes moth. *J. Econ. Entomol*. 45:20–25.

STAFFORD, F. 1971. Insects of a medieval burial. Sci. Arch. 7:6–10.

STORY, K. O. 1985. Approaches to pest management in museums. Smithsonian Institution, pp. 33–38.

TITSCHACK, E. 1922. Beiträge zu einer Monographie der Kleidermotte, *Tineola bisselliella* Hum. Leipzig: Borntraeger.

TRANYIER, R. M. M., SCHUMACHER, R. K., and LAU, D. M. 1994. Oviposition site selection by Tineola bisselliella, Tinea spp (Lepidoptera: Tineidae) and *Anthrenus flavipes* (Coleoptera: Dermestidae). *J. Stor. Prod. Res.* 30:321–329.

TREMATERRA, P., and FONTANA, F. 1996. Monitoring of webbing clothes moth *Tineola bisselliella* (Hummel), by sex pheromone. *Anz Schädlingskunde Pflanzenschutz Umweltschutz* 69:119–121.

VANLAERHOVEN, S. L., and ANDERSON. G. S. 1999. Insect succession on buried carrion in two biogeoclimatic zones of British Columbia. *J. Foren. Sci*. 44:32–43.

WOODROFFE, G. E. 1953. An ecological study of the insects and mites in the nests of certain birds in Britain. *Bull. Entomol. Res*. 44:739–772.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs: New Jersey.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

COMMUNICATION ECOLOGY OF WEBBING CLOTHES MOTH: 2. IDENTIFICATION OF SEMIOCHEMICALS MEDIATING ATTRACTION OF ADULTS TO LARVAL HABITAT

STEPHEN TAKÁCS, GERHARD GRIES,* and REGINE GRIES

Center for Environmental Biology
Department of Biological Sciences
Simon Fraser University
Burnaby, B.C., V5A 1S6, Canada

(Received November 14, 2000; accepted April 2, 2001)

Abstract—Our objective was to identify the semiochemicals that mediate attraction of the webbing clothes moth (WCM), *Tineola bisselliella* (Lepidoptera: Tineidae), to suitable larval habitat. Coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses of Porapak Q-captured bioactive volatiles from horseshoe crab, and dried but untanned vertebrate pelts revealed numerous EAD-active volatiles. These volatiles were identified by comparative GC-mass spectrometry and GC-EAD analyses of natural and synthetic compounds. A blend of 28 synthetic candidate semiochemicals attracted both male and female WCM. Experiments deleting various components determined that saturated aldehydes—but not unsaturated aldehydes, saturated hydrocarbons, saturated alcohols, or ketones—were essential for blend attractiveness. A blend of nonanal, the single most attractive aldehyde, in combination with geranylacetone was more attractive to WCM than the 28-component blend or dried, untanned animal pelt. Selection of larval habitat resides more with male than female WCM, as indicated by stronger EAD responses from male than female antennae to habitat-derived semiochemicals, and more selective and early response to habitat cues by males than females. Exploitation of nonanal and geranylacetone as resource-derived semiochemicals by both adult WCM and its larval parasitoid, *Apanteles carpatus*, is an example of convergent semiochemical parsimony.

Key Words—*Tineola bisselliella*, Lepidoptera, Tineidae, semiochemicals, larval-habitat location, host-habitat location, nonanal, geranylacetone, coupled gas chromatographic-electroantennographic detection, GC-EAD.

*To whom correspondence should be addressed.

0098-0331/01/0800-1547$19.50/0

INTRODUCTION

Insects use short- and long-range, habitat-derived semiochemicals to locate larval or host habitat. Most such semiochemicals have been identified for phytophagous, predatory or parasitic insects (e.g., Borden, 1982; Vet, 1983; Lindroth et al., 1988; Lin and Phelan, 1991; Nottingham et al., 1991; Leal et al., 1994; Byers, 1995; Takács et al., 1997; Macias-Samano, 1998; DeLury et al., 1999). Very few have been determined for scavenging and detritus-feeding insects like the webbing clothes moth (WCM), *Tineola bisselliella* (Hum.) (Lepidoptera: Tineidae) (e.g., Wilson, 1940, Kan and Waku 1985; Detmers et al., 1992; Gerard et al., 1993; Tranyier et al., 1994). Long-range attraction (Kennedy, 1974) of WCM to larval habitat has been demonstrated (Takács et al., 2001). We report identification of the semiochemicals that mediate this attraction.

METHODS AND MATERIALS

Capture and Analyses of Habitat-derived Semiochemicals. Dried but untanned pelt (150 cm^2) of various vertebrates or dry, formaldehyde-preserved horseshoe crab, *Limulus polyphemus*, (Table 1) as potential larval habitat were aerated for one week in a cylindrical Pyrex^{TM} glass chamber or extracted with dichloromethane (Cl_2CH_2) (Takács et al., 2001). Five pelt-min of volatile collection equalled 2 μl of volatile extract. Aliquots (1 μl) of Porapak Q-captured volatiles or Cl_2CH_2 extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a Hewlett Packard (HP) 5890A gas chromatograph equipped with a fused silica column (30 m $\times$ 0.25 or 0.32 mm ID) coated with DB-5, DB-210, or DB-23 (J & W Scientific, Folsom, CA). Full scan electron impact (EI) mass spectra of EAD-active compounds were obtained by coupled GC-mass spectrometry (MS), using a Varian Saturn II Ion Trap GC-MS and a HP 5985B GC-MS, respectively, each fitted with the DB-210 or DB-5 column referred to above.

Experimental Insects and Arena Bioassay Experiments. Wild WCM adults were collected and reared for bioassays as previously described (Takács et al., 2001). Tactic responses of WCM to test stimuli were assessed in a closed Plexiglas^{TM} arena (Takács et al., 2001). Twenty-five male or 25 female moths per replicate were released from a Petri dish in the center of the arena after a 30-min acclimatization period. After 12 hr, moths captured on the sticky disks associated with test stimuli were recorded as responders; all other moths were removed and classed as non-responders. Numbers of captured moths were analysed by the Wilcoxon's paired sample test ($\alpha = 0.05$) (Zar, 1984).

Treatments and test insects for 23 experiments are documented in Table 2. Experiments 1 and 2 tested captured volatiles from pelts of red squirrel, *Tamiasciurus*

hudsonicus, for attractiveness. Experiments 3–6 compared attractiveness of a complex blend of synthetic candidate semiochemicals (Table 1) with that of a solvent control (experiments 3, 4) or red squirrel pelt volatiles (experiments 5, 6). To determine essential semiochemicals, experiments 7–15 tested the complex synthetic blend versus blends lacking certain classes of organic chemicals (Byers, 1992). An attractive but less complex blend plus geranylacetone–the latter identified after completion of preceding experiments—was then tested versus the complex blend (experiment 16). Taking results of experiments 7–16 into account, experiments 17–21 tested various blends of (mainly) aldehydic candidates. Experiments 22 and 23 tested a 2-component synthetic blend (nonanal plus geranylacetone) versus an attractive natural pelt. In experiments 22 and 23, captures of moths in bait stations were recorded hourly for 12 hr and once again after 24 hours.

Capture and Quantitative Comparison of Natural and Synthetic Kairomones. In experiment 24, volatiles from the following sources (4 replicates) were captured for 24 hr on Porapak Q: 1) muskrat pelt as used in experiments 22 and 23; 2) filter paper impregnated with equivalent amounts of synthetic chemicals tested in experiments 22 and 23; and 3) filter paper. Aeration and extraction procedures were as described above. Aliquots of 1.5 pelt-min equivalents of Porapak Q-captured volatiles, with piperitone added as an internal standard, were subjected to GC analysis, employing a Hewlett Packard (HP) 5890A GC equipped with a fused silica column (30 m $\times$ 0.25 mm ID) coated with DB-5 (J & W Scientific). Mean proportions of nonanal and geranylacetone relative to the internal standard were compared with the Kruskal Wallis test ($\alpha = 0.05$) followed by a Tukey-type nonparametric test ($\alpha = 0.05$) (Zar, 1984).

RESULTS

GC-EAD analyses of volatiles from horseshoe crab and dried but untanned animal pelts including red squirrel, muskrat (*Ondatra zibethica*), beaver (*Castor canadensis*), coyote (*Canis latrans*), mink (*Mustela vison*), red fox (*Vulpes fulva*), and bobcat (*Lynx rufus*) revealed 32 EAD-active volatiles (Figures 1, 2; Table 1). Responses from male antennae were consistently stronger than those from female antennae (Figures 1, 2). Chemical structures were assigned to EAD-active compounds based on their comparable retention indices, EI mass spectra, and EAD activity with synthetic compounds.

Volatiles from red squirrel pelts and a blend of 28 synthetic candidate semiochemicals (SB1; Table 1) were highly and equally attractive to both sexes (Figure 3; experiments 1–6). Responses by females did not significantly differ between the complex blend (SB1) and blends lacking either unsaturated aldehydes (SB2), hydrocarbons (SB3), or alcohols (SB4), but males consistently preferred certain incomplete blends over SB1 (Figure 4; experiments 7–12). Both males and

TABLE 1. LIST OF EAD-ACTIVE COMPOUNDS PORAPAK Q CAPTURED FROM DRIED BUT UNTANNED PELTS AND DRY FORMALDEHYDE-PRESERVED HORSESHOE CRABS

Compound labels (Figures 1, 2)	EAD active compounds[a,b,c]	Mean amount (ng) in natural pelts[d] (15 pelt min equiv)	Designation of synthetic blend[e]													
			SB1	SB2	SB3	SB4	SB5	SB6	SB7	SB8	SB9	SB10[f]	SB11[g]	SB12	SB13	SB14[h]
1	6:Ald	20.0												+		
2	7:Ald	35.0												+		
3	8:Ald	55.0												+		
4	9:Ald	80.0	+	+	+	+		+	+	+		+	+	+	+	+
5	10:Ald	20.0	+	+	+	+		+	+	+						
6	11:Ald	4.0	+	+	+	+		+	+	+						
7	13:Ald	6.0	+	+	+	+		+	+	+						
8	14:Ald	5.0	+	+	+	+		+	+	+	+					
9	15:Ald	0.8	+	+	+	+		+	+		+					
10	16:Ald	1.0	+	+	+	+		+	+		+					
11	17:Ald	0.7	+	+	+	+		+	+		+					
12	18:Ald	0.1	+	+	+	+		+	+		+					
13	8:OH	10.0	+	+	+		+									
14	9:OH	10.0	+	+	+		+									
15	10:OH	12.0	+	+	+		+									
16	11:OH	200.0	+	+	+		+									
17	12:OH	10.0	+	+	+		+									
18	13:OH	70.0	+	+	+		+									
19	14:OH	3.0	+	+	+		+									
20	15:OH	2.0	+	+	+		+									
21	16:OH	0.3	+	+	+		+									

22	17:OH	0.5	+	+	+		+								
23	18:OH	0.1	+	+	+		+								
24	14:Hy	20.0	+	+		+	+								
25	15:Hy	100.0	+	+		+	+								
26	16:Hy	100.0	+	+		+	+								
27	20:Hy	20.0	+	+		+	+								
28	21:Hy	0.7	+	+		+	+								
29	2-11:One	4.0	+		+	+	+								
30	E2-9:Ald	9.0	+		+	+	+								
31	E2-10:Ald	11.0	+		+	+	+								
32	GA	1.0						+					+	+	

[a] Compound abbreviations: 6:Ald = hexanal; 7:Ald = heptanal; 8:Ald = octanal; 9:Ald = nonanal; 10:Ald = decanal; 11:Ald = undecanal; 13:Ald = tridecanal; 14:Ald = tetradecanal; 15:Ald = pentadecanal; 16:Ald = hexadecanal; 17:Ald = heptadecanal; 18:Ald = octadecanal; 8:OH = octanol; 9:OH = nonanol; 10:OH = decanol; 11:OH = undecanol; 12:OH = dodecanol; 13:OH = tridecanol; 14:OH = tetradecanol; 15:OH = pentadecanol; 16:OH = hexadecanol; 17:OH = heptadecanol; 18:OH = octadecanol; 14:C = tetradecane; 15:C = pentadecane; 16:C = hexadecane; 20:C = eicosane; 21:C = heneicosane; 2-11:One = 2-undecanone; *E*2-9:Ald = *E*2-nonenal; E2-10:Ald = *E*2-decenal; GA = Geranylacetone. 6:Ald, 7:Ald, 8:Ald and GA were identified while bioassay experiments were already under way, and were thus not components of SB1.

[b] All synthetic compounds were >90% chemically pure.

[c] Source of chemicals: saturated primary alcohols purchased from Sigma Chemical Corp. (St. Louis M.); unsaturated aldehydes purchased from Bedoukian (Danbury CT); saturated aldehydes and ketone synthesized by pyridinium chloro-chromate reaction of respective alcohols.

[d] Mean amount calculated from samples of dry, untanned animal pelts (150 cm^2) including red squirrel, muskrat, beaver, coyote, mink, red fox and bobcat, and dry, formaldehyde preserved horseshoe crab.

[e] 75 pelt minute equivalents used except for d, e, f and h.

[f] 500 pelt minute equivalents of 9-Ald used to approximate total quantity of all aldehydes in sample.

[g] 75 pelt minute equivalents of 9-Ald used to approximate the quantity of 9-Ald in sample.

[h] 720 pelt minute equivalents used of 9-Ald (3.5 μg) and geranylacetone (44 ng).

TABLE 2. SUMMARY OF EXPERIMENTS INVOLVING WEBBING CLOTHES MOTHS (*T. bisselliella*), AND STIMULI TESTED IN ARENA BIOASSAY EXPERIMENTS 1–23. TEN REPLICATES WERE TESTED FOR EACH EXPERIMENT

	Experimental insects		Experimental treatments[a,b]	
Exp.	Sex	Mating status	Treatment 1	Treatment 2
1	Female	Mated	Volatiles of squirrel pelt	Pentane
2	Male	Unknown	Volatiles of squirrel pelt	Pentane
3	Female	Mated	SB1	Hexane
4	Male	Unknown	SB1	Hexane
5	Female	Mated	SB1	Volatiles of squirrel pelt
6	Male	Unknown	SB1	Volatiles of squirrel pelt
7	Female	Mated	SB1	SB2
8	Male	Unknown	SB1	SB2
9	Female	Mated	SB1	SB3
10	Male	Unknown	SB1	SB3
11	Female	Mated	SB1	SB4
12	Male	Unknown	SB1	SB4
13	Female	Mated	SB1	SB5
14	Male	Unknown	SB1	SB5
15	Male	Unknown	SB1	SB6
16	Male	Unknown	SB1	SB7
17	Male	Unknown	SB8	SB9
18	Male	Unknown	SB7	SB10
19	Male	Unknown	SB7	SB11
20	Male	Unknown	SB11	SB12
21	Male	Unknown	SB13	SB6
22	Female	Mated	SB14	Muskrat pelt
23	Male	Unknown	SB14	Muskrat pelt

[a] For experiments with solvent controls, both treatments received the same amount of solvent ranging from 15–25 μl. For experiments 1, 2, 5, and 6, 75 pelt-min of Porapak Q captured volatiles from pelts of red squirrel were tested. In experiments 22 and 23, untanned muskrat pelt was 150 cm^2 in size.
[b] Chemical composition of synthetic blends as in Table 1.

females were more strongly attracted to SB1 than to a blend lacking all saturated aldehydes (SB5) (Figure 4; experiments 13, 14), and males were equally attracted to SB1 and to a blend of saturated aldehydes (SB6) (Figure 4; experiment 15). However, males preferred a blend of saturated aldehydes plus geranylacetone (SB7) over SB1 (Figure 4; experiment 16) and a blend of C8 to C14 aldehydes (SB8) over a blend of C14–C18 aldehydes (SB9) (Figure 5; experiment 17). Males also preferred nonanal at high dose (SB10) over a blend of aldehydes including nonanal (SB6) (Figure 5; experiment 18). Nonanal at low dose (SB11) was as attractive as the blend of nonanal plus higher homologues (SB6) or nonanal plus lower homologues (SB12) (Figure 5; experiments 19, 20). Finally, males preferred nonanal plus geranylacetone (SB13) over unsaturated aldehydes including nonanal (SB6) (Figure 5; experiment 21).

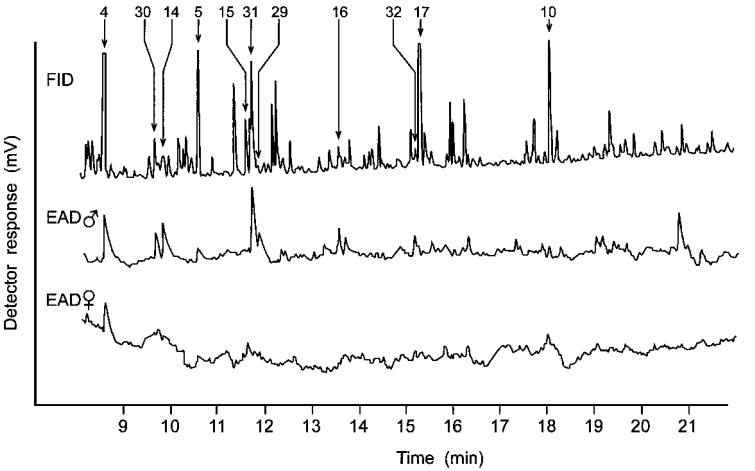


FIG. 1. Representative flame ionization detector (FID) and electroantennographic detector (EAD: ♂ or ♀ *T. bisselliella* antenna) responses to aliquots of 2.5 pelt-min equivalents of Porapak Q-collected volatiles from five museum specimens of horseshoe crab. Chromatography: Hewlett Packard 5890A equipped with DB-5 coated column; linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector temperature: 240°C; temperature program: 1 min at 50°C, 20°C/min to 70°C then 7.5°C/min to 280°C. Numbers above FID peaks correspond to identified components in Table 1.

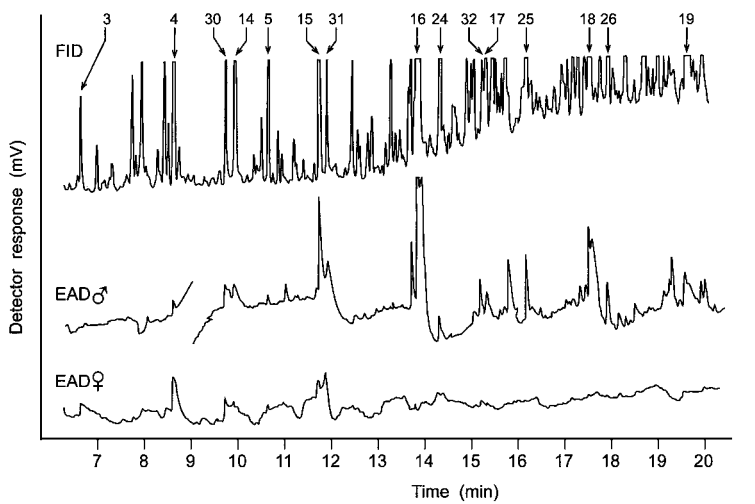


FIG. 2. Representative flame ionization detector (FID) and electroantennographic detector (EAD: ♂ or ♀ *T. bisselliella* antenna) responses to aliquots of 2.5 pelt-min equivalents of Porapak Q-collected volatiles from red squirrel pelt. Chromatography as in Figure 1. Numbers above FID peaks correspond to compounds in Table 1.

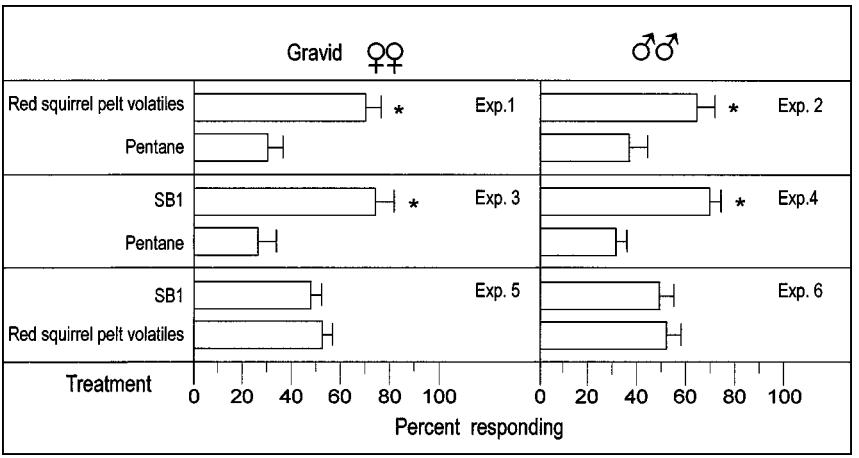


FIG. 3. Responses of adult *T. bisselliella* to test stimuli (Table 1) in binary choice arena bioassay experiments 1–6. For each experiment, asterisks indicate a significant preference for a particular treatment [Wilcoxon paired-sample test ($P < 0.05$)]. Nonresponders as follows: Exp. 1, 54 +/− 5%; Exp. 2, 39 +/− 6%; Exp. 3, 52 +/− 7%; Exp. 4, 42 +/− 8%; Exp. 5, 38 +/− 7%; Exp. 6, 33 +/− 5%.

Both females and males were more strongly attracted to the 2-component semiochemical blend of nonanal plus geranylacetone (SB14) than to natural muskrat pelt (Figure 5; experiments 22, 23). Males responded to test stimuli significantly earlier than did females (Figure 6).

When synthetic nonanal and geranylacetone released from filter paper, or natural nonanal and geranylacetone emanating from muskrat pelt, were captured on Porapak Q and quantified, amounts of nonanal were similar but more geranyl acetone was found emanating from muskrat pelt (Figure 7). This indicates that in experiments 22 and 23 nonanal and geranylacetone were released at quantities equivalent to, or less than those emanating from muskrat pelt, and that superior attractiveness of the synthetic blend was due to blend quality.

DISCUSSION

Our analyses show that nonanal and geranylacetone are key semiochemicals that mediate attraction of male and female WCM to larval habitat. The equal response of adult WCM to red squirrel pelt volatiles and a blend of 28 synthetic candidate semiochemicals (SB1) at equivalent quantities (Figure 3; experiments 5, 6), indicates that essential semiochemicals were present in SB1. Testing SB1 versus blends lacking unsaturated aldehydes (SB2), saturated hydrocarbons (SB3),

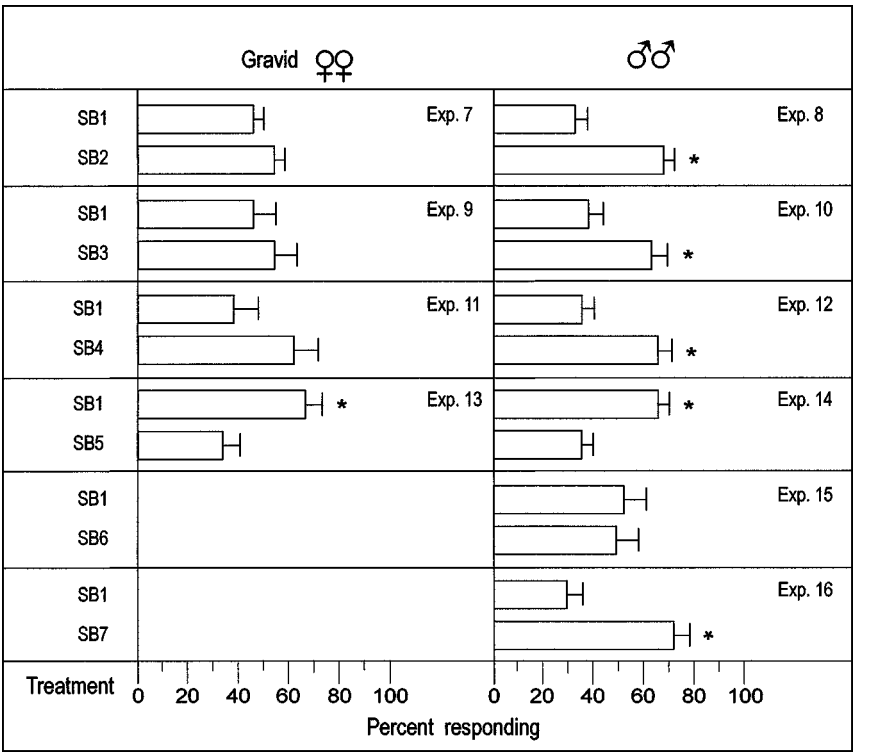


FIG. 4. Responses of adult *Tineola bisselliella* to test stimuli (Table 2) in binary choice arena bioassay experiments 7–16. For each experiment, asterisks indicate a significant preference for a particular treatment [Wilcoxon paired-sample test ($P < 0.05$)]. Non-responders as follows: Exp. 7, 52 +/− 6%; Exp. 8, 21 +/− 3%; Exp. 9, 52 +/− 8%; Exp. 10, 18 +/− 2%; Exp. 11, 58 +/− 9%; Exp. 12, 34 +/− 6%; Exp. 13, 26 +/− 8%; Exp. 14, 40 +/− 5%; Exp. 15, 42 +/− 8%; Exp. 16, 48 +/− 5%.

saturated alcohols (SB4), or saturated aldehydes (SB5) revealed that blend attractiveness required the presence of saturated aldehydes (Figure 4; experiments 7–14). The single most attractive aldehyde (nonanal) and attractiveness-enhancing geranylacetone were determined in bioassays with only males (Figure 4, experiments 15, 16; Figure 5, experiments 17–21), because males were generally more responsive and discriminating than females (Figure 4; experiments 7–14).

The prediction by a simulation model (Takács, unpublished data) that selection of larval habitat resides more with male than female WCM is supported by long-range attraction of males to larval habitat (Takács et al., 2001), and by our findings that: 1) habitat-derived stimuli elicited stronger responses by male than female antennae (Figures 1, 2); 2) males responded more selectively to habitat

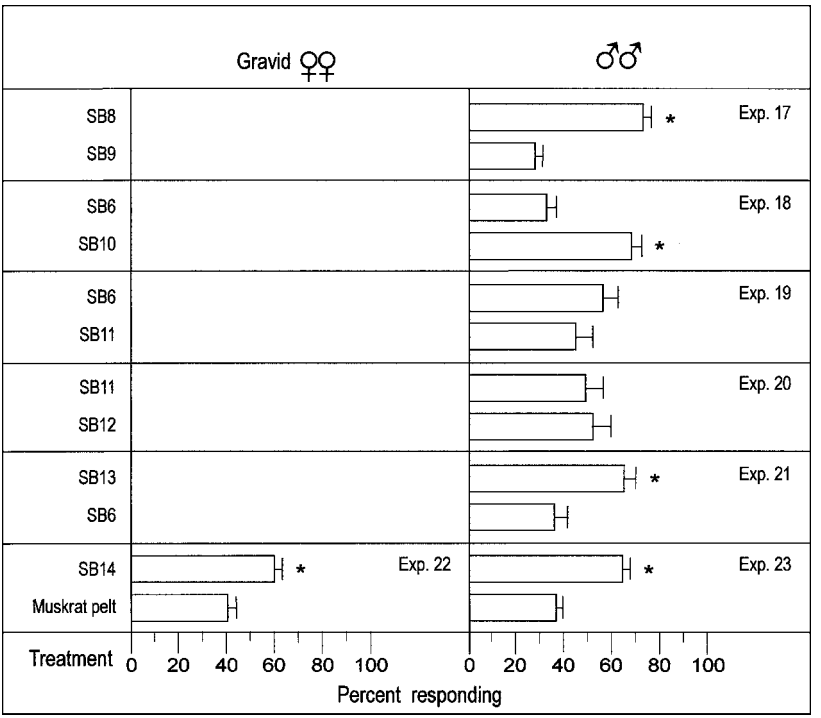


FIG. 5. Responses of adult *T. bisselliella* to test stimuli (Table 2) in binary choice arena bioassay experiments 17–23. For each experiment, asterisks indicate a significant preference for a particular treatment [Wilcoxon paired-sample test ($P < 0.05$)]. Non-responders as follows: Exp. 17, 37 +/− 5%; Exp. 18, 35 +/− 7%; Exp. 19, 50 +/− 6%; Exp. 20, 26 +/− 4%; Exp. 21, 41 +/− 7%; Exp. 22, 14 +/− 4%; Exp. 23, 13 +/− 5%.

cues (Figure 4, experiments 7–12); and 3) that males sought larval habitat earlier in the scotophase than females (Figure 6; experiments 22, 23). Habitat selection by males could enhance their potential mating success. It may place them at or near resources essential for resource-seeking females (Emlen and Oring, 1977).

The identification of nonanal and geranylacetone as key semiochemicals for both adult WCM (this study) and its larval parasitoid, *Apanteles carpatus* (Takács et al., 1997), provides an example of convergent semiochemical parsimony (Blum, 1996). Nonanal and geranylacetone obviously emanate from natural resources like dry carrion or untanned animal pelt in quantities (Figure 7) sufficient to serve both WCM and *A. carpatus* as habitat-derived cues.

Progressive colonization of temporary microhabitat by scatophagous and necrophagous insects seems to be associated with progressive changes in the composition of habitat-derived odorants (Bornemissza, 1957; Reed, 1958; Parker,

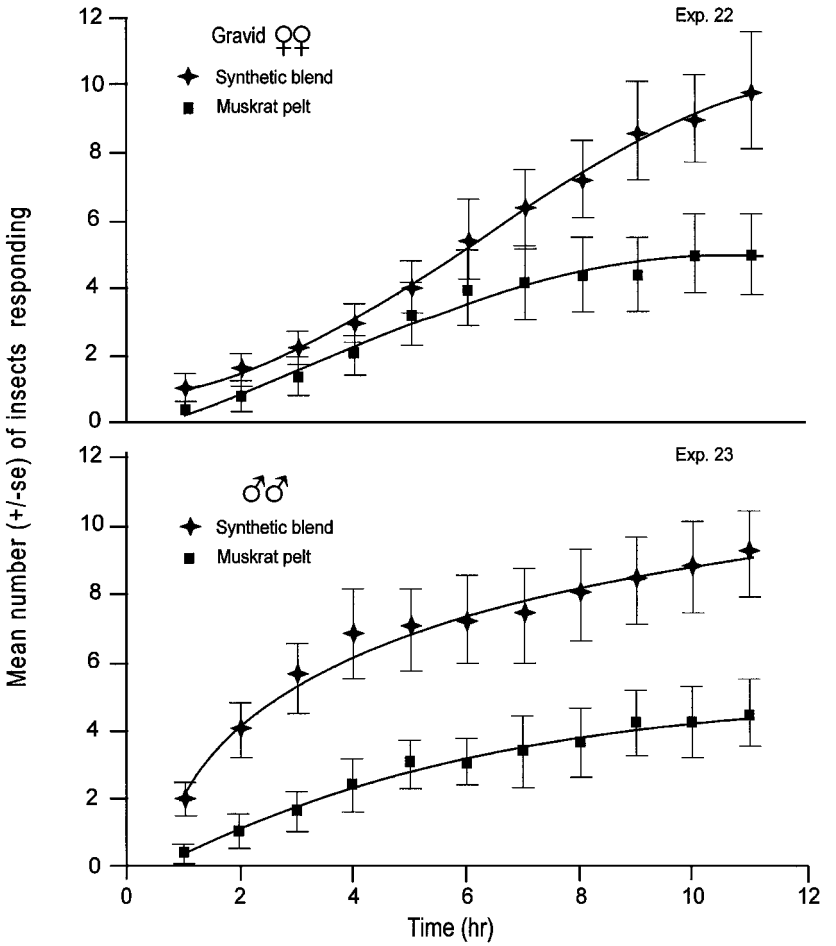


FIG. 6. Cumulative captures of adult *T. bisselliella* in binary choice arena bioassay experiments 22 and 23 on traps baited with a synthetic blend of nonanal plus geranylacetone (SB14, Table 1) or with dry, untanned muskrat pelt (150 cm^2). Experimental replicates commenced at the onset of the scotophase.

1970; Stafford, 1971; Rodriguez and Bass, 1983; Early and Goff, 1986; Anderson and VanLaerhoven, 1996; Takács et al., 1997; VanLaerhoven and Anderson, 1999). We hypothesize that respective odorants are characterized, in part, by their oxidative state, and that aldehydes predominate in the final dry stage of carrion. If true, female WCM may utilize the relative proportion of alcohols and aldehydes to assess suitability of larval habitat. Antennal response (Figures 1, 2) to, and behavioral deterrence by alcohols (Figure 4; experiments 11, 12) suggest that alcohols

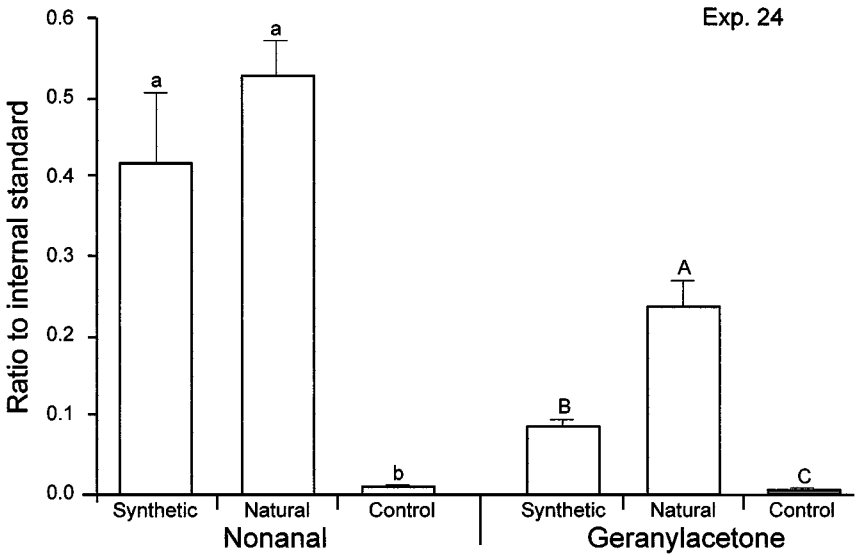


FIG. 7. Relative quantities of Porapak Q-captured synthetic and natural nonanal and geranylacetone disseminating from filter paper or muskrat pelt (150 cm^2). For each chemical, bars with different letters are significantly different [Kruskal Wallis test with Tukey-type non-parametric multiple comparison ($P < 0.05$)]. Note: Filter paper was provisioned with the equivalent amount of nonanal and geranylacetone that emanated from natural pelt during 24 hr of aeration in preceding studies.

signal sub-optimal larval habitat. Olfactory recognition of semiochemicals both from sub-optimal and optimal larval habitat by WCM may represent a fail-safe mechanism to select the microhabitat most suitable for larval development. Alternatively, a sub-optimal habitat at the time of oviposition may become optimal as the larvae develop.

Acknowledgments—We thank, H. D. Pierce, Jr. for assistance in volatile collections, S. Halford, D. Baber, J. Moldenado and L. Bertram for collecting and rearing *T. bisselliella*, Anne Embra from the Langley Spinners and Weavers Guild for supplying larval habitat, and J. H. Borden and A. S. Harestad for review of the manuscript. This study was supported, in part, by an industrially oriented research grant (IOR) from the Natural Sciences and Engineering Research Council of Canada (NSERC) with S. C. Johnson & Son, Inc. as the industrial partner, and by a Frank A. Linville Graduate Scholarship in Olfaction from Simon Fraser University to S. T.

REFERENCES

ANDERSON, G. S., and VANLAERHOVEN, S. L. 1996. Initial studies on insect succession on carrion in South-western British Columbia. *J. Foren. Sci.* 41:617–625.

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c:722–725.

BLUM, M. S. 1996. Semiochemical parsimony in the Arthropoda. *Annu. Rev. Entomol.* 41:353–374.

BORDEN, J. H. 1982. Aggregation pheromones in bark beetles, pp. 74–139, *in* J. B. Mitton, and K. B. Sturgeon (eds.). North American Conifers: A System for the Study of Evolutionary Biology. University of Texas Press: Austin.

BORNEMISSZA, G. F. 1957. An analysis of arthropod succession in carrion and the effect of its decomposition on the soil fauna. *Aust. J. Zool.* 5:1–12.

BYERS, J. A. 1992. Optimal fractionation and bioassay plans for the isolation of synergistic chemicals: the subtractive-combination method. *J. Chem. Ecol.* 18:1603–1621.

BYERS, J. A. 1995. Host tree chemistry affecting colonization in bark beetles, pp. 154–213, *in* R. T. Cardé, and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman and Hall: New York.

DELURY, N. C., GRIES, R., GRIES, G., JUDD, G. J. R., and KHASKIN, G. 1999. Moth scale-derived kairomones used by egg-larval parasitoid *Ascogaster quadridentata* to locate eggs of its host, *Cydia pomonella. J. Chem. Ecol.* 25:2419–2431.

DETMERS, B., WOHLGEMUTH, R., and SCHNEIDER, E. 1992. Über die Wirkung von Steinkleekraut und Patschuliölgranulat auf die Kleidermotte *Tineola bisselliella* Hum. (Lepidoptera: Tineidae). *Anz. Schädlingskunde Pflanzenschutz Umweltschutz.* 65:81–88.

EARLY, M., and GOFF, M. L. 1986. Arthropod succession patterns in exposed carrion on the island of O'Ahu, Hawaiian Islands, USA. *J. Med. Entomol.* 23:520–531.

EMLEN, S. T., and ORING, L. W. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science.* 197:215–223.

GERARD, P. J., PERRY, N. B., RUF, L. D., and FOSTER, L. M. 1993. Antifeedant and insecticidal activity of compounds from *Pseudowintera colorata* (Winteraceae) on the webbing clothes moth, *Tineola bisselliella* (Lepidoptera: Tineidae) and the Australian carpet beetle, *Anthrenocerus australis* (Coleoptera: Dermestidae). *Bull. Entomol. Res.* 83:547–552.

KAN, E., and WAKU, Y. 1985. Analysis of oviposition preference in the webbing clothes moth, *Tineola bisselliella* Hum. (Lepidoptera: Tineidae). *Appl. Ent. Zool.* 20:322–330.

KENNEDY, J. S. 1974. Changes in the patterning of behavioural sequences, pp. 1–6, *in* L. B. Brown (ed.). Experimental Analysis of Behaviour. Springer-Verlag: New York.

LEAL, W. S., ONO, M., HASEGAWA, M., and SAWADA, M. 1994. Kairomone from dandelion, *Taraxicum officinale* attractant for scarab beetle. *J. Chem. Ecol.* 20:1697–1704.

LIN, H., and PHELAN, P. L. 1991. Identification of food volatiles attractive to *Glischrochilus quadrisignatus* and *Glischrochilus fasciatus* (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:2469–2480.

LINDROTH, R. L., SCRIBNER, J. M., and HSIA, M. T. S. 1988. Chemical ecology of the tiger swallowtail: mediation of host use by phenolic glycocides. *Ecology* 52:814–822.

MACÍAS-SÁMANO, J. E., BORDEN, J. H., GRIES, R., PIERCE, H. D. JR., GRIES, G., and KING, G. G. S. 1998. Primary attraction of the fir engraver, *Scolytus ventralis. J. Chem. Ecol*. 24:1049–1075.

NOTTINGHAM, S. F., HARDIE, J., DAWSON, G. W., HICK, A. J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1991. Behavioural and electrophyiological responses of aphids to host and non-host volatiles. *J. Chem. Ecol.* 17:1231–1243.

PARKER, G. A. 1970. Sperm competition and its evolutionary effect on copula duration in the fly, *Scatophaga stercoraria. J. Insect Physiol.* 16:1301–1328.

REED, H. B. JR. 1958. A study of dog carcass communities in Tennessee, with special reference to the insects. *Am. Mid. Nat.* 59:213–245.

RODRIGUEZ, W. C., and BASS, W. M. 1983. Insect activity and its relationship to decay rates of human cadavers in east Tennessee. *J. Foren. Sci.* 28:423–432.

STAFFORD, F. 1971. Insects of a medieval burial. *Sci. Arch.* 7:6–10.

TAKÁCS, S., GRIES, G., and GRIES, R. 1997. Semiochemical-mediated location of host habitat by *Apanteles Carpatus* (Say) (Hymenoptera: Braconidae), a parasitoid of clothes moth larvae. *J. Chem. Ecol.* 23:459–472.

TAKÁCS, S., GRIES, G., and GRIES, R. 2001. Communication ecology of webbing clothes moth: 1. Semiochemical-mediated location and suitability of larval habitat. *J. Chem. Ecol.* 27:1535–1546.

TRANYIER, R. M. M., SCHUMACHER, R. K., and LAU, D. M. 1994. Oviposition site selection by *Tineola bisselliella, Tinea* spp (Lepidoptera: Tineidae) and *Anthrenus flavipes* (Coleoptera: Dermestidae). *J. Stor. Prod. Res.* 30:321–329.

VANLAERHOVEN, S. L., and ANDERSON, G. S. 1999. Insect succession on buried carrion in two biogeoclimatic zones of British Columbia. *J. Forensic Sci.* 44:32–43.

VET, L. E. M. 1983. Host-habitat location through olfactory cues by *Leptopilina clavipes* (Hartig) (Hymenoptera: Eucoilidae), a parasitoid of fungivorous *Drosophila*: The influence of conditioning. *Neth. J. Zool.* 33:225–248.

WILSON, H. F. 1940. Lures and traps to control clothes moths and carpet beetles. *J. Econ. Ent.* 33:651–653.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs: New Jersey.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

CAPTURE RATES OF THE EUROPEAN PINE SAWFLY, *Neodiprion sertifer*, IN PHEROMONE TRAPS, WITH SPECIAL REGARD TO EFFECTS OF WIND SPEED

FREDRIK ÖSTRAND,[1,*] OLLE ANDERBRANT,[1] PETER JÖNSSON,[2,3] and PÄIVI LYYTIKÄINEN-SAARENMAA[1,4]

[1]*Dept. of Ecology, Lund University, Ecology Building, SE–223 62 Lund, Sweden*
[2]*Dept. of Physical Geography, Lund University, Sölvegatan 13, SE–223 62 Lund, Sweden*

(Received September 5, 2000; accepted April 7, 2001)

Abstract—Males of the European pine sawfly, *Neodiprion sertifer* Geoffr., were marked and released downwind from pheromone traps, baited with 100 μg of the sex pheromone (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecyl acetate. Males were released 5 m downwind from one trap, or downwind from five traps, 50 m or 200 m away. The average capture rates after 24 hr were 21.5%, 17.7% and 3.8%, respectively. The capture rate was highest at moderate wind speeds (1–2 m/sec) in the 50 m experiments, whereas it decreased above wind speeds of 1.5 m/sec in the 200 m experiments. With no precipitation and $>13.5^{\circ}$C during overcast, wind speed is presumably the most important climatic factor for *N. sertifer* males flying upwind to a pheromone source. Travel time, the elapsed time form take-off to landing on the trap, varied considerably, and the shortest recorded travel times were 1, 6 and 45 min for the 5, 50, and 200 m experiments, respectively. The trap efficiency *i.e.*, number of captured males per number of males that landed on the trap, was estimated at 52% in the 5 m experiments. The sampling range after 24 hr was calculated at approximately 400 m by regression analysis. The combination of the males' flight ability during upwind progress and their longevity (12 days), suggests a potentially large seasonal sampling range of the traps used in this study.

Key Words—Diprionidae, Hymenoptera, sampling range, mark-release-capture.

*To whom correspondence should be addressed.
[3]Present address: Malmö University, SE–205 06 Malmö, Sweden.
[4]Present address: University of Joensuu, Faculty of Forestry, P.O. Box 111, FIN- 80101 Joensuu, Finland.

0098-0331/01/0800-1561$19.50/0

INTRODUCTION

Traps baited with species-specific pheromones are becoming common tools for monitoring insect pests, but the correspondence between catches and damage is often poor (Trumble, 1996; Lyytikäinen-Saarenmaa et al., 1999), partly because of an unknown sampling range of the trap. The sampling range is the maximum range from which insects can be shown to reach an attractive (odorant) source within a given time period (Wall and Perry, 1987). Another definition used for describing the action range of a pheromone trap is attraction range, the maximum distance over which insects can be shown to direct their movement to a source (Wall and Perry, 1987). The sampling range will increase with time until it levels off, whereas the attraction range is constant.

By performing well designed mark-release-capture experiments, one can determine how many insects are captured from different distances within a given time, and how fast the insects reach a trap. The influence of weather on trap catch can also be determined. Studies of this kind are rare, but should be useful when designing a monitoring program.

The European pine sawfly, *Neodiprion sertifer* Geoffr. (Hymenoptera: Diprionidae), is one of the most harmful defoliators of pines in Europe (Austaråa et al., 1987; Day and Leather, 1997). Many pine sawflies use sex pheromones for mate attraction (e.g., Anderbrant, 1999). On average, one *N. sertifer* female contains ca. 10 ng of the sex pheromone precursor, (2*S*,3*S*,7*S*)-3,7-dimethyl-2-pentadecanol (diprionol) (Wassgren et al., 1992). Males respond to the female-released pheromone (either acetate or propionate of the alcohol), both in electrophysiological recordings (Hansson et al., 1991) and in field trapping (Anderbrant et al., 1992a).

Östrand et al., 2000 showed that *N. sertifer* responded to synthetic pheromone 200 m away in the field; males directed their behavior, and also took off faster when stimulated by pheromone. Here, we report the capture rates of *N. sertifer* males released at various distances from pheromone traps, how weather affects capture rates, travel times of males flying upwind to a pheromone source, and the behavior of males landing on the pheromone trap.

METHODS AND MATERIAL

Study Site and Local Pine Sawfly Population. The study was performed in August and September, from 1996 to 1998, 35 km east of Lund, southernmost Sweden. To reduce the influence of native females, the experiments were carried out in a 210 × 210 m plot inside a 28 ha plantation of 2 to 3 m tall birch, *Betula pendula* Roth., bordered on the north and west by plantations of Scots pine, *Pinus sylvestris* L., 15 to 20 m tall. Small plots of larch, *Larix decidua* Mill., approximately 0.8 m tall, were present within the birch plantation.

To monitor the local population of *N. sertifer*, four Lund-I sticky traps (Anderbrant et al., 1989) were hung in a square, approximately 350 m from each corner of the experimental plot. Traps were baited with 100 μg of the attractive isomer, (2*S*,3*S*,7*S*)- 3,7-dimethyl-2-pentadecyl acetate (99% stereochemically pure), prepared at the Mid Sweden University (Högberg et al., 1990; Anderbrant et al., 1992a), and released from a cotton roll, Celluron® No. 2 (Paul Hartmann, S.A., France). Monitoring began at the end of July in 1996 and 1997, and baits were renewed every fortnight.

Insects. *Neodiprion sertifer* larvae were collected in June 1996 and 1997 near Valdemarsvik, Östergötland, Sweden, and in 1998, outside Filipstad, Värmland, Sweden. The larvae were reared in cardboard boxes, standing outdoors in a sun- and rain-protected area, and given water daily and pine twigs when needed. After having spun cocoons, males to be used in the experiments were separated from females.

During the first three release experiments in 1996, males were not marked because the four monitoring traps caught very few wild males. As the catches of wild *N. sertifer* males increased, we marked the males before release by painting a dot on the pronotum with a white instant marker (Edding 780, extra fine). In 1997, we also used silver and gold (PILOT, SC, extra fine). In 1998, the pronotum was divided into four fields. By painting one to three dots of seven different water-based and water-resistant colors, there were enough combinations for releasing individually marked males.

Males emerged indoors and, after marking, they were kept individually for $\leq$4 days at 4–8°C in plastic test tubes (i.d. 9.5 mm, 55 mm long) containing a piece of soft moistened paper and capped with perforated corks to allow for air exchange. On experimental days, males were kept in a cooling bag until release.

Longevity Study. The longevity of marked and unmarked males was determined in 1998. After emergence, the males were placed individually in plastic jars, 5 $\times$ 5 $\times$ 7 cm high, each containing a fresh pine shoot wrapped in moistened paper. The jars were put in plastic boxes, 22 $\times$ 30 $\times$ 12 cm, placed on wooden boards lying on the ground in the experimental plot. The plastic boxes were covered with a rain shelter. Two series each of 15 marked and 15 unmarked males were run, and the results were pooled. Living males were counted each day until all had died. The temperature inside the boxes was recorded throughout the study.

Experimental Design. Males were released 50 or 200 m downwind of five Lund-II pheromone traps (Anderbrant et al., 1989) (intertrap distance 25 m), or 5 m downwind of one trap (Östrand et al., 2000). At 50 m and 200 m, we used five traps to minimize the effect of shifts in wind direction. The traps were placed about 1.7 m above ground. New baits (100 μg) were used in each experiment; each bait released approximately 45 μg of the pheromone during the first day (calculated from Anderbrant et al., 1992b).

Males were released individually from open test tubes. In the first two experiments in 1996, up to 100 tubes were placed vertically in a test tube rack ($33 \times 12 \times 6$ cm) resting on a cardboard platform and attached to a wooden pole (ca. 1.7 m above ground). In the following experiments, the test tubes containing the males were opened and stacked horizontally in a plastic jar (i.d. 11 cm, 13 cm height) covered on the outside with black opaque plastic. Five holes (5 mm) were drilled in the lid around which a white cardboard platform (35×35 cm) was attached. After leaving the test tubes, the males moved towards the light coming through the holes in the lid. If >40 males were released, the test tubes were divided into two jars attached to one platform each and placed ca. 0.5 m from each other.

To avoid interference, pheromone sources were removed from the four monitoring traps before the experiments started. The prevailing wind direction was determined with a wind vane at 9 m above ground, and the platforms and the traps were re-positioned every experimental day so that males always were released downwind of the trap(s).

One or two observers stood behind the platforms and recorded which males took off. Another observer recorded incoming males in the traps. In the 5 m experiments, one person stood upwind of the trap and recorded the behavior of males landing on the trap. To allow behavioral observation of males as they entered these traps in 1997 (see Östrand et al., 2000), we used a sticky bottom with glue only in a central 4×4 cm square (below the dispenser). Males were observed until they took off again or became stuck on the glue. After 3–4 hr, we exchanged the bottom for one with glue on the entire surface during the remaining hours of the experiment. In 1998, we only used the regular bottoms fully coated with glue. Data from 1997 and 1998 on landing and take-off were coupled with observations in 1998 on trap efficiency to create a flow chart describing the sequence of behaviors during orientation and capture. Capture rates were based on the number of males that had left the take-off platform within 24 hr.

Because males seldom flew straight from the platform to the trap, and gusty wind transported them out of sight of the observer, it was impossible to measure the ground speed of orienting males. Instead, we calculated the elapsed time from take-off to landing = travel time. The exact time for landing could not be recorded when patrolling the five traps in the 50 m and 200 m experiments, and instead we present a range. The median of such ranges was used for statistical analysis of travel time. For instance the median travel time of a male reaching the trap within 6–9 min was 7.5 min.

Weather Recording. An automatic weather station was placed in the experimental plot. Air temperature and relative humidity were recorded at 2 m above ground with copper-constantan thermocouples with a 0.5 mm diameter and Hygromer® (Rotronic Bassersdorf, Switzerland), respectively. Incoming short wave radiation (335–2000 nm, Wm^{-2}) was measured 2 m above ground (Kipp and Zonen, Delft, The Netherlands). Wind speed and direction were recorded at

2 m and 9 m above ground with cup-wheel anemometers and wind vanes (R. M. Young, Traverse City, Michigan). Mostly, the sensors were scanned every 30 sec, and the data were stored as 30 min averages in a CR10 micro logger (Campbell Scientific, Logan, Utah). During the 5 m experiments and all experiments in 1998, the scanning interval was 2 sec, and averages were stored every second min.

In 1998, wind speed and direction, together with temperature, were recorded only at 2 m above ground. A copper-constantan thermocouple was used for recording the temperature in the plastic boxes during the longevity study. Wind speed recorded 2 m above ground was used in the capture-wind speed regression analysis.

Statistical Tests. The proportions of males of different age taking flight were compared with a *G*-test, and the longevity of marked and unmarked males was compared with Student's *t*-test (Sokal and Rohlf, 1995). Polynomial (second order) or linear regression analyses were used for evaluating the relationship between capture-wind speed (with and without temperature included), and capture-distance. Total capture rates were used in the statistical evaluations, except in the capture-distance regression, where captures were expressed per trap. Before regression analysis, the capture rates and distance were arcsin $\sqrt{}$ (proportion capture) and log transformed, respectively. The maximum sampling ranges were obtained by calculating the x-intercept at $y = 0$ for each of the three sampling times 1, 2.5, and 24 hr (Schlyter, 1992), and confidence intervals following Sokal and Rohlf, 1995.

RESULTS

Of 1961 males released on 28 days, 82.7% took off (range 62.1–96.6%) (Table 1). Take-off percentage was positively correlated with mean temperature during the first 3–5 hr of the experiments ($r^2 = 0.217$, $P = 0.01$, $N = 28$). The total catch in the two outermost traps in the 200 m experiments was higher than in the two innermost or the central trap, from left to right 7, 2, 5, 2, 7, whereas it was the opposite in the 50 m experiments: 13, 20, 28, 18, 7. From the release point, the angle of separation for the outermost traps was 64° and 32° in the 50 m and 200 m experiments, respectively.

Capture Rates. The capture rates after 24 hr in the 5, 50, and 200 m experiments were on average 21.5, 17.7, and 3.8%, respectively (Table 1). Captures varied with wind speed at all three distances. In the 50 m experiments, the recaptures were the highest at intermediate wind speeds, 1-2 m/s (Figure 1B). In the 200 m experiments, the captures decreased at wind speeds above 1.5 m/s (Figure 1C). In the 5 m experiment, which made use of only one trap, there was no significant relationship ($P > 0.2$ for linear and polynomial regressions) between capture and wind speed (Figure 1A). The recorded temperature varied from 13.4–25.4°C. In one instance, no males were captured when the temperature was low and the

TABLE 1. NUMBER OF *N. sertifer* MALES THAT WERE RELEASED, TOOK OFF AND WERE CAPTURED AFTER 24 HR. MEAN VALUES OF TEMPERATURE AND WIND SPEED (2 M ABOVE GROUND) CALCULATED FROM 30 MIN AVERAGES DURING THE TIME THE TRAPS WERE UNDER OBSERVATION (TYPICALLY 3–5 H). BOLD FIGURES = MEAN ± SD

Date	No. released	Take-off (%)	Recapt % (*N*)	Temp (°C)	Wind speed (m/s)
5 m					
6 Sep. 97	36	72.2	11.5 (3)[a]	19.0	2.7
17 Sep. 97	87	94.3	11.0 (9)	17.5	4.0
24 Sep. 97	87	90.8	50.6 (40)	16.0	2.3
26 Sep. 97	87	62.1	11.1 (6)	15.6	1.4
29 Sep. 97	59	96.6	28.1 (16)	17.7	2.0
30 Sep. 97	52	88.5	13.0 (6)	16.2	3.7
7 Sep. 98	63	90.5	19.3 (11)	19.2	1.2
10 Sep. 98	45	88.9	17.5 (7)	19.6	2.6
			21.5 ± 14.1[b]		
50 m					
14 Aug. 96	40	80.0	37.5 (12)[c]	19.6	1.4
20 Aug. 96	53	96.2	27.5 (14)[c]	25.4	1.8
25 Sep. 96	68	69.1	2.1 (1)	13.4	1.7
20 Sep. 97	84	71.4	26.7 (16)	14.0	2.0
28 Sep. 97	77	80.5	25.8 (16)	17.2	0.9
19 Aug. 98	50	88.0	13.6 (6)	16.8	3.0
20 Aug. 98	71	88.7	6.3 (4)	19.8	2.7
25 Aug. 98	52	78.8	9.8 (4)	16.8	3.0
30 Aug. 98	85	83.5	8.5 (6)	18.0	0.5
8 Sep. 98	84	92.9	20.5 (16)	21.2	2.1
			17.7 ± 11.6		
200 m					
17 Aug. 96	69	92.8	4.7 (3)[c]	23.9	0.6
3 Sep. 96	71	78.9	7.1 (4)	19.3	1.0
6 Sep. 96	79	75.9	0	15.1	1.7
14 Sep. 96	82	68.3	8.9 (5)	14.6	0.7
17 Sep. 96	86	84.9	0	13.9	2.4
19 Sep. 96	65	93.8	6.6 (4)	14.3	1.4
18 Sep. 97	63	88.9	1.8 (1)	14.6	3.1
27 Aug. 98	84	76.2	3.1 (2)	16.2	1.5
1 Sep. 98	98	71.4	2.9 (2)	17.5	1.2
2 Sep. 98	84	77.4	3.1 (2)	16.4	1.3
			3.8 ± 3.0		

[a] Capture figure after 2.5 hr.
[b] Excluding 6 Sep 97.
[c] Unmarked males released.

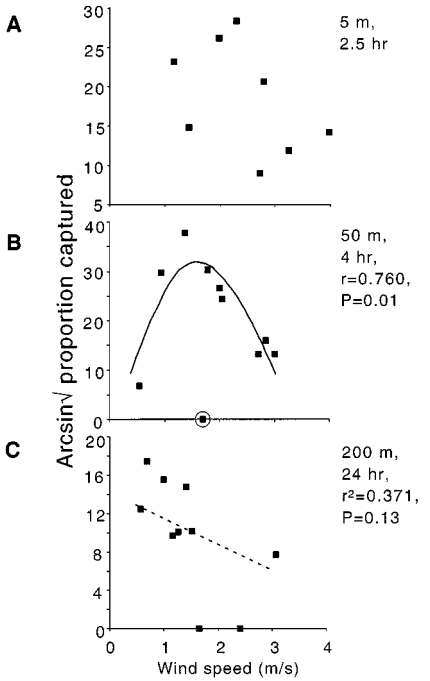


FIG. 1. Capture of male *N. sertifer* at different wind speeds when released at different distances from traps. Wind speed calculated from 30 min averages in the 50 and 200 m experiments, and 2 min averages in the 5 m experiments, except for the data on 24 Sept 97 when only 30 min averages were available. A. No significant relation. B. Encircled point is from 25 Sep 1996, when both temperature and incoming short wave radiation were low: 13.4°C and 172 W/m^2, respectively. Excluding this date, the data fit the bell-shaped function, $y = -10.8 + 48.1\,x - 13.9\,x^2$. C. $y = 14.3 - 2.5\,x$.

weather was cloudy (Table 1, Figure 1B). Otherwise, there was no relationship between capture and temperature, and the capture-wind speed regressions did not improve when temperature data were included.

Four and eight late-coming males were captured after the initial 24 hr of sampling in 1997 and 1998, respectively. Nine of these males were captured approximately 48 hr after release, and the remaining three males after 72 hr. Eight of these males were two days old, and one was five days old when captured.

Sampling Range. At all three distances, most males were recaptured within the first 3 hr of sampling (Figure 2). The calculated maximal distances at which a trap can be expected to catch males after 1, 2.5, and 24 hr of sampling were 190 m (95% confidence interval, 70–630 m), 290 m (C.I. 90–1250), and 396 m (C.I. 140–1600), respectively (Figure 2).

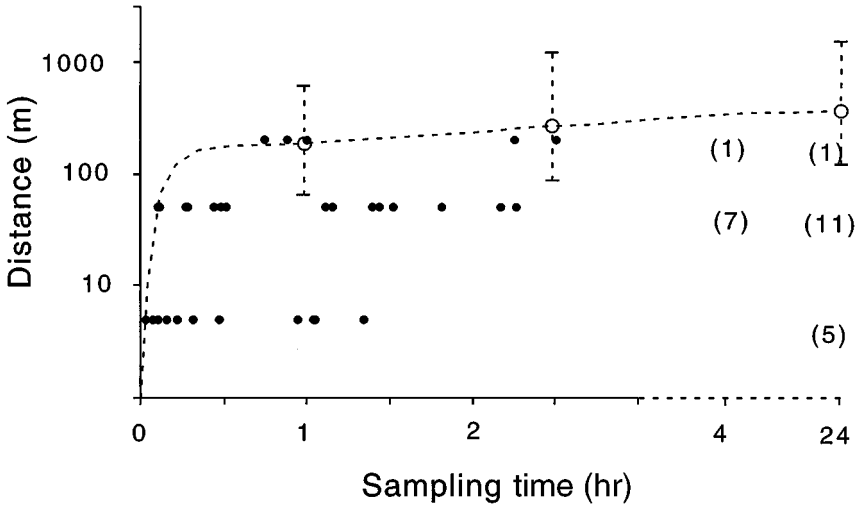


FIG. 2. Relationship between distance from pheromone traps and travel times of males caught in 1998 during the first 3 hr in the 5 m ($N = 12$), 50 m ($N = 16$), and 200 m ($N = 5$) experiments, respectively (black dots). Also shown (in parenthesis) are additional catches after 4 hr (not recorded in 5 m exp.) and 24 hr. Calculated maximal sampling distances (i.e., the expected sampling ranges of traps), with 95% confidence intervals, after 1 hr, 2.5 hr, and 24 hr of sampling are shown as open circles. The dashed line illustrates the theoretical relationship between time and sampling range.

Travel Time. When considering the distance, the time elapsed from take-off to landing was comparable for the fastest males at each of the three distances (Table 2); it took the sawflies 1 min or more to reach the trap in the 5 m experiment. Increasing the distance by a factor of 10 or 40, the shortest travel times increased to 6–10 min or 40–50 min, respectively (Table 2).

Males in the 50 m experiment in 1998 tended to reach the traps faster with increasing wind speed, $r^2 = 0.404$, $P = 0.065$, $N = 9$ (1 day old males with travel time $\leq$45 min, wind speed calculated from 2 min averages). Occasionally males landed in birch trees before reaching a trap, resulting in longer travel time.

Longevity and Effects of Age. Marked and unmarked *N. sertifer* males lived 11.4 ± 4.3, and 12.3 ± 5.2 days (mean $\pm$ SD), respectively, $t_{(58)} = 0.73$, $P > 0.05$, with a range of 2–25 days. The mean recorded temperature during the experiment was 13.6 ± 4.0°C, range 4.0–25.3°C, $N = 1344$, based on 30-min averages from 28 days.

Fewer males took off as age increased from 0 days (released the day they emerged) to 2 days; $G = 7.3$, $P < 0.05$, $N = 594$ in 1997, and $G = 8.2$, $P < 0.05$, $N = 639$ in 1998. There were no trends in the capture of males of different ages.

TABLE 2. TRAVEL TIMES (RANGES) FOR THE FIVE FASTEST MALES AT EACH OF THE THREE DISTANCES IN 1998. IN THE 200 M EXPERIMENTS, THE TRAVEL TIMES FOR ALL CAPTURED MALES THAT COULD BE IDENTIFIED ARE PRESENTED. THE AVERAGE WIND SPEED (RECORDED AT 2 M ABOVE GROUND) DURING EACH FLIGHT IS SHOWN TOGETHER WITH THE MALE'S AGE. ZERO DAY OLD MALES EMERGED ON THE SAME DAY THEY WERE RELEASED

5 m			50 m			200 m		
Travel time (min)	Wind speed (m/s)	Age (days)	Travel time (min)	Wind speed (m/s)	Age (days)	Travel time (min)	Wind speed (m/s)	Age (days)
0.8–3.0	0.8	0	≤6	3.2	1	39–50	1.3	1
1.4–3.0	1.3	4	≤7	3.2	1	44–62	1.7	1
1.0–3.0	2.5	0	9–17	1.8	0	51–68	1.9	0
1.7	1.1	0	9–19	1.9	0	139–170	1.3	1
3.1	1.2	0	≤17	2.8	1	194–205	1.2	1

In one of the 5 m experiments in 1998, 15.4 % of males aged 0 days were captured after 24 hr, and 19.4% of males aged 4 days were captured, $G = 0.2$, $P > 0.05$, $N = 57$. However, travel time to land on or in the trap for males aged 0 days was 10.2 ± 17.0 min ($N = 10$) faster, $t_{(16)} = 2.57$, $P < 0.05$, than the time of 42.7 ± 35.4 min ($N = 8$) for males aged 4 days.

Observations at the Trap. Most males took off into the wind and then flew in broad lateral turns of several meters in amplitude (side to side). When approaching a trap, males tended to fly straighter with lateral turns of a few decimeters. The flow scheme in Figure 3 shows the sequence of behaviors by males attracted to a trap based on observations at the traps and some assumptions. In the first 2.5 hr in the 5 m exp. in 1998, 52.2% of 23 males that landed on the trap were captured. In the first 2.5 hr in the 5 m exp. in 1997 and 1998, 87% (62/71) of 130 males landing on the roof took off again. In these experiments, one male visited a trap five times without getting caught. Of the males that landed elsewhere than on the sticky bottom of the trap, we assumed that the proportion captured later on the sticky trap bottom was equal to the proportion that took flight. By allowing for a maximum of five visits without getting caught and if 52.2% of the visiting males are trapped, we obtained by iteration that 39% of the males are permanently lost from the trap's attraction range in each round. These 39% represent the males that take-off from the trap, either directly from the roof or elsewhere from the trap, and are hereafter lost (27/69.5%). In the first visit, 30.5% of the males will be caught, of which 23% of them land directly on the sticky trap bottom (Figure 3). In the next four visits, 13.0, 5.5, 2.3 and 1.0% of the males from the initial visit will be caught.

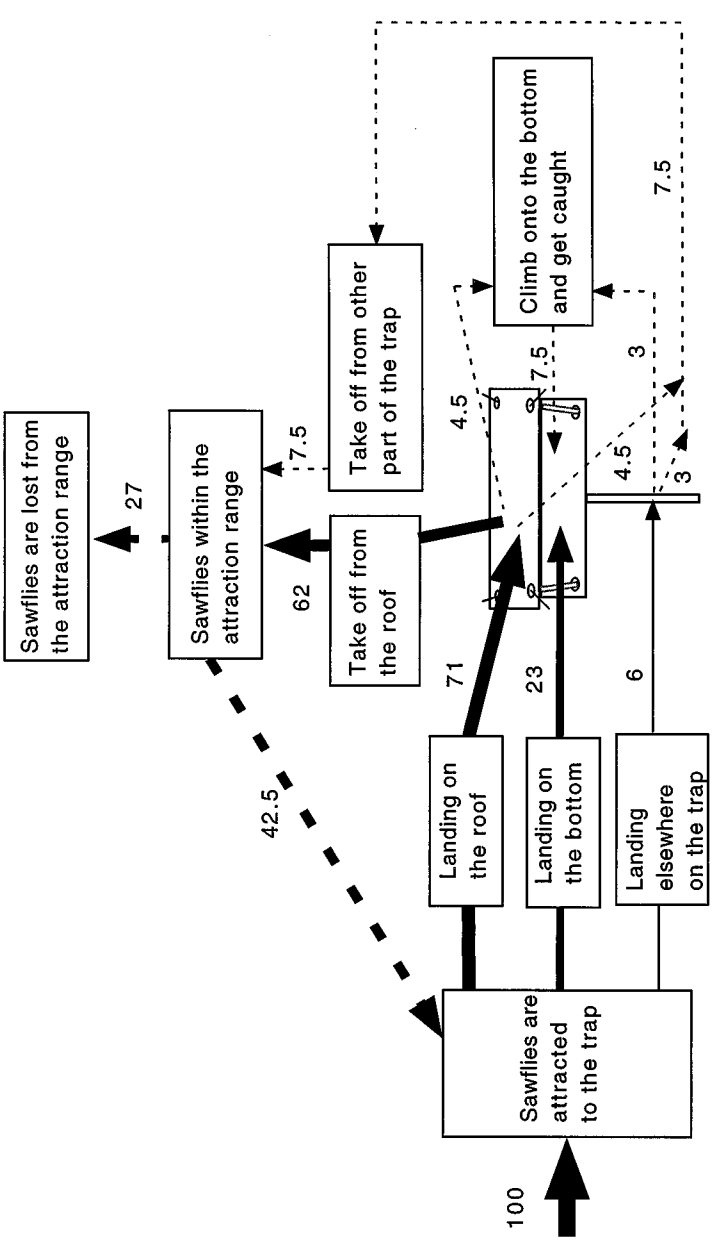


FIG. 3. Flow-scheme showing the capture of *N. sertifer* males landing on different parts of a pheromone trap. All figures in percentage, and at the start 100 males are attracted to the trap. Solid arrows are based on observations at the trap, and dashed arrows are based on assumptions. See text for more information.

DISCUSSION

Capture Rates and Sampling Range. Although a rough estimate, the 400 m sampling range, after 24 hr for the Lund-II traps baited with 100 μg of synthetic pheromone, is the first calculated sampling range of a trap for pine sawflies. The theoretical sampling range-time curve (Figure 2) will level off towards the maximum sampling range of a trap when sampling for longer than 24 hr, and will probably reach at least 500–600 m (Östrand and Anderbrant, unpublished data).

Captures in the 200 m experiments during the first 3.5–5 hr are similar to the 76% capture of the total capture of sweet potato weevils in the first 10.5 hr of a 27 hr period when *Cylas formicarius* (Summers) were released 10–1000 m from pheromone-baited traps (Sugimoto et al., 1994). Similarly, 90% of fall web worms, *Hyphantria cunea* (Drury), male were caught within 12 hr of a 60 hr sampling period (Zhang and Schlyter, 1996).

Wind Speed. As wind speed increases, a pheromone plume will elongate, making it easier for insects to locate the source, whereas a further increase in wind speed will decrease the maximum distance of pheromone detection due to greater dilution of the pheromone and higher turbulence intensities (Elkinton et al., 1987). At higher wind speeds, insects will have trouble flying upwind. Temperature will also affect the plume structure (Brady et al., 1995). Male *N. sertifer* became increasingly reluctant to wing fan and take-off as wind speed 2 m above ground increases from 1.5 to 4.0 m/s (Östrand et al., 2000). Therefore, the capture rate with increasing wind speed might be bell-shaped, as we observed in the 50 m experiments (Figure 1B). Such a pattern was not apparent at the other two distances. In contrast, Wedding et al. (1995) captured *N. sertifer* in increasing numbers as wind speed rose from 1 to 3 m/s, possibly because some males were released upwind and were transported toward the traps. There are, however, several indications from other studies for an optimal wind speed with regard to odorant source finding in insects (Nottingham, 1987; Aluja and Prokopy, 1992; Brady et al., 1995; Messing et al., 1997).

Travel Time. If the shortest recorded travel times are converted to 'travel speed,' we obtain ca. 5–10 m/min for the fastest *N. sertifer* males flying upwind. This speed is lower than the 20–30 m/min recorded for gypsy moths, *Lymantria dispar* L. (Elkinton et al., 1987) and 50–75 m/min for pea moths (Wall and Perry, 1987), both at wind speeds comparable to those in our study. However, the pea moths were released in a wheat field (Wall and Perry, 1987) and the gypsy moths in a pine plantation with little understorey vegetation (Elkinton et al., 1987), whereas the relatively dense birch plantation in our study allowed the pine sawflies to land in the birches on their way towards a trap. Among tsetse flies, *Glossina pallidipes* Aust., flying upwind at 5 m/s to an odorant source 75 m away, two cohorts were observed, those arriving directly and those making stops along the way (Griffiths

et al., 1995). The high wind speed in the 50 m experiments (Table 2), might have aided the sawflies to fly faster (or make fewer stops) than in the 200 m experiments.

Longevity and Effects from Age. Longevity in pine sawflies is temperature-dependent (Mallach, 1974). In Canada *N. sertifer* males lived on average 20 days at 18°C (range 4–32°C) and 25 days at 15°C (Lyons and Griffiths, 1962). In their 18°C treatment, an unexplainably longer longevity was recorded than the 11–12 day duration at 13.6 ± 4°C in our study. Male and female white pine sawflies, *Neodiprion pinetum* (Norton), lived 3–4 days at a constant temperature of 23°C (Rauf and Benjamin, 1980), whereas *Neodiprion fulviceps* Cresson lived only 2–3 days in the laboratory (Wagner et al., 1986). The combination of the males' flight ability during upwind progress and their longevity (12 days) suggest a potentially large sampling range of the Lund-I traps used in this study.

Moths often show a peak in response to pheromone at intermediate ages (McNeil, 1991 and references therein). However, although the amplitudes of EAG's recorded from *N. sertifer* increased until day 4 after emergence (Hansson et al., 1991), no trend could be seen in the capture of males of different ages in our study. In contrast, more 2 day old gypsy moth males were captured than 1 day old males (Elkinton and Cardé, 1980). In general, it seems as if the insect increases its sensitivity to the pheromone with increasing age, while the flight ability decreases (e.g., Haniotakis and Pittara, 1994; Shirai, 1998). In a short-lived species like *N. sertifer*, the 'optimal' age to locate a pheromone source is, therefore, about 1–2 days.

Observations at the Trap. The 52.2% catch efficiency obtained in this study is comparable with the highest proportions of pea moths captured by Lewis and Macaulay (1976) with different types of traps. In their study, there was no way of knowing if some moths returned and were caught later. In our study, males were marked, and many could be observed taking off several times before being trapped. The efficiency of the Lund-II trap may seem low, but it has the advantage of low catches when unbaited (Anderbrant et al., 1989). In a mark-release-capture experiment using the bark beetles *Ips typographus* L., Helland et al. (1984) recorded that some beetles were constantly lost from the close vicinity of a pheromone trap, and concluded that this was one explanation for pheromone traps having low catch efficiency.

Acknowledgments—We thank Karin Johnson, Johanna Skobe and Thomas Johansson for excellent technical assistance in the field, Christine Achberger, Marie Ekström and Kristina Blennow for running the weather station, Fredrik Schlyter and Neil J. Vickers for review of an earlier version of the manuscript, and Erik Hedenström and Hans-Erik Högberg for kindly providing the pheromone. The research was funded by the Swedish Council for Forestry and Agricultural Research, Stiftelsen Futura and the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD programme "Pine sawfly pheromones for sustainable management of European forests (PHERODIP)," CT 95-0339. The study does not necessarily reflect the Commission's views and in no way anticipates its future policy in this area.

REFERENCES

ALUJA, M., and PROKOPY, R. J. 1992. Host search behaviour by *Rhagoletis pomonella* flies: Inter-tree movement patterns in response to wind-borne fruit volatiles under field conditions. *Physiol. Entomol.* 17:1–8.

ANDERBRANT, O. 1999. Sawflies and seed wasps, pp. 199–226, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. CAB International, Wallingford.

ANDERBRANT, O., LÖFQVIST, J., JÖNSSON, J., and MARLING, E. 1989. Effects of pheromone trap type, position and colour on the catch of the pine sawfly *Neodiprion sertifer* (Geoffr.) (Hym., Diprionidae). *J. Appl. Entomol.* 107:365–369.

ANDERBRANT, O., LÖFQVIST, J., HÖGBERG, H.-E., HEDENSTRÖM, E., WASSGREN, A.-B., BERGSTRÖM, G., BENGTSSON, M., and MAGNUSSON, G. 1992a. Field response of the pine sawfly *Neodiprion sertifer* to the pheromone (2S, 3S, 7S)- diprionyl acetate and its stereoisomers. *Entomol. Exp. Appl.* 74:83–90.

ANDERBRANT, O., BENGTSSON, M., LÖFQVIST, J., and BAECKSTRÖM, P. 1992b. Field response of the pine sawfly *Neodiprion sertifer* to controlled release of diprionyl acetate, diprionyl propionate and trans-perillenal. *J. Chem. Ecol.* 18:1707–1725.

AUSTARÅ, Ø., ORLUND, A., SVENDSRUD, A., and VEIDHAL, A. 1987. Growth loss and economic consequences following two years defoliation of *Pinus silvestris* by the pine sawfly *Neodiprion sertifer* in West-Norway. *Scand. J. For. Res.* 2:111–119.

BRADY, J., GRIFFITHS, N., and PAYNTER, Q. 1995. Wind speed effects on odor source location by tsetse flies (*Glossina*). *Physiol. Entomol.* 20:293–302.

DAY, K. R., and LEATHER, S. R. 1997. Threats to forestry by insect pests in Europe, pp. 177–205, *in* A. D. Watt, N. E. Stork, and M. D. Hunter (eds.). Forests and Insects. Chapman and Hall. London.

ELKINTON, J. S., and CARDÉ, R. T. 1980. Distribution, dispersal, and apparent survival of male gypsy moths as determined by capture in pheromone-baited traps. *Environ. Entomol.* 9:729–737.

ELKINTON, J. S., SCHAL, C., ONO, T., and CARDÉ, R. T. 1987. Pheromone puff trajectory and upwind flight of male gypsy moths in a forest. *Physiol. Entomol.* 12:399–406.

GRIFFITHS, N., PAYNTER, Q., and BRADY, J. 1995. Rates of progress up odour plumes by tsetse flies: A mark-release video study of the timing of odour source location by *Glossina pallidipes. Physiol. Entomol.* 20:100–108.

HANIOTAKIS, G. E., and PITTARA, I. S. 1994. Response of *Bactrocera* (*Dacus*) *oleae* males (Diptera: Tephritidae) to pheromones as affected by concentration, insect age, time of day, and previous exposure. *Environ. Entomol.* 23:726–731.

HANSSON, B. S., VAN DER PERS, J. N. C., HÖGBERG, H.-E., HEDENSTRÖM, E., ANDERBRANT, O., and LÖFQVIST, J. 1991. Sex pheromone perception in male pine sawflies, *Neodiprion sertifer* (Hymenoptera; Diprionidae). *J. Comp. Physiol. A.* 168:533–538.

HELLAND, I. S., HOFF, J. M., and ANDERBRANT, O. 1984. Attraction of bark beetles (Coleoptera: Scolytidae) to a pheromone trap. Experiment and Mathematical models. *J. Chem. Ecol.* 10:723–752.

HÖGBERG, H.-E., HEDENSTRÖM, E., WASSGREN, A.-B., HJALMARSSON, M., BERTRÖM, G., LÖFQVIST, J., and NORIN, T. 1990. Synthesis and gas chromatographic separation of the eight stereoisomers of diprionol and their acetates, components of the sex pheromone of pine sawflies. *Tetrahedron* 46:3007–3018.

LEWIS, T., and MACAULAY, E. D. M. 1976. Design and elevation of sex-attractant traps for pea moth, *Cydia nigricana* (Steph.) and the effect of plume shape on catches. *Ecol. Entomol.* 1:175–187.

LYONS, L. A., and GRIFFITHS, K. J. 1962. Observations on the development of *Neodiprion sertifer* (Geoff.) within the cocoon (Hymenoptera: Diprionidae). *Can. Entomol.* 94:994–1001.

LYYTIKÄINEN-SAARENMAA, P., ANDERBRANT, O., LÖFQVIST, J., HEDENSTRÖM E., and HÖGBERG, H.-E. 1999. Monitoring the European pine sawfly population densities with pheromone traps in young pine plantations. *For. Ecol. Manage.* 124:113–121.

MALLACH, N. 1974. Zur Kenntnis der kleinen Kiefern-Buschhornblattwespe, *Diprion* (*Microdiprion*) *pallipes* (Fall.) (Hym., Diprionidae). Z. *Ang. Ent.* 75:134–173.

MCNEIL, J. N. 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps.*Annu. Rev. Entomol.* 36:407–430.

MESSING, R. H., KLUNGNESS, L. M., and JANG, E. B. 1997. Effects of wind on movement of *Diachasmimorpha longicaudata*, a parasitoid of tephritid fruit flies, in a laboratory flight tunnel. *Entomol. Exp. Appl.* 82:147–152.

NOTTINGHAM, S. F. 1987. Anemotactic strategy and optimum flight conditions for host-plant finding in the cabbage root fly, *Delia radicum* (L.) (Diptera: Anthomyiidae). *Bull. Entomol. Res.* 77:271–277.

ÖSTRAND, F., ANDERBRANT, O., and JÖNSSON, P. 2000. Behaviour of male pine sawflies, *Neodiprion sertifer*, released downwind from pheromone sources. *Entomol. Exp. Appl.* 95:119–128.

RAUF, A., and BENJAMIN, D. M. 1980. The biology of the white pine sawfly, *Neodiprion pinetum* (Hymenoptera: Diprionidae) in Wisconsin. *The GT Lakes Entomol.* 13:219–224.

SCHLYTER, F. 1992. Sampling range, attraction range, and effective attraction radius: Estimates of trap efficiency and communication distance in coleopteran pheromone and host attractant systems. *J. Appl. Entomol.* 114:439–454.

SHIRAI, Y. 1998. Laboratory evaluation of flight ability of the Oriental corn borer, *Ostrinia furnacalis* (Lepidoptera: Pyralidae). *Bull. Entomol. Res.* 88:327–333.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry, the Principles of Practice of Statistics in Biological Research. W. H. Freeman, New York.

SUGIMOTO, T., SAKURATANI, Y., SETOKUCHI, O., KAMIKADO, T., KIRITANI, K., and OKADA, T. 1994. Estimations of attractive area of pheromone traps and dispersal distance, of male adults of sweet potato weevil, *Cylas formicarius* (Fabricius) (Coleoptera, Curculionidae). *Appl. Entomol. Zool.* 29:349–358.

TRUMBLE, J. T. 1996. Integrating pheromones into vegetable crop production, pp. 397–410, *in* R. T. Cardé and A. K. Minks (eds.). Insect Pheromone Research: New Directions, Chapman and Hall. New York.

WAGNER, M. R., MCCULLOUGH, D. G., and DI MATTEO J. M. 1986. Life history of *Neodiprion fulviceps* (Cresson) a ponderosa pine feeding sawfly (Hymenoptera: Diprionidae). *Proc. Entomol. Soc. Wash.* 88:221–226.

WALL, C., and PERRY, J. N. 1987. Range of action of moth sex-attractant sources. *Entomol. Exp. Appl.* 44:5–14.

WASSGREN, A.-B., ANDERBRANT, O., LÖFQVIST, J., HANSSON, B. S., BERGSTRÖM, G., HEDENSTRÖM, E., and HÖGBERG, H.-E. 1992. Pheromone related compounds in pupal and adult female pine sawflies, *Neodiprion sertifer*, of different age and in different parts of the body. *J. Insect Physiol.* 38:885–893.

WEDDING, R., ANDERBRANT , O., and JÖNSSON, P. 1995. Influence of wind conditions and intertrap spacing on pheromone trap catches of male European pine sawfly, *Neodiprion sertifer*. *Entomol. Exp. Appl.* 77:223–232.

ZHANG, Q.-H., and SCHLYTER, F. 1996. High captures and long sampling range of pheromone traps for fall web worm moth *Hyphantria cunea* (Lepidoptera: Arctiidae) males. *J. Chem. Ecol.* 22:1783–1796.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

DUAL CHEMICAL BARRIERS PROTECT A PLANT AGAINST DIFFERENT LARVAL STAGES OF AN INSECT

J. ALAN A. RENWICK,[1,*] WENQING ZHANG,[2] MEENA HARIBAL,[1] ATHULA B. ATTYGALLE,[3] and KIMBERLY D. LOPEZ[1]

[1]*Boyce Thompson Institute, Tower Road, Ithaca, NY 14853*

[2]*Institute of Entomology & State Key Laboratory for Biological Control, Zhongshan University Guangzhou 510275, China*

[3]*Department of Chemistry & Chemical Biology, Cornell University, Ithaca, NY 14853*

(Received February 23, 2001; accepted April 16, 2001)

Abstract—The host plants of the native American butterfly, *Pieris napi oleracea*, include most wild mustards. However, garlic mustard, *Alliaria petiolata*, a highly invasive weed that was introduced from Europe, appears to be protected from this insect. Although adults will oviposit on the plant, most larvae of *P. n. oleracea* do not survive on garlic mustard. We used feeding bioassays with different larval stages of the insect to monitor the isolation and identification of two bioactive constituents that could explain the natural resistance of this plant. A novel cyanopropenyl glycoside (**1**), alliarinoside, strongly inhibits feeding by first instars, while a flavone glycoside (**2**), isovitexin-6″-D-β-glucopyranoside, deters later instars from feeding. Interestingly, the first instars are insensitive to **2**, and the late instars are little affected by **1**. Furthermore, differential effects of dietary experience on insect responses suggest that **1** acts through a mechanism of post-ingestive inhibition, whereas **2** involves gustatory deterrence of feeding.

Key Words—Pieris, pieridae, crucifer, feeding deterrent, feeding inhibition, flavonoid, garlic mustard, Alliaria.

INTRODUCTION

Plants are chemically defended from insect herbivores by the presence of repellents, oviposition deterrents, feeding deterrents, and toxins. However, some herbivores inevitably adapt to these defenses and may eventually specialize by utilizing the "defensive compounds" to recognize their host plants (Ehrlich and Raven, 1964; Lindroth, 1988). In the Cruciferae (=Brassicaceae), glucosinolates and their

*To whom correspondence should be addressed. e-mail: jar14@cornell.edu

0098-0331/01/0800-1575$19.50/0

hydrolysis products act as a first line of defense against generalist insects, but some of these compounds have become signals for host finding and acceptance for many specialists (Feeny, 1977; Chew, 1988; Chew and Renwick, 1995). Some crucifers, however, produce additional compounds that protect them from specialists. For example, butterflies of the imported cabbageworm, *Pieris rapae* L. are deterred from ovipositing on *Erysimum cheiranthoides* and *Iberis amara* by the presence of cardenolides and cucurbitacin glycosides, respectively (Sachdev-Gupta et al., 1990; Huang et al., 1993). Larvae of this butterfly also are deterred from feeding on these plants by the same and similar compounds (Sachdev-Gupta et al., 1993a,b). The presence of such bioactive chemicals may have contributed to the rapid proliferation of many introduced plants in North America. For example, garlic mustard, *Alliaria petiolata*, a particularly invasive crucifer that was introduced from Europe late in the 19th century, is relatively free of herbivory (Nuzzo, 1993). The native butterflies, *Pieris napi oleracea* and *P. virginiensis*, oviposit on this plant, but their larvae usually do not survive (Bowden, 1971; Chew, 1981). In this study, experiments were conducted to examine the basis for the failure of *P. n. oleracea* larvae to develop on garlic mustard. Two chemicals responsible for inhibiting feeding were identified, and a unique system of selective resistance against different larval stages was discovered.

METHODS AND MATERIALS

Plants. Alliaria petiolata seedlings were obtained from various natural sites in the vicinity of Ithaca, New York, and transplanted into 10 cm cord pots of artificial soil (Cornell Mix A) in an air-conditioned greenhouse that was maintained at *ca* 25°C. Supplementary lighting was provided by 400-W multivapor high intensity discharge lamps.

Chemical Extraction. After 4-6 weeks, foliage from rosette plants was harvested and extracted with boiling ethanol for 5 min. The ethanolic extract was evaporated to dryness under reduced pressure, and lipids were removed by extracting with n-hexane. The defatted residue was dissolved in water and the aqueous solution was partitioned 3 times with 1-butanol. The combined butanol extract and the post-butanol water extract were concentrated under reduced pressure and dissolved in methanol/water mixtures for bioassays.

Insects. P. n. oleracea larvae for bioassays were from a colony started with insects that were collected by Dr. F. S. Chew (Tufts University) in Vermont and maintained in the laboratory at *ca*. 25°C. The colony was renewed or supplemented with field-collected butterflies on an annual basis. Oviposition occurred on cabbage plants in greenhouse cages, and the larvae were reared on cabbage (*Brassica oleracea* L var Golden Acre). Naïve neonates (no experience with a plant) were

obtained by allowing butterflies to oviposit on a strip of Parafilm that was wrapped around a beaker immediately below a cabbage leaf, on which butterflies made tarsal contact (Webb and Shelton, 1988).

Bioassays. Choice bioassays for feeding deterrent activity were conducted with early 4th instar larvae, using 1.5 cm diameter cabbage leaf discs as test substrate. Each side of test discs was treated with 20 μl of solution to provide a total of 0.1 gram leaf equivalents (gle) per disc, whereas pure solvent was applied to control discs. The tests were conducted in waxed paper ice cream cups (250 ml), each containing a paraffin wax layer at the bottom, which was covered with moist filter paper. Two treated and two control discs were arranged alternately in each cup and supported on insect pins. Four larvae were placed in the center of each cup, which was covered with a perforated plastic lid. After a period of 6 hr in the dark, the remaining area of each disc was measured with a LiCor-3100 area meter. The area consumed was obtained by subtracting the remaining area of each eaten disc from the average measurement of all discs that showed no signs of feeding. Feeding inhibition was expressed as a feeding deterrent index (FDI): $\mathrm{FDI} = 100 \times (\mathrm{C} - \mathrm{T})/(\mathrm{C} + \mathrm{T})$, where C is the area of control discs consumed and T is the area of treated discs consumed (Dimock et al., 1991). An FDI value of zero means that there is no preference, a positive value means that there is a preference for the control, and a negative value means a preference for the treatment. Subsequent bioassays with 2nd, 3rd, and 5th instars were conducted in the same manner. The time period for these assays was adjusted to allow for different consumption rates of the different instars. In each case, assays were terminated when approximately 50% of the control discs had been consumed.

For neonate assays, one treated and one control disc were pinned at the same height near the center of each cup so that they overlapped and larvae could readily move from one disc to the other. Five larvae were introduced to the overlapping area and the assays were continued for 24 hr, when measurable feeding had usually occurred. To determine the effects of diet on larval responses to the active compounds, eggs were allowed to hatch on a wheat germ diet (Bell et al., 1979) in styrofoam cups (250 ml), which were kept in an incubator at 27°C until the larvae at the different stages were used for assays.

Chemical Isolation. Semi-preparative HPLC was performed by using a C_{18} Bondex 10 (Phenomenex) column, 300 × 7.8 mm, at a flow rate of 2 ml/min. Subsequent analytical separation and isolation of pure compounds was accomplished by using a Luna C_{18} column, 250 × 4.6 mm (Phenomenex) at a flow rate of 1 ml/min. For both HPLC systems, a gradient of water and acetonitrile was used: CH_3CN 0% at time 0, 15% at 5 min, 30% at 25 min, and 10% at 30 min. The HPLC effluent was monitored by a diode array detector at 254 nm and at 218 nm.

RESULTS AND DISCUSSION

Previous studies have shown that water-soluble fractions from ethanolic extracts of *A. petiolata* foliage contain both oviposition stimulants and deterrents for *P. n. oleracea* butterflies, and the deterrent compounds could be extracted into butanol. Despite the presence of these unidentified deterrents, acceptance for oviposition could be attributed to the overwhelming effect of allylglucosinolate that is present as a stimulant (Huang et al., 1995). In this study, we tested for the presence of feeding inhibitors in similar extracts of rosette leaves of *A. petiolata*, before and after partitioning with 1-butanol. In choice bioassays with 4th instar larvae, we found that butanol extracts have strong antifeedant activity. Cabbage leaf discs treated with 0.1 gram leaf equivalents (gle) of the butanol extract were consumed by larvae significantly less than control discs that were treated with solvent alone (FDI $= 35.74 \pm 4.94$, $N = 10$). Reverse phase HPLC analysis of the butanol fraction using a water-acetonitrile gradient gave a chromatogram which showed 4 significant peaks (Figure 1). The UV-spectra corresponding to all but the first of these peaks were typical of flavonoids. Four major fractions (A–D), each of which included material associated with one major peak, were collected for bioassays. Feeding by 4th instar larvae of *P. n. oleracea* was deterred only by the fraction B (Figure 2a).

Additional bioassays with newly hatched larvae indicated that the total butanol extract of *A. petiolata* was even more inhibitory to neonates than to the 4th instars (mean FDI 54.6 vs 35.7, $N = 10$) . However, the fraction B, which was most inhibitory to 4th instars, was relatively inactive against neonates. Instead,

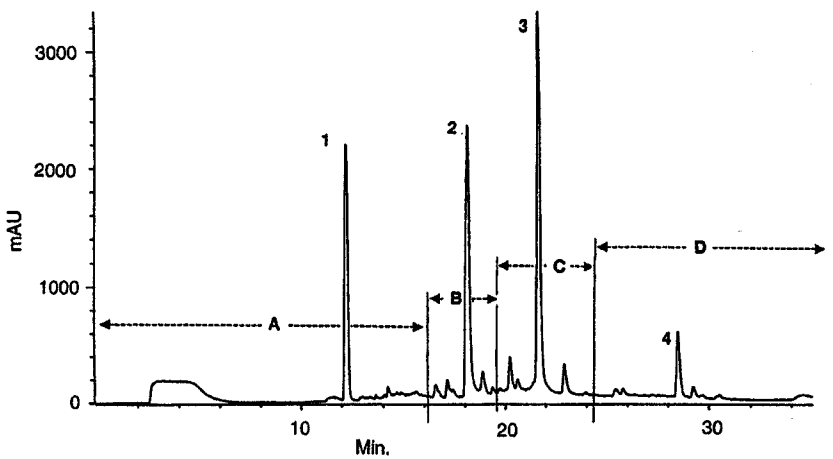


FIG. 1. High pressure liquid chromatogram of the 1-butanol fraction obtained from aqueous extract of *Alliaria petiolata*, with UV monitoring at 218 nm.

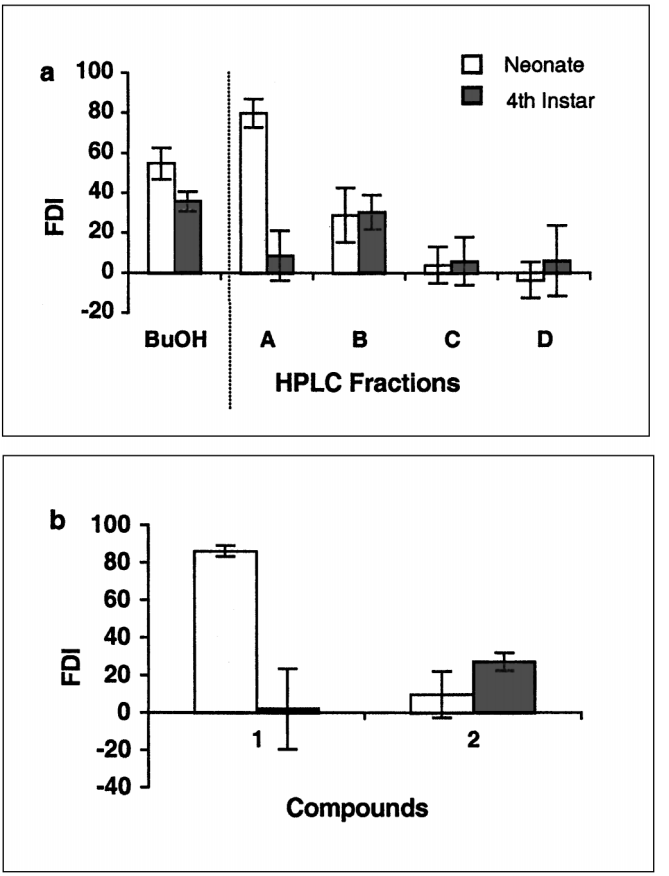


FIG. 2. Feeding inhibition (Feeding Deterrent Index, mean ± SE, $N = 10$) of neonate and 4th instar *P. napi oleracea* by **a**: total butanol extract (BuOH) and individual HPLC fractions, and **b**: compounds **1** and **2** isolated from these fractions by HPLC, at concentrations of 0.1 gram leaf equivalents/disc.

the fraction A that showed no activity against 4th instars was most inhibitory to neonates (Figure 2a). The major compound in each active fraction (**1** from A and **2** from B) was isolated by HPLC and tested for activity against both 1st and 4th instars. Compound **1** inhibited feeding by neonates, whereas compound **2** was active against the 4th instars (Figure 2b).

To determine the stage of development at which larval sensitivity changes from one compound to the other, we measured the responses of larvae to each compound at each stage in their development. Batches of larvae were reared from egg to 5th instar on cabbage or on wheat germ diet, and groups of larvae were

removed for assays at each stage. Previous work with *Pieris rapae* showed that larval responses to feeding deterrents can depend on dietary experience, and specific constituents of wheat germ are responsible for suppressing development of sensitivity to deterrents Huang and Renwick, 1995, 1997). Our experiments with *P. n. oleracea* now show that the larval response of this species to compound **1** is unaffected by diet, but sensitivity to the feeding inhibitor decreases steadily to zero by the time larvae molt to the 4th instar (Figure 3a). In contrast, the larval response to compound **2** is clearly dependent on diet. Larvae reared on wheat germ diet

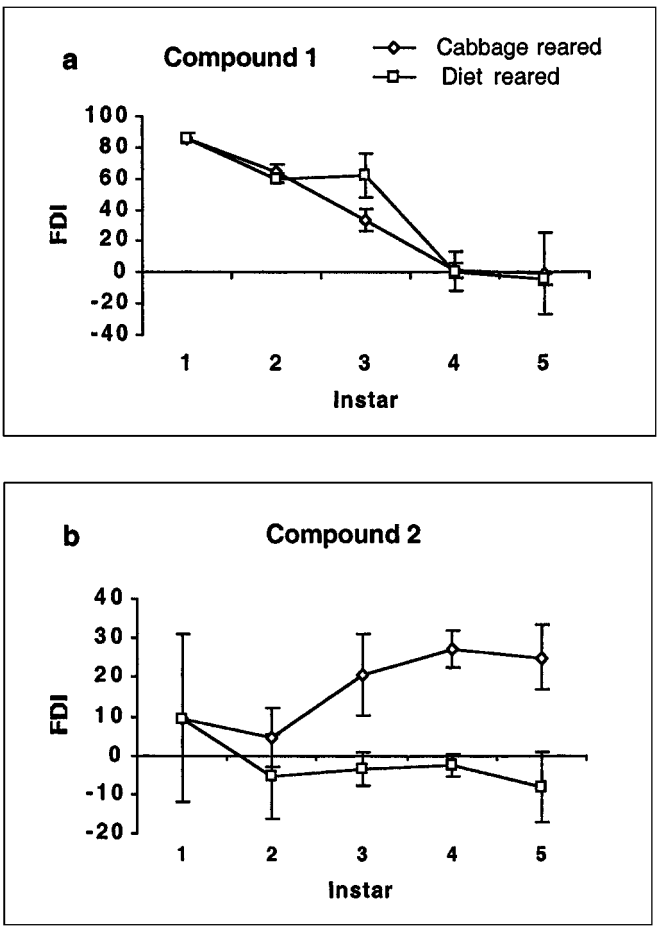


FIG. 3. Feeding inhibition by compounds **1** and **2** on different instars of cabbage-reared and wheat germ-reared larvae of *P. napi oleracea* (Feeding Deterrent Index, mean ± SE, $N = 10$).

remain insensitive to compound **2** throughout their development, whereas larvae feeding on cabbage develop sensitivity to reach a maximum during the last two instars (Figure 3b).

Compound **2** has been identified previously as a flavone glycoside, isovitexin 6"-*O*-β-D-glucopyranoside (Figure 4) (Haribal and Renwick, 1998). Compound **1** was collected by preparative HPLC of butanol extracts to provide mg quantities of material for spectral analysis, resulting in its identification as 4-β-D-pyranoglucosyloxyl-2(Z)-propene-1-nitrile (Haribal et al, 2001) (Figure 4).

Behavioral observations during bioassays provided clues about the mechanisms involved in the rejection of *A. petiolata* as a food plant. When neonate larvae were presented with a cabbage disc that was treated with a butanol extract of *A. petiolata* foliage or with isolated compound **1**, limited nibbling at the surface was observed, without any visible damage. However, some ingestion was evident from the green color visible in the larval gut, which is translucent at this stage. Similarly, when neonates were placed on the surface of a rosette leaf of *A. petiolata*, about one-quarter to one-third of the gut became green within a period of 4 hr, after which the larvae became motionless and made no further attempt to feed. In contrast, those 4th instars that rejected *A. petiolata* rosettes or cabbage discs treated with compound **2** made no attempt to feed or even to bite the plant tissue. (Note: the FDIs reflect combined data from larvae that fed to some extent and those that rejected treated discs outright). This observation would indicate that no ingestion

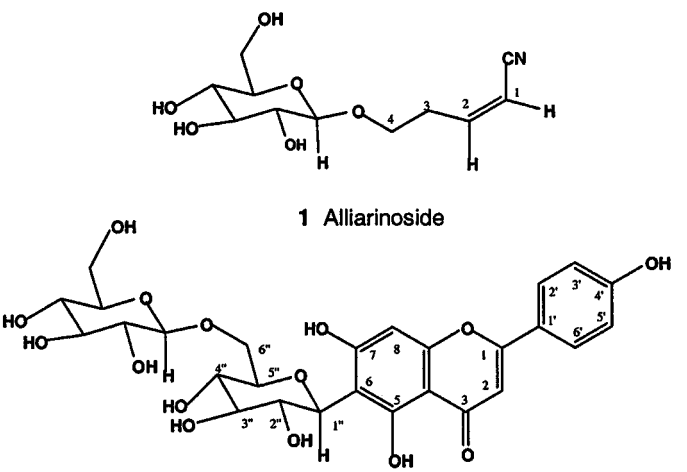


FIG. 4. Structures of compounds **1** and **2** identified as feeding inhibitors from *Alliaria petiolata.*

occurred before rejection by these individuals, whereas extensive palpation with the mouthparts would suggest that taste was involved in their rejection behavior (unpublished data).

Two distinct mechanisms appear to be involved in the resistance of garlic mustard to these pierid caterpillars. Compound **1** inhibits feeding by early instars through an apparent post-ingestive feedback mechanism (Frazier, 1991), whereas compound **2** acts as a direct feeding deterrent that is perceived by taste receptors on the mouthparts of late instars. To our knowledge, this is the first report of such selective barriers in a plant against different larval stages of an insect herbivore. The phenomenon of differential larval responses appears to be a result of simultaneous development of post-ingestive tolerance and taste sensitivity as the larvae feed and develop on their host plants.

Exploitation of garlic mustard as a potential host plant by *P. n. oleracea* is apparently thwarted by the presence of these two unrelated chemicals. Compound **1** effectively blocks feeding by first instars, so that any development of larvae from hatching eggs is unlikely. In addition, the potential migration of late instars from neighboring host plants is likely to fail as a result of the deterrent activity of compound **2**. Although it is clear that the response of such larvae to the deterrent will depend on their previous diet, experience on a preferred host such as cabbage is likely to result in relatively high sensitivity.

Subsequent studies have resulted in the identification of additional flavonoids in foliage of *A. petiolata*, but these do not appear to play a significant role in protecting the plant from *P. napi oleracea*. However, seasonal and population variation in content of the active constituents could potentially affect the susceptibility of garlic mustard to herbivory. (Haribal and Renwick, 2001).

Acknowledgments—We thank Drs. Paul Feeny, Daniel Papaj, and Erich Städler for reviews of early versions of this manuscript. We are also indebted to members of the Feeny group for constructive discussion and suggestions. Supported in part by NSF grant No. DEB-9419797 to JAAR.

REFERENCES

BELL, R. A., OWENS, C. D., SHAPIRO, M., and TARDIF, J. R. 1979. Development of mass rearing technology, pp. 599–633, *in* C. C. Doane and M. L. McManus (eds.). The Gypsy Moth: Research Toward Integrated Pest Management. USDA-FS Technical Bulletin 1584.

BOWDEN, S. R. 1971. American white butterflies (Pieridae) and English food plants. *J. Lepidopterists' Soc.* 25:6–12.

CHEW, F. S. 1981. Coexistence and local extinction in two *Pieris butterflies. Am. Nat.* 118:655–672.

CHEW, F. S. 1988. Searching for defensive chemistry in the Cruciferae, or, do glucosinolates always control interactions of Cruciferae and their potential herbivores and symbionts? No! pp. 81–112, *in* K. C. Spencer (ed.). Chemical Mediation of Coevolution, Academic Press, San Diego.

CHEW, F. S., and RENWICK, J. A. A. 1995. Host-plant choice *in Pieris* butterflies, pp. 214–238, *in* R. T. Cardé and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman and Hall, New York.

DIMOCK, M. B., RENWICK, J. A. A., RADKE, C. D., and SACHDEV-GUPTA, K. 1991. Chemical constituents of an unacceptable crucifer, *Erysimum cheiranthoides*, deter feeding by *Pieris rapae*. *J. Chem. Ecol.* 17:525–533.

EHRLICH, P. R., and RAVEN, P. H. 1964. Butterflies and plants: A Study in Coevolution. *Evolution* 18:586–608.

FEENY, P. 1977. Defensive ecology of the Cruciferae. *Ann. Missouri Bot. Gard.* 64:221–234.

FRAZIER, J. L. 1991. How animals perceive secondary plant compounds, pp. 89–134, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, San Diego.

HARIBAL, M., and RENWICK, J. A. A. 1998. Isovitexin 6″-*O*-β-D-Glucopyranoside: A Feeding Deterrent to *Pieris napi oleracaea* from *Alliaria petiolata*. *Phytochemistry* 47:1237–1240.

HARIBAL, M., and RENWICK, J. A. A. 2001. Seasonal and population variation in flavonoid and alliarinoside content of *Alliaria petiolata*. *J. Chem. Ecol.* 27:1603–1612.

HARIBAL, M., YANG, Z., ATTYGALLE, A. B., RENWICK, J. A. A., and MEINWALD, J. 2001. A cyanoallyl glucoside from *Alliaria petiolata* as a feeding deterrent for larvae of *Pieris napi oleracea*. *J. Nat. Products* 64:440–443.

HUANG, X. P., and RENWICK, J. A. A. 1995. Chemical and experiential basis for rejection of *Tropaeolum majus* by *Pieris rapae* larvae. *J. Chem. Ecol.* 21:1601–1617.

HUANG, X. P., and RENWICK, J. A. A. 1997. Feeding deterrents and sensitivity suppressors for *Pieris rapae* larvae in wheat germ diet. *J. Chem. Ecol.* 23:51–70.

HUANG, X. P., and RENWICK, J. A. A., and SACHDEV-GUPTA, K. 1993. Oviposition stimulants and deterrents regulating differential acceptance of *Iberis amara* by *Pieris rapae* and *P. napi oleracaea*. *J. Chem. Ecol.* 19:1645–1663.

HUANG, X. P., RENWICK, J. A. A., and CHEW, F. S. 1995. Oviposition stimulants and deterrents control acceptance of *Alliaria petiolata* by *Pieris rapae* and *P. napi oleracea*. *Chemoecology* 5/6:79–87.

LINDROTH, R. L. 1988. Adaptations of mammalian herbivores to plant chemical defenses, pp. 415–445, *in* K. C. Spencer (ed.). Chemical Mediation of Coevolution, Academic Press, San Diego.

NUZZO, V. 1993. Distribution and spread of the invasive biennial *Alliaria petiolata* (garlic mustard) in North America, pp. 137–145, *in* W. N. McKnight (ed.). Biological Pollution: The Control and Impact of Invasive Exotic Species. Indiana Acad. Sci., Indianapolis.

SACHDEV-GUPTA, K., RENWICK, J. A. A., and RADKE, C. D. 1990. Isolation and identification of oviposition deterrents to cabbage butterfly, *Pieris rapae*, from *Erysimum cheiranthoides*. *J. Chem. Ecol.* 16:1059–1067.

SACHDEV-GUPTA, K., RADKE, C. D., RENWICK, J. A. A., and DIMOCK, M. B. 1993a. Cardenolides from *Erysimum cheiranthoides*: Feeding Deterrents to *Pieris rapae* larvae. *J. Chem. Ecol.* 19:1355–1369.

SACHDEV-GUPTA, K., RADKE, C. D., and RENWICK, J. A. A. 1993b. Antifeedant activity of cucurbitacins from *Iberis amara* against larvae of *Pieris rapae*. *Phytochemistry* 33:1385–1388.

WEBB, S. E., and SHELTON, A. M. 1988. Laboratory rearing of the imported cabbageworm. *Bull. N.Y. Food and Life Sci.* 122:1–6.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

SEASONAL AND POPULATION VARIATION IN FLAVONOID AND ALLIARINOSIDE CONTENT OF *Alliaria petiolata*

MEENA HARIBAL* and J. ALAN. A. RENWICK

Boyce Thompson Institute, Tower Road, Ithaca, NY 14853, USA

(Received December 19, 2000; accepted April 7, 2001)

Abstract—*Pieris napi oleracea*, an indigenous butterfly in North America, lays eggs on *Alliaria petiolata*, an invasive weed that was introduced from Europe. However, larval development on plants from different sources varies considerably. *A. petiolata* is a compulsive biennial, and its foliage is rich in apigenin flavonoids. We compared the chemistry of different vegetative forms from different populations in the vicinity of Ithaca, NY throughout the year. Significant differences occurred in the number of apigenin derivatives in different populations and vegetative forms, and seasonal variations in the amounts of these compounds were found. We have previously isolated two major compounds, alliarinoside [(2*Z*)-4-(β-D-glucopyranosyloxy)-2-butenenitrile] (**1**) and isovitexin-6-*O*″-β-D-glucoside (**3**), which negatively affect development of *P. napi oleracea* larvae. Comparative analyses of these compounds in two populations throughout the year showed that their concentrations reached maxima twice annually. Foliage is almost devoid of flavonoids in June-July. Thus, variation in the chemistry of the plant may account for observed variation in development rates and survival of the larvae. Several apigenin compounds were isolated and identified by spectral studies.

Key Words—*Alliaria petiolata*, *Pieris*, flavonoids, variation, seasonal, cyanoglucoside, Cruciferae.

INTRODUCTION

Alliaria petiolata, a native crucifer of Europe is quickly spreading in the eastern united states, replacing the native flora in various habitats. It was first reported on Long Island about 150 years ago, and is now distributed throughout many states from Maine to North Carolina in the south, and west to Wisconsin and Illinois (Nuzzo, 1993). Previously, a few C-glycosides were reported from *A. officinalis*

*To whom correspondence should be addressed. e-mail: mmh3@cornell.edu

(=*petiolata*) by Paris and Delaveau (1962). We have reported isovitexin 6″-*O*-β-D-glucopyranoside (3) from the foliage of *A. petiolata* as a compound that deters feeding by 4th instar larvae of *Pieris napi oleracea* (Harris), an indigenous butterfly of the north eastern USA (Haribal and Renwick, 1998). More recently, from aqueous extracts of *A. petiolata*, we identified (2*Z*)-4-(β-D-glucopyranosyloxy)-2-butenenitrile (alliarinoside) (**1**) as a feeding inhibitor/deterrent for first instar larvae (Haribal et al., 2001). However, field observations suggest that some populations of *P. napi oleracea* can survive on selected populations of *A. petiolata* (Chew, 1980; Courant et al., 1994; Chew et al., 1996). We have found considerable variation in alliarinoside and flavonoid content in different sources of the plant, and, in the present paper, we investigated variation in flavonoid content throughout the year, in different populations, and in different leaf forms to help explain observed variation in *P. napi oleracea* survival.

METHODS AND MATERIALS

Plant Material. To examine geographic differences, samples of *Alliaria petiolata* were collected from Tompkins County, in the neighborhood of Ithaca, NY, including Eastern Heights, Dryden Lake, and Salmon Creek areas. Additional samples were obtained from seeds collected in Massachusetts by F. S. Chew. Seedlings of these were grown in a greenhouse and in garden plots in June 1995. Both cauline and rosette leaves of the same plants were collected whenever available. Thirteen samples of rosette and 12 of cauline leaves randomly collected from each site (1-3 samples per site) were included for chemical analysis.

To monitor seasonal variation, the plants were studied throughout the year from Dryden Lake and Salmon Creek areas. Three samples of each leaf type were randomly collected every month (except in February and March, when only two samples were collected) from each location according to availability. Cauline leaves were present only from late May to July and rosette leaves were scarce in June and July.

Sample Preparation and HPLC Analyses. 0.25 g of fresh young leaf material were boiled in 5 ml of EtOH for 5 min and the resulting mixture was blended using a Tissue Tearor before dilution with water to 10 ml. After centrifugation, 6 ml of supernatant solution were evaporated to dryness and redissolved in 6 ml of water to give a concentration of 0.05 gram leaf equivalent (gle) per 2 ml. Suspended particles were removed by filtering through 4 μ nylon filters. Samples of 0.05 gle were directly injected onto the HPLC, fitted with a C_{18} RP column (C_{18} Bondex 5 μ, 300 $\times$ 7.0 mm, Phenomenex) using a water-acetonitrile gradient at a flow rate of 2 ml/min (0-5 min 0% acetonitrile; 25% acetonitrile at 40 min; 100% acetonitrile at 50 min), monitored by a Photodiode Array Detector at 218 and 254 nm.

Spectroscopic Methods. ^{1}H NMR spectra were recorded at 399.99 MHz, and ^{13}C NMR at 25.59 MHz in DMSO-d_6 or in acetone-d_6 unless otherwise stated, on a Varian instrument. ESI mass spectra were recorded on a Micromass (Manchester, UK) Quattro 1 instrument and the samples were introduced by a direct infusion method.

Quantification and Statistical Methods. The total absorption unit (AU) value was calculated as area under each peak (at 218 nm) to compare concentrations. Quantities of individual components of rosette leaves and cauline leaves from the same plant and from different plants in the same season were compared using the Student t-test. Mean values from the seasonal samples collected at Dryden Lake and Salmon Creek were also compared by the Student t-test. Only data for rosette leaves were used for this analysis.

RESULTS

The water-soluble fraction contained primarily highly polar compounds, including several flavonoid glycosides, which were indicated by their UV spectra obtained with the diode array detector. Eight chromatographic peaks, one corresponding to alliarinoside (**1**), and seven flavonoids, designated compounds **2–8** (Figure 1) with increasing retention time were analyzed. Considerable variation

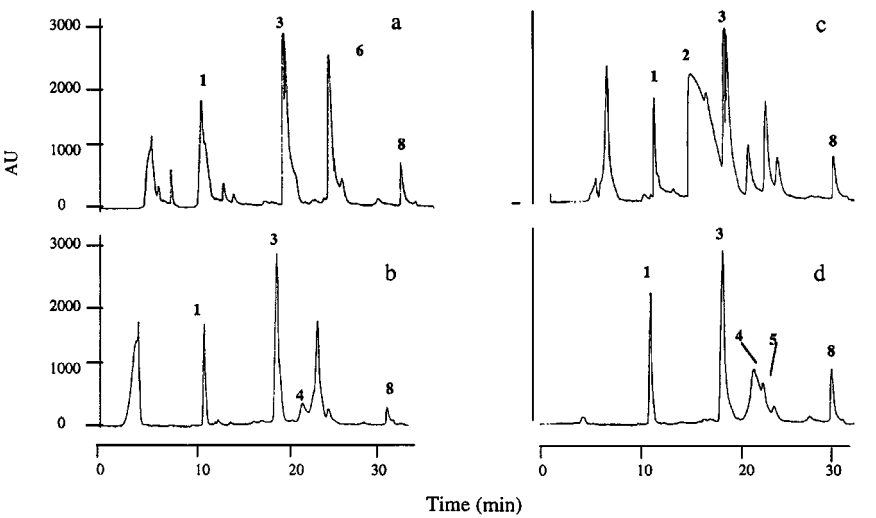


FIG. 1. HPLC chromatograms of extracts (0.05gle) of rosette leaves of *A. petiolata* from a) Eastern Heights, b) East Ithaca recreation way, c) Salmon Creek, d) from plants collected as seed in Massachusetts and grown in a greenhouse (UV absorptions monitored at 218 nm).

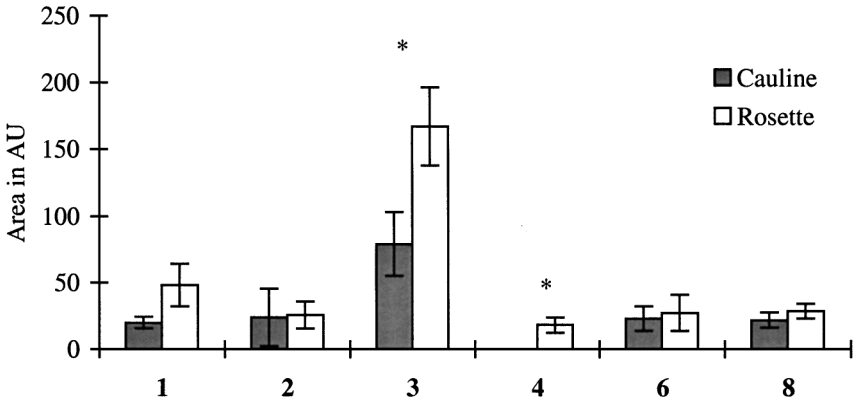


FIG. 2. Mean concentrations ± SE ($N = 13$ for rosette and $N = 12$ for cauline leaves) expressed in area ×1000 AU of alliarinoside (**1**) and flavonoids (**2–8**) in cauline and rosette leaves of *A. petiolata*. Integration values obtained based on absorption at 218 nm.

in flavonoid and in alliarinoside content (Figure 1 is a representative example of 4 samples) in the plants from different populations and in the different vegetative types of foliage (Figure 2) were found. Most flavonoids were flavones, but, in a few cases, kaempferol derivatives were detected. In some young leaves, anthocyanins were also present. Seven major flavonoids were detected. Most samples contained alliarinoside (**1**), flavonoids **2**, **3**, and **8**, at retention times of 10.8, 17.3, 19.5, and 32.5 min, respectively, at varying concentrations. The identification of compounds **1** and **3** has already been reported (Haribal and Renwick, 1998; Haribal et al., 2001). On the basis of its spectral data, compound **2** was identified as isoorientin-6″-*O*-*β*-D-glucopyranoside (Chulia and Mariottee, 1985). The ^{1}H NMR spectrum of **2** showed satellite peaks (at δ 5.00 and 4.66) for anomeric protons of hexose sugars, suggesting that a structural isomer of compound **2** was present at 5% of compound **2**. Other flavonoids **4**, **5**, **6** and **7** eluted at 21.6, 22.7, 23.7, and 29.0 min. Compound **6** has been identified as 6‴-*O*-sinapoyl-isovitexin-6″-*O*-*β*-D-glucopyranoside (Haribal et al., 1999). Compounds **4** and **5** were found to be isomers of **3** as shown by ESIMS ($[M + H]^+$ 595), but their fragmentation patterns were quite different. Since only small samples were available, no further structural analysis could be carried out. Peak **8** was found to represent a mixture of di-sinapoyl derivatives of an apigenin C-glycoside (MW 1008). Their complex ^{1}H NMR spectrum indicated that these may be structural isomers that have yet to be completely identified.

The rosette leaves contained higher concentrations of all compounds than did cauline leaves. The concentration of **1** in rosette leaves was not significantly different from that in the cauline leaves ($P = 0.064$), but levels of **3** (isovitexin 6″-*O*-*β*-D-glucopyranoside) were significantly higher ($P < 0.05$) (Figure 2). On

the basis of UV calibration curves, we estimated that the highest concentration of **3** in rosette leaves was 12–15 mg ($2.0–2.5\times10^{-5}$ mol) per gm fresh wt. Compound **4** was present in many populations of rosette leaves, but was absent from cauline leaves (Figure 2). The concentration of **1** could not be accurately assessed, since its highly hygroscopic nature made accurate weighing difficult.

The plants from the Dryden Lake area had fewer flavonoids than did those from Salmon Creek or from any other areas we investigated. Foliage from the Dryden Lake population contained **2**, **3** and **8**, as well as **7**, which was absent in the specimens from Salmon Creek (Figure 3). The plots of mean concentrations of flavonoids showed a bimodal temporal pattern in a given season for most of the compounds in both populations, but the patterns were not synchronized for the two populations. Flavonoid concentrations reached maxima around December–February and in May. Around June–July, when plants were senescing, most of the flavonoids were at low levels or undetectable. Only the flavonoid **8** was present throughout the year, and little temporal variation in its abundance occurred in the population from the Dryden Lake area (Figure 4). However, in the population from Salmon Creek, **8** reached higher concentrations when no other flavonoids were present. There were no differences in content of alliarinoside ($P = 0.41$, Student *t*-test) and isovitexin 6″-*O*-β-D-glucopyranoside ($P = 0.45$, Student *t*-test) (Figure 5) between the two the populations throughout the year, although these compounds reached maximal concentrations at different times. Also, **2** (Figure 6) was more abundant in the population from Salmon Creek area than in that from Dryden Lake area ($P < 0.0001$).

*Spectral Data for Flavonoids **2**, **4**, **5**, **7** and **8**.*

*Compound **2***. (*Isoorientin-6″-O-β-D-glucopyranoside*). UV λ_{max} at 272, 336 nm; Positive-ion ESIMS *m/z* at 611 $[M + 1]^+$; ^{1}H NMR (DMSO-d_6): δ 7.45 (1H, dd, $J = 8.2$, 1.6 Hz), 7.41 s, 6.82 (1H, d, $J = 8.2$ Hz), 6.82 s, 6.73 s, 4.98 (1H, d, J = 6.8 Hz), 4.64 (1 H, d, J = 8.2 Hz), 3.30(1H, m), 3.67(1H, m), 3.88(1H, m), 3.14(1H, m) and 2.9-3.6 (complex multiplets for other sugar protons).

*Compound **4***. UV λ_{max} at 265, and 332 nm; Positive-ion ESIMS: *m/z* at 595 $[M + 1]^+$ 449 ($[M - 145]^+$, loss of $C_9H_5O_2$), 396, 344, 329, 284 (base peak, $[M - 145–163]^+$, loss of $C_6H_{11}O_5$ and $C_9H_5O_2$), 256, 201, 142 and 101.

*Compound **5***. UV λ_{max} at 265, and 332 nm; Positive-ion ESIMS: *m/z* at 595 $[M+1]^+$ 449 ($[M-145]^+$, loss of $C_9H_5O_2$), 372, 344, 328, 284 ($[M-145–163]^+$, loss of $C_6H_{11}O_5$ and $C_9H_5O_2$), 209, 119 (base peak).

*Compound **7***. UV λ_{max} 265, 362 nm; Positive-ion ESIMS: *m/z* at 611 $[M + 1]^+$; a diglycoside of kaempferol.

*Compound **8***. (*Di-O-sinapoyl-isovitexin 6″-O-β-D-glucopyranoside derivative*). UV λ_{max} 265, 325 nm; Positive-ion ESIMS: *m/z* at 1009 $[M + 1]^+$.

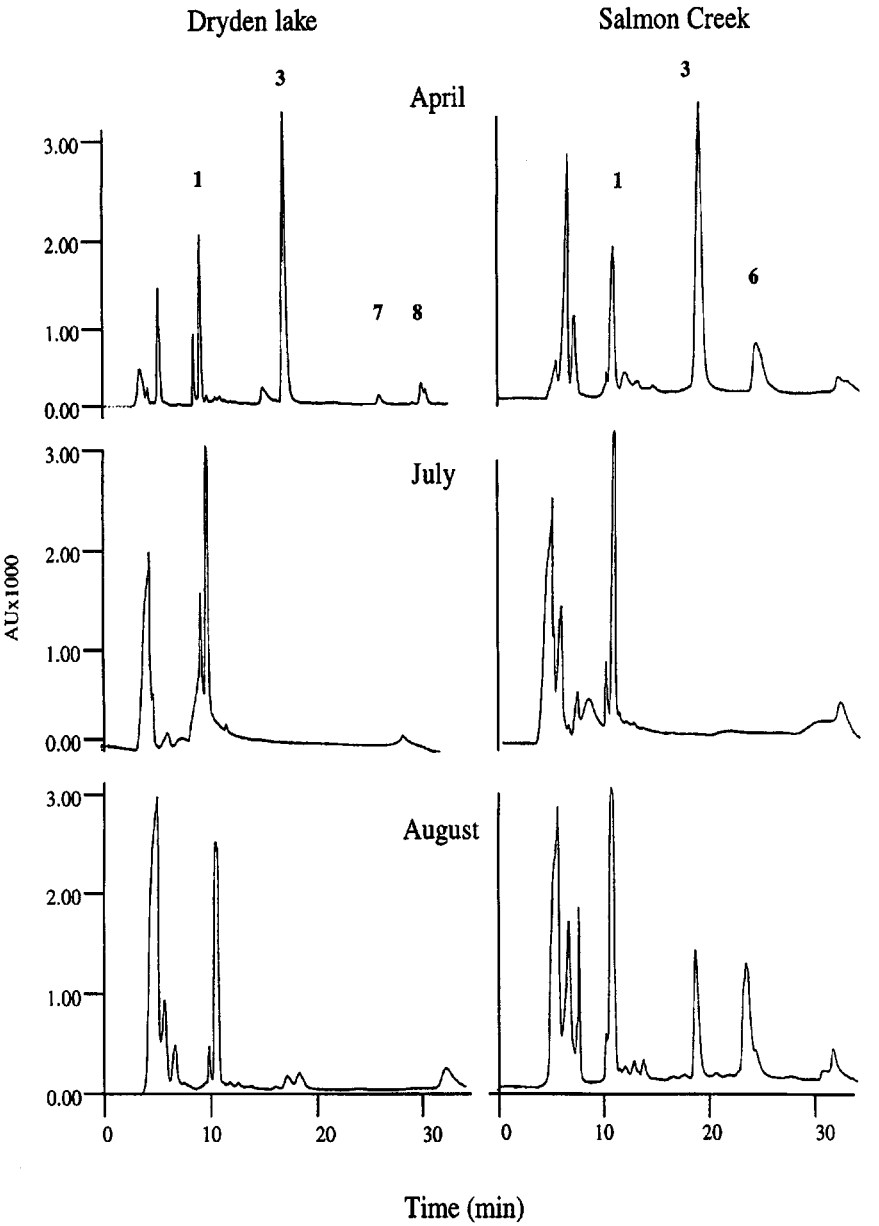


FIG. 3. HPLC chromatograms of extracts (0.05gle) of rosette leaves of *A. petiolata* collected from Dryden Lake and Salmon Creek in different months, monitored at 218 nm.

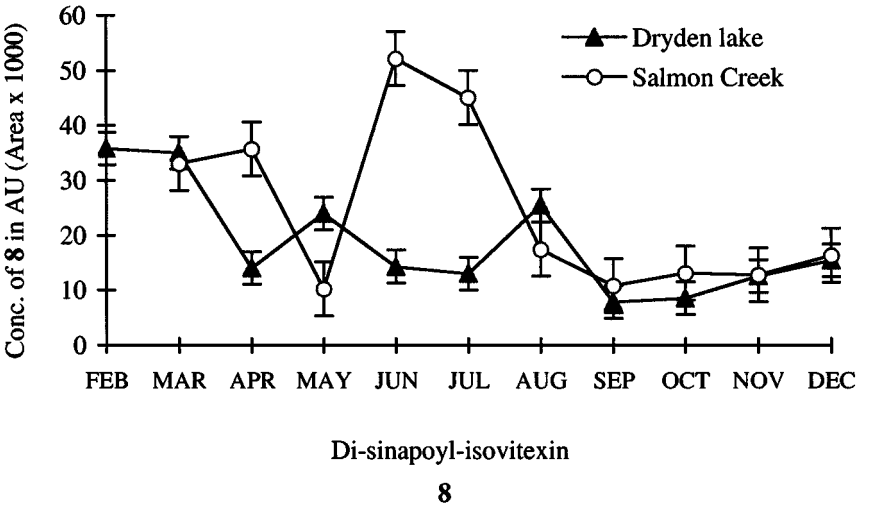


FIG. 4. Seasonal variation in concentrations (expressed in area $\times 1000$ AU) (values represent means $\pm$ SE for $N = 3$, except for Feb and Mar when only 2 replicates were collected) of **8** in Salmon Creek and Dryden Lake samples (0.05gle) of rosette leaves of *A. petiolata*. Integration values obtained for absorptions at 218 nm.

DISCUSSION

A. petiolata is a biennial (winter annual), which germinates in autumn and senesces by the following summer. It grows in different types of habitat, but prefers habitats that are in the shade of woods, along river edges, and along the roadside (Nuzzo, 1993). It is one of the first plants visible after snow-melt and it flowers by May, at which time trees and other plants are just beginning to leaf in our study area. Light, therefore, is not likely to be a direct cause of variation in individual flavonoids, although a higher total flavonoid content in May is probably related to light or day length. Plants from seeds collected in Massachusetts, when grown in greenhouse conditions, contained higher amounts of the isovitexin 6‴-*O*-sinapoyl-6″-*O*-β-D-glucopyranoside (**6**) than when grown in garden plot under natural conditions (unpublished results). Similarly, plants collected as seedlings from Eastern Heights in Ithaca produced more of **6** after cultivation in the greenhouse. Thus, **6** may be produced in response to greenhouse conditions, but a detailed investigation will be necessary to confirm this conclusion.

Several researchers have suggested that the structural diversity in flavonoid content of plants is due to genetic polymorphism and selective expression of different genes encoding for biosynthetic enzymes in response to different environmental stimuli (Chaves et al., 1993 and refs. therein). However, we did not attempt

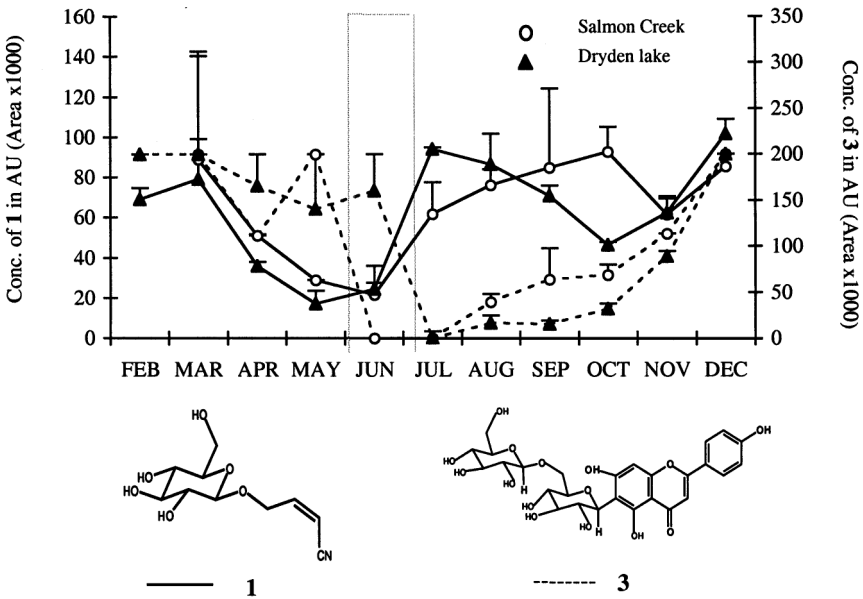
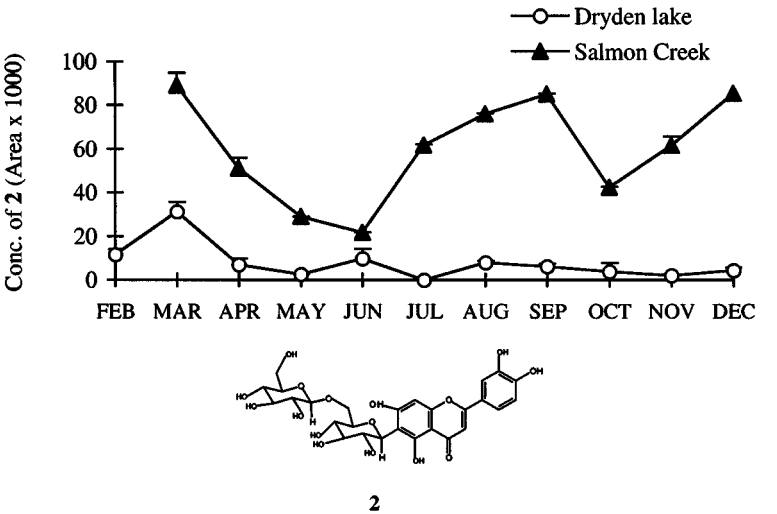


FIG. 5. Seasonal variation in concentrations expressed in area $\times 1000$ AU (values represent means $\pm$ SE for $N = 3$, except for Feb and Mar when only 2 replicates were collected) of **1** (alliarinoside) and **3** (isovitexin-6″-*O*-β-D-glucopyranoside) in extracts (0.05gle) of rosette leaves of *A. petiolata*. Integration values obtained based on absorption at 218 nm.

to confirm the role of genetics in the observed variation. The flavonoid content of plants is also known to vary quantitatively and qualitatively depending on growth stage, degree of senescence, season, and geographical location (Bohm et al., 1984; Chaves et al., 1993). Specific flavonoids are also produced in response to pathogens (Malhotra et al., 1996), wounding stress, herbivory, strong light, especially UV-B (Cuadra et al., 1997), and temperature stress (Chaves et al., 1997). Cooper-Driver et al. (1977) reported that variation in flavonoids in *Pteridium aquilinum* affects palatability to mammals and phytophagous insects such as the locust, *Schistocerca gregaria*.

A. petiolata appears to be relatively free of herbivory, except for limited slug feeding and damage by leaf miners. Flea beetles, major pests of most crucifers, generally avoid this plant (Nielsen et al., 1979). *P. napi oleracea* larvae cannot survive on *A. petiolata*, although the plant may be suitable for oviposition (Huang and Renwick, 1993). Alliarinoside inhibits feeding by neonate larvae, but later instar larvae are deterred by isovitexin 6"-*O*-β-D-glucopyranoside (Renwick et al., 2001). Szentesi (1991) and Chew et al. (1996) have shown that *P. rapae* and some populations of *P. napi* can survive on this plant. It seems likely that this occurs



2

FIG. 6. Seasonal variation in concentrations (expressed in area $\times 1000$ AU) (values represent means $\pm$ SE $N = 3$, except for Feb and Mar when only 2 replicates were collected) of **2** (isoorientine-6″-*O*-β-D-glucopyranoside) in Salmon Creek and Dryden Lake samples (0.05gle) of rosette leaves of *A. petiolata*. Integration values obtained for absorptions at 218 nm.

when levels of both alliarinoside and isovitexin 6″-*O*-β-D-glucopyranoside in the plant are below the threshold necessary for inhibition of feeding (Figure 5). When the flavonoid content is at its lowest level in June-July, the concentration of alliarinoside is at its highest (Figure 5), so that the plant has some protection mechanism at all times. Thus,variation in chemistry may explain the differential survival rates of *P. napi oleracea*, although variation in responses to these compounds by individual insects and populations may also play a role in the differential success of the species.

Acknowledgments—We wish to thank Dr. F. S. Chew for the sample of *Alliaria* seeds and for reviewing an earlier version of this manuscript. We also are grateful to Dr. A. Attygalle for running LC-MS of some of the flavonoids. This work was supported by NSF grant number DEB-9419797.

REFERENCES

BOHM, B. A., BANEK, H. M., and MAZE, J. R. 1984. Flavonoid variation in North American Menziesia (Ericaceae). *Syst. Bot*. 9:324–345.

CHAVES, N., ESCUDERO, J. C., and GUTIERREZ-MERINO, C. 1993. Seasonal variation of exudate of *Cistus ladanifer. J. Chem. Ecol*. 19:2577–2591.

CHAVES, N., ESCUDERO, J. C., and GUTIERREZ-MERINO, C. 1997. Role of ecological variables in the seasonal variation of flavonoid content of *Cistus ladanifer* exudate. *J. Chem. Ecol*. 23:579–603.

CHEW, F. S., GOODWILLIE, C., and MILBURN, N. S. 1996. Larval responses of two *Pieris* butterflies to naturalized Cruciferae. *Am. Zoologist* 29:167A.

CHEW, F. S. 1980. Food plant preferences of *Pieris* Caterpillars (Lepidoptera). *Oecologia* (Berl) 46:347–353.

CHEW, F. S. 1981. Coexistence and local extinction in two pierid butterflies. *Am. Naturalist* 118:655-672.

CHULIA, A. J., and MARIOTTEE, A. M. 1985. New C-glucosylflavones in *Gentiana pedicellata. J. Nat. Prod*. 48:480–483.

COOPER-DRIVER, G., FINCH, S., SWAIN, T., and BERNAYS, E. 1977. Seasonal variation in secondary plant compounds in relation to the palatability *of Pteridium aquilinum. Biochem. Syst. Ecol*. 5:177–183.

COURANT, A. V., HOLBROOK, A. E., VAN DER REIJDEN, E. D., and CHEW, F. S. 1994. Native pierine butterfly (Pieridae) adapting to naturalized crucifer? *J. Lep. Soc*. 48:168–170.

CUADRA, P., HARBORNE, J. B., and WATERMAN, P. G. 1997. Increases in surface flavonols and photosynthetic pigments in *Gnaphalium luteo-album* in response to UV-B radiation. *Phytochemistry* 45:1377–1383.

HARIBAL, M., and RENWICK, J. A. A. 1998. Isovitexin 6″-*O*-β-D-glucopyranoside: A feeding deterrent to *Pieris napi oleracea* from *Alliaria petiolata. Phytochemistry* 47:1237–1240.

HARIBAL, M., RENWICK, J. A. A., and ATTYGALLE, A. 1999. A new sinapoyl derivative of isovitexin 6″-*O*-β-D-glucopyranoside from *Alliaria petiolata. J. Nat. Prod*. 62:179–180.

HARIBAL, M., YANG, Z., ATTYGALLE, A. B., RENWICK, J. A. A., and MEINWALD, J. 2001. A Cyanoallyl Glucoside from *Alliaria petiolata*, as a Feeding Deterrent for larvae of *Pieris napi oleracea. J. Nat. Prod*. (in press).

HUANG, X. P., and RENWICK, J. A. A. 1993. Differential selection of host plants by two *Pieris* species: The role of oviposition stimulants and deterrents. *Entomol. Exp. Appl*. 68:59–69.

MALHOTRA, B., ONYILAGHA, J. C., BOHM, B. A., TOWERS, G. H. N., JAMES, D., HARBORNE, J. B., and FRENCH, C. J. 1996. Inhibition of tomato ringspot virus by flavonoids. *Phytochemistry* 43:1271–1276.

NIELSEN, J. K., DALGAARD, L., LARSEN, L. M., and SORENSEN, H. 1979. Host plant selection of the horseradish flea beetle *Phyllotreta armoraciae* (Coleoptera: Chrysomelidae): Feeding responses to glucosinolates from several crucifers. *Entomol. Exp. Appl*. 25:227–239.

NUZZO, V. 1993. Distribution and spread of the invasive biennial *Alliaria petiolata* (garlic mustard) in North America, pp.137–145, *in* W. N. MCKNIGHT (ed.). Biological Pollution: the Control and Impact of Invasive Exotic Species. Indiana Acad. Sci., Indianapolis.

PARIS, R. R., and DELAVEAU, P. 1962. Isolement d'un nouvel hétéroside flavonique, La alliaroside des feuilles de *l'Alliaria officinalis* Andrz., (Cruciféres). *C. R. Acad Sci*. 254:928–929.

RENWICK, J. A. A., ZHANG, W., HARIBAL, M., ATTYGALLE, A. B., and LOPEZ, K. D. 2001. Dual chemical barriers protect a plant against different larval stages of an insect. *J. Chem. Ecol*. 27:1593–1601.

SZENTESI Á. 1991. Controversial component of plant apparency in *Alliaria petiolata* Cavara & Grande (Cruciferae), pp. 237–244, *in* Á. Szentesi, and T. Jeremy (eds.). Symposia *Biologica Hungarica*: *Insects-Plants' 89* Academia Kiado, Budapest.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

DIFFERENCES IN HOST USE EFFICIENCY OF LARVAE OF A GENERALIST MOTH, *Operophtera brumata* ON THREE CHEMICALLY DIVERGENT *Salix* SPECIES

TEIJA RUUHOLA,* OLLI-PEKKA TIKKANEN, and JORMA TAHVANAINEN

Department of Biology
University of Joensuu
P.O. Box 111, FIN-80101 Joensuu, Finland

(Received November 28, 2000; accepted April 4, 2001)

Abstract—The food selection, growth, and fecundity of insect herbivores are largely dictated by the chemical composition and nutritive values of plant foliage. We studied the host-use efficiency of larvae of the generalist moth, *Operophtera brumata* (Lepidoptera: Geometridae) on three chemically divergent but nutritively similar willows (*Salix* spp.). The 4th instars were able to use the salicylate-free leaves of *S. phylicifolia* efficiently. Growth was slightly reduced on *S. pentandra*, which contained a moderate level of acetylated salicylates. The high concentration of salicylates found in the leaves of *S. myrsinifolia* seemed to provide efficient protection against non-specialized *O. brumata*. We also studied assimilation of nutrients and degradation of salicylates and other secondary compounds in the digestive tract of *O. brumata* larvae. Neither the assimilation of nitrogen nor of carbon were affected by secondary chemicals of ingested food. Salicylates were shown to be degraded to salicin and catechol, while further degradation of salicin to saligenin was rather slow. In an artificial diet experiment, we showed that two degradation products of salicylates, catechol and saligenin markedly reduced the growth of the larvae. Neither salicin nor chlorogenic acid affected larval growth. We conclude that salicylates reduced the growth of the generalist winter moth mainly by feeding deterrence caused by 6-hydroxy-2-cyclohexenone and catechol. Compared to the deleterious effects of salicylates the effects of other secondary compounds were minor.

Key Words—*Salix* spp., willow, phenolic glycosides, salicylates, *Operophtera brumata*, polyphagy, insect herbivory.

*To whom correspondence should be addressed.

INTRODUCTION

Generalist insect herbivores are able to feed on a large number of different plant species. However, they are affected by variation in leaf quality: some species are clearly better hosts than others and many species are totally avoided. The differential suitability of plant species for generalist insects is based on structural and chemical defenses and on nutrient content of plant tissues. Because the majority of individuals in aboreal insect herbivore communities are generalists, effective defense against them may benefit plants by protecting them from severe defoliation, thus saving plant resources for growth and reproduction.

Operophtera brumata L. (Lep., Geometridae) is a typical polyphagous, spring-feeding leaf-defoliator of deciduous shrubs and trees, which may use numerous plant species belonging to several different genera as hosts. In eastern Fennoscandia, *Querqus robur* L. and *Prunus padus* L. are the primary hosts of the winter moth (Tikkanen, 2000). The chemistry of the leaves of these two hosts is, however, very dissimilar. The leaves of *P. padus* contain cyanogenic glycosides (Seigler, 1998) and a low level of tannins (Julkunen-Tiitto, personal communication), whereas the leaves of *Q. robur* are rich in tannic compounds (Feeny, 1968, 1970). During occasional outbreaks *O. brumata* can feed on many suboptimal host species including several willow species (*Salix* spp.) (Kirsten and Topp, 1991; Tikkanen et al., 1998, 1999, 2000). Since willows are common and abundant shrubs and trees in early successional deciduous forests in northern Europe (Hämet-Ahti et al., 1998; Skvortsov, 1999), where in many places the main hosts of *O. brumata* can be quite scarce, willows are potential alternative hosts of *O. brumata* (Tikkanen et al., 1998, 1999, 2000).

It is known that the palatability of different willow species to leaf-defoliators is largely determined by the chemical composition of the leaves. Salicylates deter feeding of many generalist insect herbivores (Tahvanainen et al., 1985; Lindroth et al., 1988; Lindroth and Peterson, 1988), but, can stimulate oviposition (Denno et al.,1990; Kolehmainen et al., 1994) and feeding of specialists (Matsuda and Matsuo, 1985; Smiley et al., 1985; Tahvanainen et al., 1985; Rank, 1992; Kolehmainen et al., 1995).

We studied the host use efficiency of a generalist moth, *O. brumata*, on three chemically different willow species; *Salix pentandra* (L.), *Salix phylicifolia* (L.) and *Salix myrsinifolia* (Salisb.). These species are common throughout Scandinavia (Hämet-Ahti et al., 1998; Skvortsov, 1999) and grow side by side on the banks of ditches, rivers, and other moist, disturbed habitats. However, the chemical composition and herbivore load of these willows are dissimilar. *Salix myrsinifolia* leaves contain a high level of non-acetylated salicylates (Julkunen-Tiitto, 1989; Rank et al., 1998) and a small number of generalist herbivores feed upon its leaves (Sipura, 1999, 2000). *Salix pentandra* contain a moderate level of acetylated salicylates (Rank et al., 1998; Julkunen-Tiitto, 1989), chlorogenic

acid and flavonoids (this study). Both *S. myrsinifolia* and *S. pentandra* are favored hosts for some specialized insect herbivores (Kolehmainen et al., 1994, 1995; Rank et al., 1998). *Salix phylicifolia* is practically devoid of salicylates but rich in flavonoids (Rank et al., 1998; Sipura et al., personal communication), and leaves of this mild tasting willow are readily eaten and sometimes heavily defoliated by generalist insect species (Tahvanainen et al., 1985; Sipura, 1999, 2000).

A potential defense compound may act in three ways: it may decrease the consumption, it may reduce assimilation efficiencies and digestion, and it may act as a toxin (Scriber and Slansky, 1981). Degradation of secondary chemicals generally increases the toxicity of compounds, e.g. cleavage of the sugar moiety from cyanogenic glycosides produces highly toxic HCN (Wink, 1997). Salicylates have been shown to be activated via degradation (Clausen et al., 1989). Degradation of salicortin, acetylsalicortin and tremulacin produce salicin and 6-hydroxy-2-cyclohexenone (6-HCH) which is oxidized further to catechol (Mattes et al., 1987; Clausen et al., 1989; Julkunen-Tiitto and Meier, 1992). Both 6-HCH and catechol are effective components of salicylates in their role of defending plants against herbivores (Clausen et al., 1989; Reichardt et al., 1990). Chlorogenic acid, which was found in considerable levels in the leaves of *S. myrsinifolia* and *S. pentandra* in our study, is shown to be oxidized to chlorogenoquinone by polyphenoloxidase (PPO) when leaf tissue is damaged and cell compartmentalization is ruptured (in Felton et al., 1989). PPOs (EC 1.14.18.1 or EC 1.10.3.2.) catalyze hydroxylation of monophenols and oxidation of diphenols to respective quinones (Mayer, 1987). Orthoquinones have strong oxidizing and alkylating activities and can bind to nucleophilic NH_2 and SH groups of aminoacids, peptides and proteins (Constabel, 1999), leading to reduction in the nutritive quality of foliage and in the growth of lepidopteran larvae (Felton et al., 1989)

The purpose of our study was to examine how the chemistry and nutritive status of the leaves of three chemically dissimilar willows affect feeding and growth of a generalist insect herbivore. More particularly, we wanted to find out how salicylates influence the growth of larvae: whether they act as antifeeding deterrents or whether they reduce the assimilation efficiency of nutrients. We analyzed the nitrogen and carbon content of the leaves of these willow species and the frass produced by larvae feeding on the same leaves. Furthermore, we studied the degradation of secondary chemicals, especially the degradation of salicylates, by analyzing the chemical content of the leaves and the frass. In addition, by rearing larvae on an artificial diet containing different potential defense compounds, we studied which of the secondary compounds reduced feeding and growth of larvae in the "real" leaf experiment. The ecological and evolutionary consequences of the interaction between chemically variable plant groups, such as willows, and the common polyphagous insect herbivore are discussed briefly.

METHODS AND MATERIALS

Plant Material. In mid-June 1999, leaves of *S. pentandra*, *S. myrsinifolia* and *S. phylicifolia* were collected from two-year-old clones from Siikalahti, in Parikkala, eastern Finland. These willow species were growing in mixed stands on the bank of an embankment road. Because the vegetation had been cut two years previously, all willow shrubs were of the same age. We randomly selected and marked 12 individual willow shrubs of each species.

Insects. The laboratory stock of larvae of *O. brumata* used in these experiments was reared on leaves of *P. padus* until the larvae reached the 4th instar. The population originated from 200 individuals collected in the summer of 1997 from a *P. padus* forest stand situated near Siikalahti.

Experimental Procedure of a "Real Leaf" Experiment. Fresh leaves were split into two parts, the main vein was removed and the leaf-halves were weighed. To obtain as homogeneous leaf material as possible, the first mature leaf counted from the shoot tip was always selected (a first fully expanded leaf). One leaf-half was offered to a single larva and the other was dried at room temperature and stored at -20°C until analyzed. Larvae feeding on *P. padus* were transferred on the leaves of the new host plants 10 h before the actual experiment, in order to rid the digestive system of these larvae of the remnants of *P. padus* leaves. After this pre-feeding period, the 4th instars were weighed and placed in petri dishes (one larvae/dish) with a new leaf-half. On the following day, new fresh leaf-halves were cut and offered to the larvae. These larvae were reared for 36 h at 16°C with an 18:6 L:D photoperiod. Thereafter, the larvae were weighed and allowed to continue feeding on fresh leaf halves for an additional 24 h to obtain enough frass for analyses. The frass and the remains of the leaf-halves were dried at room temperature and weighed. After drying, the frass was stored at -20°C until analyzed. The leaves of one individual willow were used for feeding two different larvae. The leaves used for feeding the first larva were analyzed for their secondary chemical content, and the leaves used for feeding the second larva, were analyzed for their nitrogen and carbon contents. Relative growth rates (RGRs), relative consumption rates (RCRs), apparent digestibility (AD), and water content of leaves were determined for the whole set of data.

Secondary Chemistry. Dried and frozen leaf samples were put into Falcon tubes containing 20 ml of cold methanol, and the samples were incubated for 15 min in an ice bath. After incubation, the samples were extracted 2×2 min with methanol ($20 + 10$ ml) using an Ultra-Turrax homogenizer (Janke & Kunkel, IKA Labortecnik, Staufen, Germany). The leaf extracts were filtered and evaporated to dryness in a vacuum evaporator. The residues were redissolved in 10 ml of methanol, and the samples were divided into 1 and 2 ml aliquots. The methanol was reduced to dryness with gaseous nitrogen and the samples were stored at -20°C until used for HPLC-analyses and butanol tests. The frass samples were

extracted by the same method except that 4 + 4 ml of methanol were used for extraction, and the samples were redissolved in 4 ml of methanol and divided into 0.5 ml, 1.0 ml and 1.8 ml aliquots.

The samples were analyzed by HPLC (Hewlett-Packard, Palo Alto, CA). The device, elution gradient, and conditions were as described by Julkunen-Tiitto et al. (1996). The compounds were identified by comparing their spectra and retention times to those of reference compounds. The compounds were quantified using the response factors of reference compounds. 2′-*O*-Acetylsalicin, diglucoside of salicin and an unknown salicin derivative were calculated using the response factor for salicin, a putative diacetylsalicortin found in *S. pentandra* leaves was calculated using the response factor for 2′-*O*-acetylsalicortin, and three salicortin derivatives of *S. myrsinifolia* were calculated using the response factor for salicortin. From the chromatogram of *S. pentandra*, we found six rather large peaks of some unknown flavonoids. The levels of these compounds were calculated using the response factor for quercetin. The levels of condensed tannins were determined from the methanol extracts by means of the butanol-HCl-test (Hagerman, 1995).

The unknown salicylates were tentatively identified by HPLC/API-ES mass spectrophotometry (HP 1100 Series LC/MSD, Hewlett-Packard, Palo Alto California) according to their molecular ion peaks. The substances were separated in an HP Hypersil ODS column (3 μm, 2.1 $\times$ 100 mm). The solvents were A: 1.5% THF and 0.25% acetic acid in dH_2O and B: 100% methanol. The elution gradient was the following: 0–5 min, 0% B; 5–10 min, 15% B; 10–20 min, 30% B; 20–30 min, 35% B; 30–40 min, 50% B; and 40–60 min, 100% B. The capillary for ES (electrospray ionization) was set to 3000 V, the nebulizer pressure was 35 psig, the temperature of the drying gas (N_2) was 350°C and the rate of flow was 12 l/min. The fragmentation voltage was 120 V, except for the fragmentation voltage for the diglucoside of salicin, which was 130 V.

Carbon and Nitrogen Analyzes. The carbon and nitrogen contents of the leaves and frass were analyzed with a CHN-analyzer (Elemental Analyzer—Mod. 1106, Carlo Erba Stumentazione). Acetanilide OAS (Elemental Microanalysis Limited) was used as the standard (C = 71.09%; H = 6.71%; N = 10.36%; O = 11.84%). The samples were analyzed in tin capsules, 6 $\times$ 4 mm D1006 (Elemental Microanalysis Limited). Three replicates were run from each leaf- and frass-sample. To check the condition of the analyzer, every 10th sample was a standard. If the deviation was too high, the analyzer was recalibrated and the samples were run again.

Experiment on Artificial Diet. We studied the effects of salicylates and chlorogenic acid on larval growth more closely in an experiment, where the 4th instars of *O. brumata* were reared on artificial diets with four different concentrations of chlorogenic acid, catechol, salicin, or saligenin. Since the isolation of salicortin and its derivatives is very laborious, we used their degradation products, catechol, salicin, and saligenin.

The highest concentration of defenses (90 μmol/g FW) used in this study was selected on the basis of an estimate of how much catechol and salicin and subsequently saligenin would be produced by a complete degradation of salicortin, the main salicylate of *S. myrsinifolia*, at the level observed in the leaves. The 2nd highest level was approximately the same as the concentrations of salicylates observed in the leaves of *S. pentandra*.

The artificial diet was produced according to the recipe of Goujet and Guilbort (Singh, 1985), with the exception that we used *S. phylicifolia* leaf powder instead of *P. padus* leaf powder and reduced the amount of agar from 5 g to 2 g (Agar bacterioloque type A, Biokar Diagnostic). Salicin (Sigma), saligenin (ICN Biomedicals Inc) and catechol (ICN Biomedicals Inc) were used at the concentrations of 0, 20, 45, and 90 μmol/g FW and chlorogenic acid (Aldrich Chemie Co, Carl Roth GmbH Co) at the concentrations of 0, 5, 20, and 45 μmol/g FW. A warm mixture of the artificial diet (2.5 ml) was poured into small petri dishes (3.5 cm) and allowed to solidify. The dishes were stored at $+4^{\circ}$C until used.

Larvae were placed in dishes on a normal artificial diet (no additional secondary metabolites) for 12 hr prior to the actual experiment. After this pre-feeding period ($+16^{\circ}$C), the larvae were weighed and transferred to experimental dishes, one larva to each dish. For every concentration of each substance studied, 10 replicate dishes were used. After 24 hours of rearing at $+16^{\circ}$C, the larvae were weighed again.

Calculation and Statistics. Relative growth rate (RGR), relative consumption rate (RCR), apparent digestibility (AD) (Waldbauer, 1968; Scriber and Slansky, 1981), water content of the leaves, and assimilation efficiencies of carbon and nitrogen were calculated by the following equations: RGR = mg biomass gained/mg initial biomass/d; RCR = mg eaten/mg initial biomass of larvae/d; AD = [(mg eaten—mg frass)/mg eaten] $*$ 100%; water content of leaves = (FW-DW/FW) $*$ 100%; assimilation efficiencies of carbon and nitrogen = [(μg ingested—μg in frass)/μg ingested] $*$ 100%. Fresh weight of the larvae was converted to dry weight by the equation: $DW = e^{(0.089 + 0.0392FW)}$. This equation is calculated from data on 57 5th instars of *O. brumata* weighing 15.5−62.8 mg ($r = 0.923$, $P < 0.001$). The larvae were dried for 24 hr at $+105^{\circ}$C and weighed again.

The differences in the levels of secondary compounds between the three willow species, were tested using a nonparametric Kruskal-Wallis test. The differences in RGR, RCR, AD, and water content as well as the differences in carbon and nitrogen contents and their assimilation efficiencies were tested using a one-way ANOVA followed by Tukey's test for multiple post hoc comparisons. The correlations of RGR with RCR, and AD and the water content of the leaves with RGR and RCR were tested by Pearson's correlation test. The correlations of the levels of secondary compounds, carbon and nitrogen content and their assimilation efficiency with RGR and RCR were also tested by Pearson's correlation test. Since the nitrogen and carbon contents were determined from leaf-halves that were different

from those used for secondary chemistry, these correlations were performed separately. Correlations were tested by including data from all three willow species and data within species. A sequential Bonferroni correction was used for evaluating correlations between secondary chemicals and RGR and RCR since they contained multiple comparisons (in whole data comparisons; $k = 8$ individual compounds, and within species comparisons; $k = 7$ individual compounds), which increases the experiment-wise rate of error (Rice, 1989). The results of the artificial diet experiment were analyzed by two-way ANOVA, but did not include chlorogenic acid since this was used at lower concentrations than the degradation products of the salicylates. The results for chlorogenic acid were tested separately using one-way ANOVA. A standard version of SPSS (1997) for Windows 8.0 (SPSS, Chicago) was used for all statistical tests.

RESULTS

Chemistry of Willow Leaves. As expected, the leaves of *S. phylicifolia, S. pentandra* and *S. myrsinifolia* were chemically very different (Tables 1–3). In these three willow species, the levels of chlorogenic acid, cinnamic acid, quercetin, myricetin, and luteolin derivatives and total salicylates, total flavonoids, and condensed tannins differed significantly ($P < 0.001$). *S. phylicifolia* (Table 1) contained a very high level of flavonoids, low levels of chlorogenic acids and condensed tannins, but no salicylates or cinnamic acid derivatives. *S. pentandra* (Table 2) contained moderate levels of salicylates, flavonoids, and chlorogenic acids and a low level of condensed tannins. *S. myrsinifolia* had a very high level of

TABLE 1. CONCENTRATIONS (MEAN ± SE) OF SECONDARY COMPOUNDS IN THE LEAVES OF *Salix phylicifolia* AND IN THE FRASS OF *O. brumata* LARVAE[a]

Compound	Concentration in leaves (mg/g DW)	Concentration in frass (mg/g DW)	Percent (%) degraded
(+)-Catechin	0.68 ± 0.11	0.37 ± 0.09	58.21 ± 11.67
Chlorogenic acid derivatives	0.97 ± 0.17	0.46 ± 0.08	66.11 ± 5.70
Ampelopsin derivatives	160.91 ± 19.70	83.60 ± 11.48	64.96 ± 5.73
Quercetin derivatives	3.42 ± 0.41	1.49 ± 0.22	71.26 ± 3.92
Myricetin derivatives	6.11 ± 0.73	1.52 ± 0.23	81.36 ± 4.09
Luteolin derivatives	2.00 ± 0.25	1.24 ± 0.25	57.21 ± 7.64
Apigenin derivative	0.62 ± 0.14	0 ± 0.00	100 ± 0.00
Total flavonoids[b]	173.12 ± 20.86	87.86 ± 11.87	65.63 ± 5.62
Condensed tannins	3.22 ± 0.29	nd	nd

[a] Percentages of degradation of compounds were calculated by the following equation: ((μg compound ingested—μg compound excreted)/μg compound ingested)*100%. $N = 11$.
[b] All flavonoids determined, nd = not determined.

TABLE 2. CONCENTRATIONS (MEAN ± SE) OF SECONDARY COMPOUNDS IN THE LEAVES OF *Salix pentandra* AND IN THE FRASS OF *O. brumata* LARVAE[a]

Compound	Concentration in leaves (mg/g DW)	Concentration in frass (mg/g DW)	Percent (%) degraded
Unknown salicylate	0.84 ± 0.11	0 ± 0.00	100 ± 0.00
Salicin	1.23 ± 0.42	23.74 ± 7.28	—
Catechol	0 ± 0.00	2.90 ± 1.00	—
2′-*O*-Acetylsalicin	1.07 ± 0.28	0 ± 0.00	100 ± 0.00
2′-*O*-Acetylsalicortin	20.10 ± 4.41	0 ± 0.00	100 ± 0.00
Diacetylsalicortin	29.11 ± 7.37	0 ± 0.00	100 ± 0.00
Total salicylates	52.32 ± 11.84	26.65 ± 8.24	73.45 ± 4.36
Chlorogenic acid derivatives	37.63 ± 2.91	24.93 ± 4.82	64.13 ± 6.66
Cinnamic acid derivatives	1.85 ± 0.28	2.21 ± 0.40	28.30 ± 11.9
Quercetin derivatives	13.78 ± 1.06	12.85 ± 1.69	80.90 ± 5.87
Myricetin derivatives	3.41 ± 0.12	3.01 ± 0.54	52.12 ± 9.32
Other flavonoids[b]	11.22 ± 0.98	1.00 ± 0.16	95.03 ± 1.04
Total flavonoids[c]	30.40 ± 1.43	17.56 ± 2.26	68.33 ± 5.58
Condensed tannins	9.26 ± 0.75	nd	nd

[a] Percentages of degradation of compounds were calculated by the following equation: ((μg compound ingested—μg compound excreted)/μg compound ingested)*100%. $N = 10$.
[b] Includes three kaemferol derivatives, one eridictyoyl derivative, and six unknown flavonoids.
[c] All flavonoids determined, nd = not determined.

salicylates, a low level of flavonoids, only a small fraction of condensed tannins, and a moderate level of chlorogenic acids (Table 3).

The main salicylates of *S. pentandra* (Table 2) were 2′-*O*-acetylsalicortin and its derivative, a putative diacetylsalicortin (531.2 *m/z* $[M + Na]^+$), which preliminarily is thought to contain two acetyl groups attached to a glucose moiety (Ruuhola et al., unpublished results). *S. pentandra* also contained low levels of salicin, 2′-*O*-acetylsalicin, and an unknown derivative of salicin (possibly an isomer of diglucoside of salicin). The main salicylate of *S. myrsinifolia* was salicortin. The leaves also contained salicin, diglucoside of salicin (471 *m/z* $[M + Na]^+$), and three derivatives of salicortin (Table 3). Salicortin derivative-1 (585.2 *m/z* $[M + Na]^+$) has provisionally been calculated to contain two 1-hydroxy-6-oxo-2-cyclohexen moieties (Tegelberg and Julkunen-Tiitto, 2001). Salicortin derivative-2 (871.2 *m/z* $[M + Na]^+$) is assumed to consist of two salicortin moieties (Julkunen-Tiitto and Sorsa, 2001). Salicortin derivative-3 was an isomer of salicortin derivative-2. As the main flavonoids, *S. phylicifolia* contained ampelopsin and its derivatives, but also quercetin, myricetin, luteolin derivatives, and one apigenin derivative (Table 1). In addition, *S. phylicifolia* contained (+)-catechin, which was absent from the leaves of *S. pentandra* and *S. myrsinifolia*. *S. pentandra* contained quercetin, myricetin

TABLE 3. CONCENTRATIONS (MEAN ± SE) OF SECONDARY COMPOUNDS IN THE LEAVES OF *Salix myrsinifolia* AND IN THE FRASS OF *O. brumata* LARVAE[a]

Compound	Concentration in leaves (mg/g DW)	Concentration in frass (mg/g DW)	Percent (%) degraded
Diglucoside of salicin	2.49 ± 0.27	0.00 ± 0.00	100 ± 0.00
Salicin	2.06 ± 0.24	81.60 ± 6.04	—
Salicortin	112.64 ± 6.88	0.00 ± 0.00	100 ± 0.00
Salicortin derivative-1	7.54 ± 1.38	0.00 ± 0.00	100 ± 0.00
Salicortin derivative-2	1.08 ± 0.13	0.00 ± 0.00	100 ± 0.00
Salicortin derivative-3	0.91 ± 0.11	0.00 ± 0.00	100 ± 0.00
Total salicylates	125.85 ± 7.77	81.60 ± 6.04	66.49 ± 3.38
Chlorogenic acid derivatives	48.99 ± 3.50	0.00 ± 0.00	100 ± 0.00
Cinnamic acid derivatives	4.52 ± 0.36	1.62 ± 0.25	82.39 ± 2.10
Quercetin derivatives	3.78 ± 0.46	0.00 ± 0.00	100 ± 0.00
Luteolin derivatives	1.75 ± 0.18	2.94 ± 0.44	13.53 ± 11.85
Total flavonoids[b]	5.53 ± 0.56	2.89 ± 0.44	72.44 ± 4.15
Condensed tannins	0.29 ± 0.09	nd	nd

[a] Percentages of degradation of compounds were calculated by the following equation: ((μg compound ingested—μg compound excreted)/μg compound ingested)*100%. $N = 10$.
[b] All flavonoids determined, nd = not determined.

and kaemferol derivatives, one eridictyoyl derivative, and six unknown flavonoids (Table 2). The flavonoids of *S. myrsinifolia* consisted of quercetin and luteolin derivatives (Table 3).

Nutritive Values of Leaves and Assimilation Efficiencies of Carbon and Nitrogen. The leaves of the willow species differed significantly both in their digestibility (AD; $F_{2,29} = 3.495$, $P = 0.037$) and water content ($F_{2,29} = 3.587$, $P = 0.034$). The AD of the leaves of *S. myrsinifolia* was higher than the AD of the leaves of *S. phylicifolia* (Figure 1a). However, the differences in ADs between *S. myrsinifolia* and *S. pentandra* and between *S. pentandra* and *S. phylicifolia* were not significant. The water content of leaves tended to be higher in *S. myrsinifolia* than in *S. phylicifolia*; Tukey's HSD test, which is a conservative test, did not find the differences that were detected by ANOVA.

The nitrogen contents of the leaves of the different willow species did not differ significantly ($F_{2,29} = 1.337$, $P = 0.278$). By contrast, the carbon content of the leaves showed significant differences ($F_{2,29} = 6.421$, $P = 0.005$); the carbon content of *S. phylicifolia* leaves was significantly higher than that of *S. pentandra* leaves or *S. myrsinifolia* (Figure 1b). In spite of the lower AD of the *S. phylicifolia* leaves, the assimilation efficiency of nitrogen ($F_{2,29} = 1.225$, $P = 0.308$) or carbon ($F_{2,29} = 1.915$, $P = 0.166$) did not differ in larvae grown on different willows (Figure 1c).

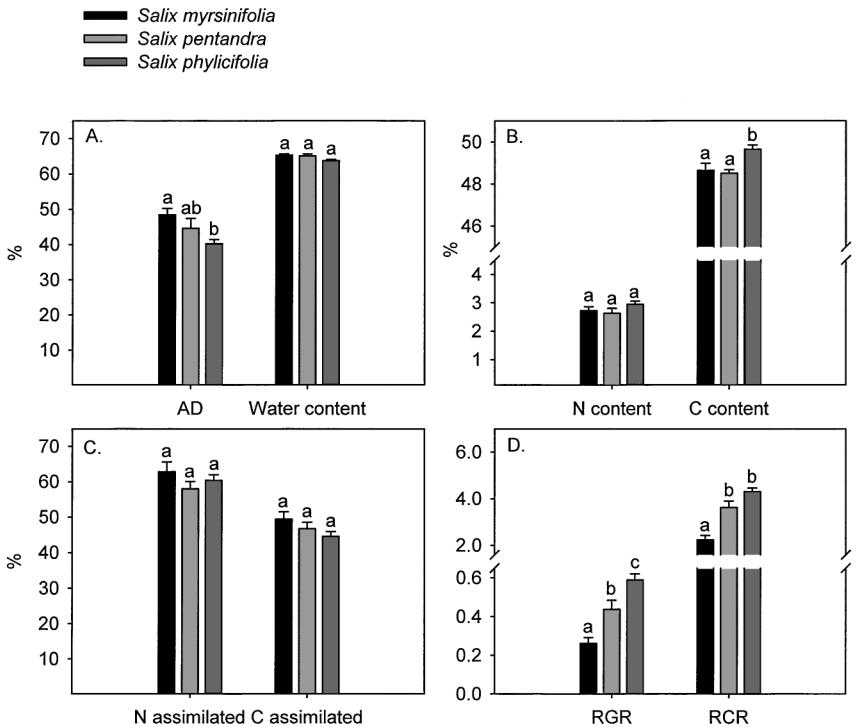


FIG. 1. AD and water (A), N and C (B) contents of the leaves of *S. pentandra*, *S. myrsinifolia* and *S. phylicifolia*. Assimilation efficiencies of N and C (C), RGR- and RCR-values of *O. brumata* larvae reared on these three willow species (D). The data were tested by one-way ANOVA followed by Tukey's HSD test for multiple comparisons (SPSS, 1997). Cases with statistically significant differences ($P < 0.05$) are identified by different letters.

Feeding and Growth of Larvae. The larvae of *O. brumata* grew at very different rates on different willows ($F_{2,59} = 19.56$, $P < 0,001$). The larvae grew best on *S. phylicifolia*, and the larval growth on this species was significantly faster than on *S. pentandra* (Figure 1d). The growth of larvae was slowest on the leaves of *S. myrsinifolia* (Figure 1d), and RGR differed significantly from that of larvae feeding on *S. phylicifolia* ($P < 0.001$) or *S. pentandra* ($P < 0.05$). The growth of larvae reared on *S. pentandra* was 25% slower and on *S. myrsinifolia* 60% slower than the growth of larvae on *S. phylicifolia*.

The consumption rates of the larvae differed on the three willows studied ($F_{2,59} = 24.17$, $P < 0.001$). The relative consumption rate (RCR) tended to be higher on *S. phylicifolia* than on *S. pentandra* ($P = 0.064$), and on *S. myrsinifolia* RCR was much lower than the RCR of larvae reared on *S. pentandra* or

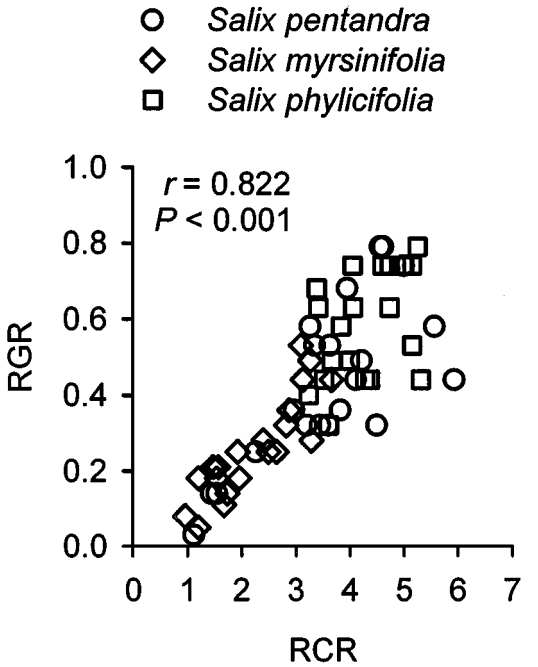


FIG. 2. Correlations between RGRs and RCRs. Comparisons were made with complete data including all three willows species (A), or within species; *S. pentandra* (B), *S. myrsinifolia* (C) and *S. phylicifolia* (D). Data were tested by Pearson's correlation test.

S. phylicifolia (Figure 1d). Furthermore, RCR and RGR correlated significantly and positively both within and between willow species (Figure 2).

Correlations between Leaf Characters and RGR and RCR. The levels of total salicylates and chlorogenic acid correlated strongly and negatively with larval growth and consumption (Figures 3a,b and 4a,b). In addition, the level of cinnamic acid correlated negatively with RGR and RCR (Figures 3c and 4c). By contrast, the level of myricetin derivatives and total flavonoids correlated positively with rate of consumption (Figures 4e and g). However, the correlation with growth rate was not significant (Figures 3e and g). The levels of quercetin and luteolin derivatives or condensed tannins did not correlate with RGR or RCR (Figures 3d,f,h and 4d,f,h). On the other hand, none of the concentrations of the secondary compounds correlated with RGR or RCR within species, although in *S. pentandra* the concentration of salicylates, chlorogenic acids, and condensed tannins tended to correlate negatively with RGR (data not shown).

When the correlation included all three willow species, digestibility of the leaves (AD) correlated negatively with RGR ($r = -0.335$, $P = 0.008$) and RCR

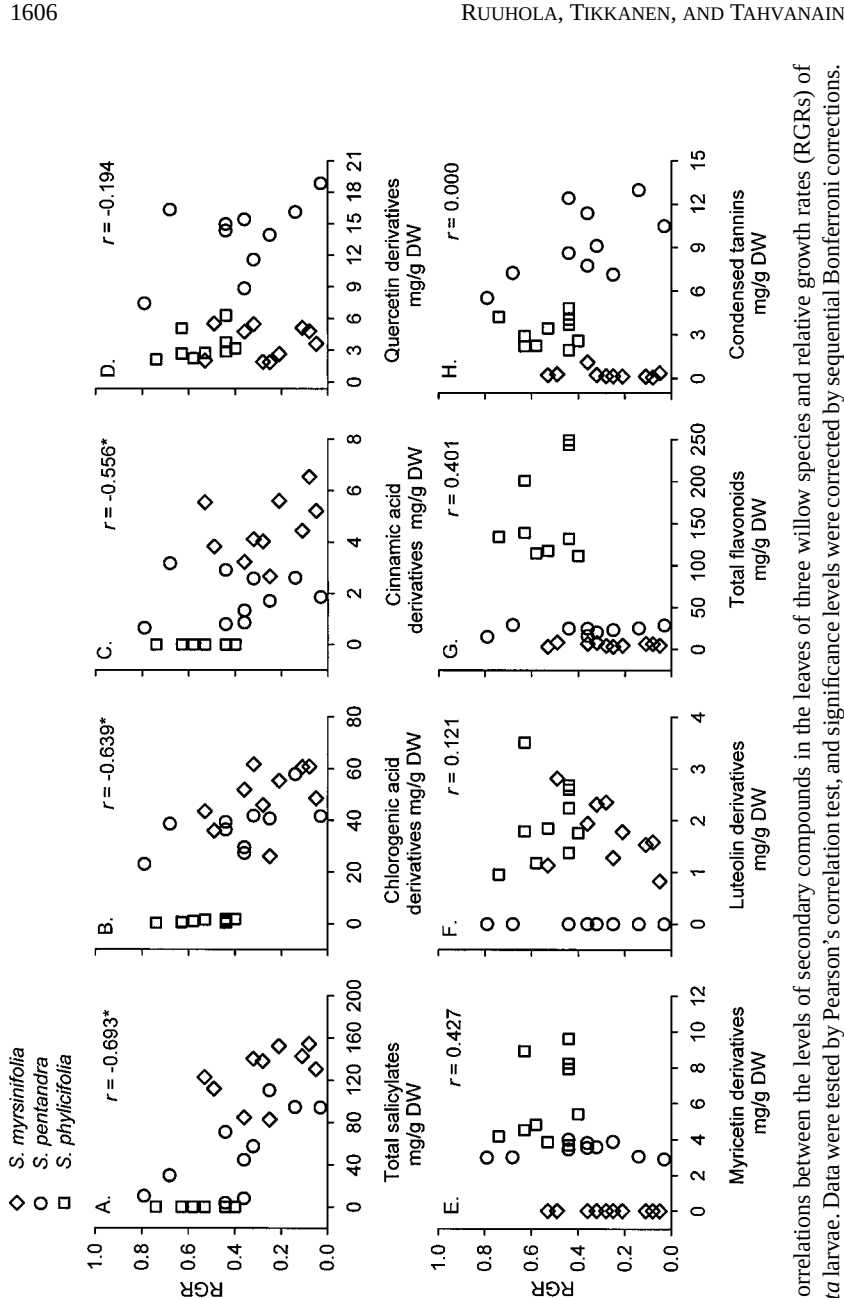


FIG. 3. Correlations between the levels of secondary compounds in the leaves of three willow species and relative growth rates (RGRs) of *O. brumata* larvae. Data were tested by Pearson's correlation test, and significance levels were corrected by sequential Bonferroni corrections. Statistically significant cases are marked by asterisks.

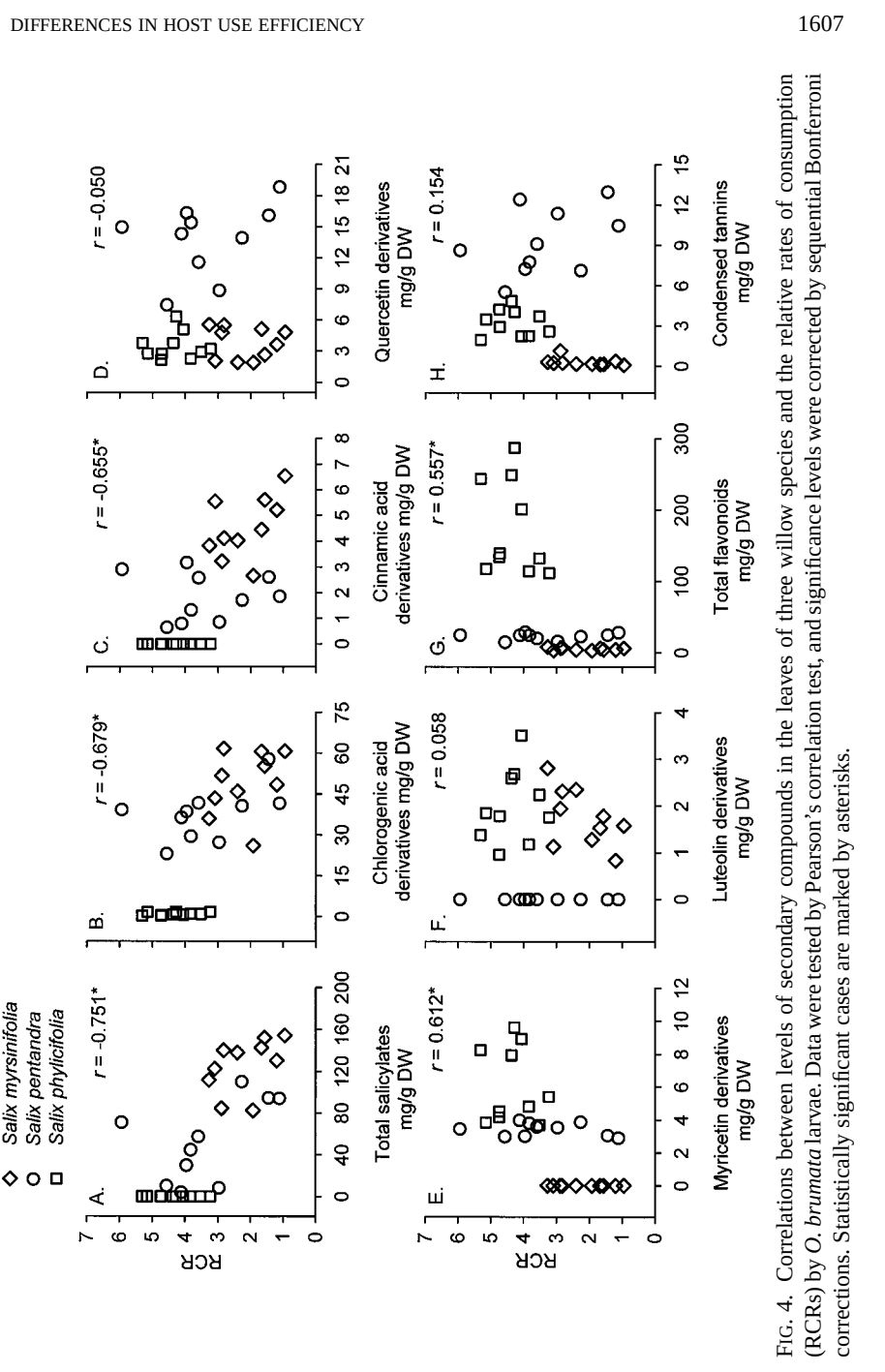


FIG. 4. Correlations between levels of secondary compounds in the leaves of three willow species and the relative rates of consumption (RCRs) by *O. brumata* larvae. Data were tested by Pearson's correlation test, and significance levels were corrected by sequential Bonferroni corrections. Statistically significant cases are marked by asterisks.

($r = -0.308$, $P = 0.015$). This is apparently due to the higher digestibility of *S. myrsinifolia* leaves as compared with *S. phylicifolia* leaves. Nevertheless, the quality of *S. myrsinifolia* leaves for *O. brumata* larvae was inferior, and the larvae grew very slowly. Neither the nitrogen nor the water content of leaves correlated with growth or consumption. The carbon content of leaves correlated significantly with RGR ($r = 0.366$, $P = 0.039$) but not with RCR ($r = 0.272$, $P = 0.132$).

Degradation of Secondary Compounds. In the digestive tract of larvae, labile salicortin, 2′-*O*-acetylsalicortin, and their derivatives were degraded completely to salicin, which was detected at high levels in the frass of larvae feeding on *S. myrsinifolia* and *S. pentandra* (Tables 2 and 3). Larvae feeding on *S. myrsinifolia* ingested on average 5.36 ± 0.57 μmol of salicortin, the main salicylate of this willow, and excreted 2.98 ± 0.31 μmol of salicin, which indicates that further metabolism of salicin was rather slow. Since we did not have separate response factors for all salicylates, we were not able to calculate the exact equimolar relationship between salicin and the other salicylates.

The frass of larvae feeding on *S. pentandra* (Table 2) contained catechol, which was not detected in the frass of larvae feeding on *S. myrsinifolia* leaves (Table 3). On the other hand, the frass of larvae reared on *S. myrsinifolia* contained

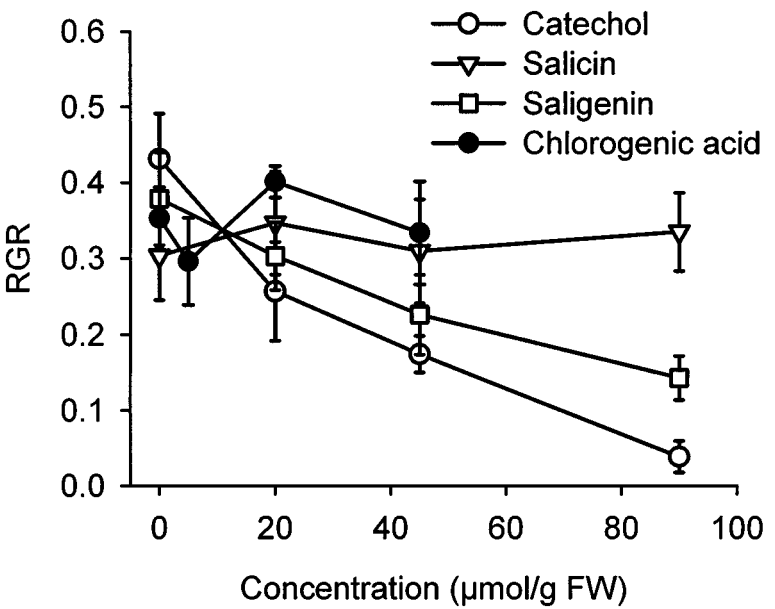


FIG. 5. Effects of increasing concentrations of catechol, saligenin, salicin, and chlorogenic acid on the relative growth rate (RGR, mean ± S.E.) of *O. brumata* larvae. $N = 10$ in each concentration of each compound.

TABLE 4. ANOVA TABLE OF THE FULL FACTORIAL ANALYSES FOR THE EFFECT OF CATECHOL, SALIGENIN AND SALICIN ON THE RELATIVE GROWTH RATE OF *O. brumata* LARVAE RAERED ON ARTIFICIAL DIET

Source of variance	*df*	MS	*F*	*P*
Model	11	0.064	4.324	<0.001
Compound	2	0.073	4.764	0.011
Concentration	3	0.110	7.180	<0.001
Compound × concentration	6	0.042	2.749	0.017

an unknown compound with a spectrum resembling that of cyclohexenone but with a longer retention time.

Chlorogenic acids, flavonoids, and cinnamic acids were degraded with different efficiencies depending on which willow species the larvae were reared on. Chlorogenic acid derivatives were degraded more efficiently on *S. myrsinifolia* than on *S. pentandra* and on *S. phylicifolia* (Tables 1–3), and cinnamic acid derivatives were degraded more efficiently on *S. myrsinifolia* than on *S. pentandra* (Tables 2 and 3). Quercetin derivatives, which were found in the leaves of all three willows, were totally absent from the frass of larvae feeding on *S. myrsinifolia* (Table 3), but in larvae feeding on *S. phylicifolia* or *S. pentandra*, quercetin derivatives were degraded 71.3% and 80.9%, respectively (Tables 1 and 2).

Growth of Larvae on Artificial Diets with Different Levels of Salicin, Saligenin, Catechol, or Chlorogenic Acid. Catechol and saligenin significantly reduced the growth rate of larvae, while salicin and chlorogenic acid did not (Figure 5). The differences between compounds and concentrations and their interactions were all significant (Table 4). With increasing levels of catechol and saligenin, the inhibition of RGR was almost linear (Figure 5).

Catechol added to an artificial diet mixture at concentrations of 20, 45, and 90 μmol/g FW resulted in 40%, 61%, and 91% reductions, respectively, in the growth of larvae. The addition of saligenin resulted in 21%, 40%, and 63% reductions, respectively, in the growth of the larvae. Thus, catechol was a more effective growth inhibitor than saligenin. We did not measure the feeding rates of the larvae, but it was clear that catechol acted as a strong feeding deterrent since larvae kept in dishes containing catechol ate very little, and at the highest concentration of catechol, larvae hardly touched the diet. This type of feeding inhibition was not detected with saligenin.

DISCUSSION

The growth and feeding of *O. brumata* larvae were retarded on both salicylate- and chlorogenic acid-rich willows, *S. myrsinifolia* and *S. pentandra*, when compared to the feeding and growth observed on the salicylate-free *S. phylicifolia*.

This retardation was obviously due to the unfavorable secondary chemistry of the leaves, since differences in the nutritive status, digestibility of leaves, and assimilation efficiencies of nitrogen and carbon cannot explain this retardation. There were no differences between *S. pentandra* and *S. phylicifolia* in water or nitrogen content or the digestibility of the leaves. Furthermore, *S. phylicifolia* leaves contained more carbon, less water, and had lower digestibility, indicating that the leaves of *S. phylicifolia* were tougher and probably contained more carbon based fibers and cellulose than did the leaves of *S. myrsinifolia*.

The strong correlation between leaf consumption and growth rate of larvae indicates that the differences between growth rates were explained by differential feeding rates of larvae reared on these three willows. In addition, the levels of the most abundant chemicals found in the leaves of *S. myrsinifolia* and *S. pentandra*, salicylates and chlorogenic acids, correlated markedly but negatively with both consumption and growth. This suggests that these compounds are potential antifeeding deterrents against *O. brumata*. Many reports have shown that salicylates act as feeding deterrents against generalist insect herbivores (Tahvanainen et al., 1985; Lindroth et al., 1988; Lindroth and Peterson, 1988); however, some reports show that chlorogenic acid slows down the growth of insect herbivores (e.g., Isman and Duffey, 1982; Felton et al., 1989; Felton and Duffey, 1991).

To clarify the question of whether the effective defensive compounds of *S. myrsinifolia* and *S. pentandra* were salicylates, chlorogenic acid, or both, we reared *O. brumata* larvae on an artificial diet. The diet contained chlorogenic acid and degradation products of salicylates in equimolar concentrations to the those observed in the leaves of *S. myrsinifolia* and *S. pentandra*. This experiment showed that the degradation products of salicylates, catechol, and saligenin, reduced the growth of *O. brumata*, while neither salicin nor chlorogenic acid significantly retarded the growth. Catechol, but not saligenin, clearly acted as a feeding deterrent. Similar results were observed by Clausen et al. (1989), who showed, that at the concentrations potentially found in the insect gut, 6-HCH and catechol significantly reduced pupal weight of *Choristoneura conflictana* (Lep.) reared on artificial diets. In addition, Lindroth and Peterson (1988) demonstrated that both salicortin and tremulacin reduced the growth of *Spodoptera eridania* (Lep.) larvae, primarily by decreasing consumption. Neither salicin nor chlorogenic acid had deleterious effects. In addition, their histopathological results showed that both salicortin and tremulacin acted as gut toxins, and they proposed that the active component was saligenin ester.

Our degradation pattern of salicylates does not conform to the model of Lindroth et al. (1988), who suggested that the degradation of salicortin and tremulacin begins with cleavage of the sugar moiety by β-glucosidase, and that the effective component in defense is cyclohexenone saligenin ester, which is detoxified by carboxyesterases. Instead, we propose that labile salicortin and 2′-*O*-acetylsalicortin and their derivatives are degraded first by foliar esterases or by

alkaline conditions to salicin and 6-HCH (see Mattes et al., 1987; Clausen et al., 1989; Julkunen-Tiitto and Meier, 1992). Salicin is then further degraded to saligenin by β-glucosidase activity (Julkunen-Tiitto and Meier, 1992). We propose that saligenin is a toxic agent of salicylates and may cause gut lesions such as those observed by Lindroth and Peterson (1988). β-Glucosidase has been shown to be ineffective in the degradation of acetylated salicylates. However, the cleavage of the acetyl moiety is achieved by esterases, although the reaction is slow (Julkunen-Tiitto and Meier, 1992). Thus, acetylation of salicylates may decrease their degradation rates in the digestive tract of insects and further decrease the toxicity of compounds. The oxidation of 6-HCH to catechol was obviously due to the alkaline condition of the midgut of *O. brumata* larvae. Catechol is a substrate of PPO (catechol oxidase; EC 1.10.3.2) and the *o*-quinone produced can bind to nucleophilic NH_2 and SH groups of amino acids and proteins (Constabel, 1999). 6-HCH is also an electrophile (Clausen et al., 1989), and thus, both catechol and 6-HCH are agents that may reduce the assimilation efficiencies of nutrients. In summary, salicylates may act in three ways: they are strong feeding deterrents, but they may also have toxic effects and reduce the assimilation of nutrients by precipitating essential amino acids and proteins. However, we emphasize that the observed growth retardation of *O. brumata* larvae was mainly a consequence of feeding deterrence.

One reason for the ineffectiveness of chlorogenic acid in retarding the growth of *O. brumata* larvae in our experiment could be the absence of active foliar PPOs in the artificial diet. However, the content of the midgut of *O. brumata* larvae is alkaline (Berenbaum, 1980), which should lead to auto-oxidation of chlorogenic acid (Felton et al., 1989). Felton and Duffey (1991) showed that chlorogenic acid added to an artificial diet decreased the growth of *Helicoverpa zea* (Lep.), but the effect was enhanced by the presence of plant peroxidases and H_2O_2. The age of the larvae may also affect their sensitivity to chlorogenic acid. For example, the growth of the 3rd and 5th instars of *Heliothis zea* (Lep.), was not affected by chlorogenic acid, while that of neonate larvae was inhibited (Isman and Duffey, 1982). Furthermore, we cannot rule out the possible synergistic effects of salicylates and chlorogenic acids.

The flavonoids, chlorogenic acid and cinnamic acid derivatives of three willow species were degraded at different rates. For example, the chlorogenic acid and quercetin derivatives that originated from *S. myrsinifolia* were decomposed more efficiently than those originating from *S. pentandra* or *S. phylicifolia*. One explanation for the dissimilar degradation efficiencies of these compounds could be the different levels of condensed tannins in the leaves of these willows; *S. myrsinifolia* had the lowest level of tannins. Tannins are able to bind proteins and thus inhibit enzymes (Goldstein and Spencer, 1985; Juntheikki and Julkunen-Tiitto, 2000). Goldstein and Spencer (1985) demonstrated that hydrolysis of the cyanogenic glycosides of *Carica papay* L. (Caricaceae) by specific β-glucosidases was inhibited

by the presence of tannins. On the other hand, the pH of the midgut of *O. brumata* is very alkaline: 9.2–9.5 (Berenbaum, 1980), and in such alkaline conditions tannin-protein complexes are thought to dissociate (Berenbaum, 1980; Zucker, 1983; Martin and Martin, 1984). It is also possible that the faster degradation rates of chlorogenic acid, cinnamic acid, and quercetin derivatives are due to the higher PPO activity of *S. myrsinifolia* leaves. We believe that secondary chemicals such as tannins may affect the degradation and thus the toxicity of other chemicals. However, we suggest that the flavonoids and the low levels of condensed tannins observed in this study did not have major negative or positive effects on larval growth when compared to the deleterious effects of salicylates.

It is intriguing to compare our results obtained with generalist *O. brumata* with the results of the experiment in which the growth of a highly specialized leaf beetle, *P. vitellinae*, was studied on the same set of willow species (Rank et al., 1998). The growth rates of *P. vitellinae* showed exactly the opposite pattern: the larvae grew fastest on salicylate-rich *S. myrsinifolia* and *S. pentandra*, and their growth was slowest on salicylate-poor *S. phylicifolia* (Rank et al., 1998). The larvae of *P. vitellinae* are assumed to utilize a glucose moiety released in the degradation of salicin as their energy source for growth (Pasteels et al., 1983; Rowell-Rahier and Pasteels, 1986). It is obvious that generalist larvae are not able to degrade salicin efficiently and utilize salicylates as an energy source.

Salicylate-rich *S. myrsinifolia* is a poor host candidate for *O. brumata*, at least in spring, when the salicylate levels are the highest (Julkunen-Tiitto, personal communication). On the contrary, a moderate level of acetylated salicylates found in the leaves of *S. pentandra* appeared not to be particularly deleterious, at least not for the 4th instars. Thus, both *S. pentandra* and *S. phylicifolia*, whose chemical compositions are, however, very dissimilar compared to the chemistry of the main hosts, *P. padus* and *Q. robur*, seem to be suitable alternative hosts for polyphagous *O. brumata*.

The ability of *O. brumata* to tolerate and to adapt to the use of hosts that are chemically so divergent and derived from different plant families is remarkable. One possible explanation for this tolerance might be found in the low β-glucosidase activity in the midgut of larvae. For example, the β-glucosidase activity observed in *Papilio glaucus canadiensis*, which is adapted to use salicylate-rich species as its host, was lower than the activity observed in *Papilio glaucus glaucus* which cannot tolerate salicylates (Lindroth, 1989). The slow further metabolism of salicin in the "real leaf" experiment and the ineffectiveness of salicin found in the artificial diet experiment support this hypothesis. In the first place, the ability to adapt to the use of chemically different hosts provides, the means for this moth to survive if dispersing neonate larvae do not succeed in settling on optimal hosts (Wint, 1983; Tikkanen, 2000). Secondly, the adaptability of *O. brumata* facilitates the opportunistic use of locally most abundant host plants (see Tikkanen, 2000).

In summary, high salicylate content of the leaves of some willow species, such as *S. myrsinifolia*, provides these plants with efficient chemical defense against many generalist insect herbivores. This resistance may significantly reduce the level of damage inflicted by generalist feeders (see Sipura, 2000) and may, in consequence, increase the overall fitness of these high salicylate willows. Plants with effective defenses may "escape" from herbivores, especially from generalist ones which are able to feed on other hosts and are not forced to take part in the "co-evolutionary arms race" (*sensu* Erlich and Raven, 1964). As a result, these plants may have more resources for competition or, if defensive chemicals are costly, they may adapt to a new life history strategy of lower growth and efficient defense (see Coley et al., 1985).

Acknowledgments—We thank the Punkaharju Forest Research Station of the Finnish Forest Research Institute for providing the facilities for the "real leaf" experiment, laboratory assistant Marja Noponen for guiding us in the use of the CHN-analyzer, Doc. Riitta Julkunen-Tiitto, Dr. Marja-Riitta Juntheikki and doctoral student Mika Sipura for their valuable comments on the manuscript. This work was supported by the Academy of Finland and the Foundation for Research of Natural Resources in Finland.

REFERENCES

BERENBAUM, M. 1980. Adaptive significance of midgut pH in larval Lepidoptera. *Am. Nat.* 115:138–146.

CLAUSEN, T. P., REICHARDT, P. B., BRYANT, J. P., WERNER, R. A., POST, K., and FRISBY, K. 1989. Chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores. *J. Chem. Ecol*. 15:2335–2346.

COLEY, P. D., BRYANT, J. P., and CHAPIN, F. S. III. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

CONSTABEL, C. P. 1999. A survey of herbivore-inducible defense proteins and phytochemicals, pp.137–166, *in* A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced plant defenses against pathogens and herbivores. Biochemistry, ecology and agriculture. APS Press, St. Paul.

DENNO, R. F., LARSSON, S., and OLMSTEAD, K. L. 1990. Role of enemy-free space and plant quality in host-plant selection by willow beetles. *Ecology* 71:124–137.

ERLICH, P. R., and RAVEN, P. H. 1964. Butterflies and plants: A study in coevolution. *Evolution* 18:586–608.

FEENY, P. P. 1968. Effect of oak leaf tannins on larval growth of the winter moth *Operophtera brumata. J. Insect. Physiol*. 14:805–817.

FEENY, P. P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–581.

FELTON, G. W., and DUFFEY, S. S. 1991. Protective action of midgut catalase in Lepidopteran larvae against oxidative plant defenses. *J. Chem. Ecol*. 17:1715–1733.

FELTON, G. W., DONATO, K., DEL VECCHIO, R. J., and DUFFEY, S. S. 1989. Activation of Plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *J. Chem. Ecol*. 15:2667–2694.

GOLDSTEIN, W. S., and SPENCER, K. C. 1985. Inhibition of cyanogenesis by tannins. *J. Chem. Ecol*. 11:847–858.

HAGERMAN, A. E. 1995. Acid butanol assay for protoanthocyanidins, pp. 24–25, *in* Tannin Analysis. Department of Chemistry, Miami University.

HÄMET-AHTI, L., SUOMINEN, J., ULVINEN, T., and UOTILA, P. 1998. Retkeilykasvio (Field flora of Finland). Finnish Museum of Natural History, Botanical Museum, Helsinki.

ISMAN, M. B., and DUFFEY, S. S. 1982. Toxicity of tomato phenolic compounds to the fruitworm, *Heliothis zea*. *Entomol. Exp. Appl*. 31:370–376.

JULKUNEN-TIITTO, R. 1989. Distribution of certain phenolics in *Salix* species (Salicaceae). PhD Thesis. Publications in Sciences, University of Joensuu, Joensuu.

JULKUNEN-TIITTO, R., and MEIER, B. 1992. Variation in growth and secondary phenolics among field-cultivated clones of *Salix myrsinifolia*. *Planta Med*. 58:77–80.

JULKUNEN-TIITTO, R., and SORSA, S. 2001. Testing drying methods for willow flavonoids, tannins and salicylates. *J. Chem. Ecol*. (In press).

JULKUNEN-TIITTO, R., ROUSI, M., BRYANT, J., SORSA, S., SIKANEN, H., and KEINÄNEN, M. 1996. Chemical diversity of Betulaceae species: Comparison of phenolics and terpenoids in northern birch species. *Trees* 11:16–22.

JUNTHEIKKI, M-R., and JULKUNEN-TIITTO, R. 2000. Inhibition of β-glucosidase and esterase by tannins from *Betula*, *Salix* and *Pinus* Species. *J. Chem. Ecol*. 26:1151–1165.

KIRSTEN, K., and TOPP, W. 1991. Acceptance of willow-species for the development of the winter moth, *Operophtera brumata* (Lep., Geometridae). *J. Appl. Entomol*. 111:457–468.

KOLEHMAINEN, J., ROININEN, H., JULKUNEN-TIITTO, R., and TAHVANAINEN, J. 1994. Importance of phenolic glucosides in host selection of shoot galling sawfly *Euura amerinae*, on *Salix pentandra*. *J. Chem. Ecol*. 20:2455–2466.

KOLEHMAINEN, J., JULKUNEN-TIITTO, R., ROININEN, H., and TAHVANAINEN, J. 1995. Phenolic glucosides as feeding cues for willow-feeding leaf beetles. *Entomol. Exp. Appl*. 74:235–243.

LINDROTH, L. R. 1989. Biochemical detoxication: Mechanism of differential tiger swallowtail tolerance to phenolic glycosides. *Oecologia* 81:219–224.

LINDROTH, R. L., and PETERSON, S. S. 1988. Effects of plant phenols on performance of southern armyworm larvae. *Oecologia* 75:185–189.

LINDROTH, R. L., SCRIBER, J. M., and HSIA, M. T. S. 1988. Chemical ecology of the tiger swallowtail: Mediation of host use by phenolic glycosides. *Ecology* 69:814–822.

MARTIN, M. M., and MARTIN, J. S. 1984. Surfactants: Their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia* 61:342–345.

MATSUDA, K., and MATSUO, H. 1985. A flavonoid, luteolin-7—glucoside, as well as salicin and populin, stimulating the feeding of leaf-beetles attacking Salicaceaous plants. *Appl. Entomol. Zool*. 20:305–313.

MATTES, B. R., CLAUSEN, T. P., and REICHARDT, P. B. 1987. Volatile constituents of balsam poplar: The phenol glycoside connection. *Phytochemistry* 26:1361–1366.

MAYER, M. M. 1987. Polyphenol oxidases in plants—recent progress. *Phytochemistry* 26:11–20.

PASTEELS, J. M., ROWELL-RAHIERT, M., BRAEKMAN, J. C., and DUPOND A. 1983. Salicin from host plants as precursors of salicyl aldehyde in defensive secretion of Chrysomeline larvae. *Physiol. Entomol*. 8:307–314.

RANK, N. E. 1992. Host plant preference based on salicylate chemistry in a willow leaf beetle (*Chrysomela aeneicollis*). *Oecologia* 90:95–101.

RANK, N. E., KÖPF, A., JULKUNEN-TIITTO, R., and TAHVANAINEN, J. 1998. Host preference and larval performance of the salicylate-using leaf beetle *Phratora vitellinae*. *Ecology* 79:618–631.

REICHARDT, P. B., BRYANT, J. P., MATTES, B. R., CLAUSEN, T. P., CHAPIN, F. S. III, and MEYER, M. 1990. Winter chemical defense of Alaskan balsam poplar against snowshoe hares. *J. Chem. Ecol*. 16:1941–1959.

RICE, W. R. 1989. Analyzing tables of statistical tests. *Evolution* 43:223–225.

ROWELL-RAHIER, M., and PASTEELS, J. M. 1986. Economics of chemical defense in Chrysomelinae. *J. Chem. Ecol*. 12:1189–1203.

SCRIBER, J. M., and SLANSKY, F. Jr. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol*. 26:183–211.

SEIGLER, D. S. 1998. Plant Secondary Metabolism Kluwer Academic Publishers, Boston.

SINGH, P. 1985. Multiple-species rearing diets, pp. 19–45, *in* P. Singh and R. F. Moore (eds.). Handbook of Insect Rearing. Elsevier Science Publishers B. V., Amsterdam.

SIPURA, M. 1999. Tritrophic interactions: Willows, herbivorous insects and insectivorous birds. *Oecologia* 121:537–545.

SIPURA, M. 2000. Herbivory on willows: Abiotic constrains and trophic interactions. PhD Dissertations in Biology, University of Joensuu, Joensuu.

SKVORTSOV, A. K. 1999. T. Erkama and H. Simola (eds.). Willows of Russia and adjacent countries. Taxonomical and geographical revision. University of Joensuu, Joensuu.

SMILEY, J. T., HORN, J. M., and RANK, N. E. 1985. Ecological effects of salicin at three trophic levels: New problems from old adaptations. *Science* 229:649–651.

TAHVANAINEN, J., JULKUNEN-TIITTO, R., and KETTUNEN, J. 1985. Phenolic glycosides govern the food selection pattern of willow feeding leaf beetles. *Oecologia* 67:52–56.

TEGELBERG, R., and JULKUNEN-TIITTO, R. 2001. Quantative changes in secondary metabolites of dark-leaved willow (*Salix myrsinifolia*) exposed to enhanced ultraviolet-B radiation. *Physiol. Plant.* In press.

TIKKANEN, O-P. 2000. Adaptation of a generalist moth, *Operophtera brumata* to host plants. PhD Dissertation in Biology, University of Joensuu, Joensuu.

TIKKANEN, O-P., CARR, T. G., and ROININEN, H. 1999. Factors influencing the distribution of a generalist spring-feeding moth, *Operophtera brumata* (Lepidoptera: Geometridae), on host plants. *Environ. Entomol*. 28:461–469.

TIKKANEN, O-P., NIEMELÄ, P., and KERÄNEN, J. 2000. Growth and development of a generalist herbivore, *Operophtera brumata*, on original and alternative host plants. *Oecologia* 122:259–536.

TIKKANEN, O-P., ROININEN, H., NIEMELÄ, P., TAHVANAINEN, J., and ZINOJEV, A. 1998. Use of host plants by *Operophtera brumata* L. (Lep., Geometridae) during the first recorded outbreak in the subcontinental boreal zone of Fennoscandia. *J. Appl. Ent*. 122:247–253.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol*. 5:229–288.

WINK, M. 1997. Special nitrogen metabolism, pp. 439–486, *in* P. M. Dey and J. B. Harborne (eds.). Plant Biochemistry. Academic Press, London.

WINT, W. 1983. The role of alternative host-plant species in the life of a polyphagous moth, *Operophtera brumata* (Lepidoptera: Geometridae). *J. Anim. Ecol*. 52:439–450.

ZUCKER, W. V. 1983. Tannins: Does structure determine function? An ecological perspective. *Am. Nat*. 121:335–365.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

EVALUATION OF VETIVER OIL AND SEVEN INSECT-ACTIVE ESSENTIAL OILS AGAINST THE FORMOSAN SUBTERRANEAN TERMITE

BETTY C. R. ZHU,[1] GREGG HENDERSON,[2,]* FENG CHEN,[2] HUIXIN FEI,[2] and ROGER A. LAINE[1]

Louisiana State University Agricultural Center
Louisiana Agricultural Experimental Station
Louisiana State University
Department of Biological Sciences[1]
and Department of Entomology,[2] *Baton Rouge LA 70803*

(Received October 9, 2000; accepted April 4, 2001)

Abstract—Repellency and toxicity of 8 essential oils (vetiver grass, cassia leaf, clove bud, cedarwood, *Eucalyptus globules*, *Eucalyptus citrodora*, lemongrass and geranium) were evaluated against the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. Vetiver oil proved the most effective repellent because of its long-lasting activity. Clove bud was the most toxic, killing 100% of termites in 2 days at 50 μg/cm^2. The tunneling response of termites to vetiver oil also was examined. Vetiver oil decreased termite tunneling activity at concentrations as low as 5 μg/g sand. Tunneling and paper consumption were not observed when vetiver oil concentrations were higher than 25 μg/g sand. Bioactivity of the 8 oils against termites and chemical volatility were inversely associated. Listed in decreasing order of volatility, the major constituents of the 8 oils were: eucalyptol, citronellal, citral, citronellol, cinnamaldehyde, eugenol, thujopsene, and both α- and β- vetivone. Vetiver oil is a promising novel termiticide with reduced environmental impact for use against subterranean termites.

Key Words—*Coptotermes formosanus*, *Vetiveria zizanioides*, clove, repellents, toxicants, natural product chemistry, vetivone, nootkatone, eugenol.

INTRODUCTION

Essential oils are natural volatile substances found in a variety of odoriferous plants. When isolated from plants, essential oils are not usually extracted as chemically pure substances, but consist of mixtures of many compounds. Terpenes,

*To whom correspondence should be addressed.

0098-0331/01/0800-1617$19.50/0

organic compounds consisting of multiples of isoprene units are the dominant constituents of essential oils. Commercially, essential oils are used in four primary ways: as odorants in fragrances, as flavor enhancers in many food products, as pharmaceuticals, and as insecticides. Particular emphasis has been placed on their antimicrobial, antifugal, antitumor, and insecticidal actions (Franzios et al., 1997).

In recent years, essential oils have received much attention as potentially useful bioactive compounds against insects. Insecticidal activity of essential oils has been shown against cockroaches (Ahmad et al., 1995; Ngoh et al., 1998), mosquitoes (Watanabe et al., 1993), stored-product insects (Shaaya et al., 1991), ticks (Lwande et al., 1999) and house flies (Singh and Singh, 1991). Bultman et al. (1979) tested 42 tropical African woods and suggested that volatile allelochemicals from the wood might be one of the major factors for their natural resistance to *Coptotermes formosanus* Shiraki. Other studies confirmed that some essential oils, such as that extracted from cedarwood (Adams, 1991), *Litsea cubeba* (Lin and Yin, 1995a) and *Cinnamomum* spp. (Lin and Yin, 1995b), were naturally repellent to termites. Some essential oil components from resistant wood species also have been reported to be toxic to termites, including chamaecynone, an acetylenic terpenoid from *Chamaecyparis pisidera* D. Don (Saeki et al., 1973), 7-methyljuglone, a naphthoquinone from *Diospyros virginiana* L. (Carter et al., 1978), and torreyal from, *Torreya nucifera* Sieb. et Zucc., Kaya wood (Ikeda et al., 1978). A number of diverse terpenoids in essential oils, such as citronellal, citral, gernaniol and eugenol have repellent activity against the American cockroach (Ngoh et al., 1998) and the Formosan subterranean termite (Cornelius et al., 1997 and Sharma et al., 1994). Scheffrahn et al. (1988) isolated ferruginol, manool, and nezukol from bald cypress, *Taxodium distichum* L. wood, which acted principally as a feeding deterrent to *C. formosanus*. Nagnan and Clement (1990) reported on the topical toxicity of geranyllinalool to termites. Grace and Yamamoto (1994) found that Formosan subterranean termites avoided feeding on Alaskan yellow cedar. A major extractive in Alaskan yellow cedar is a sesquiterpene, nootkatone (Erdtman and Hirose, 1962). Recently, we found this chemical also was a component of vetiver grass oil and demonstrated it to have strong repelling activity against the Formosan subterranean termite (Zhu et al., 2001). In this study, the toxicity and repellency of 8 selected essential oils to the Formosan subterranean termite were evaluated and the volatility of their major compounds were compared by gas chromatography-mass spectrometry (GC-MS).

METHODS AND MATERIALS

Oils of vetiver (*Vetiveria zizanioides*, Haiti), cassia leaf (*Cinnamomum cassia*, Chinese), clove bud (*Eugenia caryophyllata*, Indonesia), cedarwood (*Juniperus virginiana*, Virginia), *Eucalyptus globulus*, *Eucalyptus citrodora*, lemongrass

(*Cymbopogon citrates*, East Indian) and geranium (*Geranium pelergonium graveolens*, Bourbon) were purchased from The Good Scents Company (TGSC, Oak Creek, WI). Cinnamaldehyde, thujopsene, eugenol, eucalyptol, citronellal, citral and citronellol were purchased from the Fluka Company (Milwaukee, WI). Vetivones (α- and β-) were kindly provided by Professor Ekkhard Winterfeldt (Institut für Organische Chemie, Technische Universität, Berlin).

Formosan subterranean termites were collected in a fallen Oak tree from New Orleans, Louisiana, and stored in a 250 liter container at 24-28°C in the laboratory. The mean weight of a worker termite (3^{rd} instar or older) was 1.2 ($\pm$0.01) mg.

Repellency Assay. The testing procedure was modified from Lewis et al. (1978). A Petri dish (5 cm in dia. and 1 cm high containing 20 ml air) was used for testing the toxicity and repellency of the essential oils against workers of the Formosan subterranean termite. Essential oils were dissolved in ethyl alcohol and prepared in a series of dilutions for testing. One ml of hot agar solution (1.5 g agar in 100 ml H_2O) was spread evenly in the bottom of each dish and allowed to cool. This ensured adequate moisture for the termites and helped to hold the sand in place that was layered above it. Blasting sand (fine, #4) was autoclaved for 30 min and oven-dried. The top half agar layer was covered with 1 g of treated sand and a piece of treated filter paper (Whatman #1; 0.5 cm $\times$ 1 cm). The other half was covered with 1 g of untreated sand and a piece of untreated filter paper (0.5 cm $\times$ 1 cm). Enough sand was used to cover the agar completely, but thin enough to not conceal the termites once introduced. Three concentrations of treated sand/filter paper were evaluated for each of the 8 oils tested (100 μg/g sand or 10 μg/cm^2, 250 μg/g sand or 25 μg/cm^2 and 500 μg/g sand or 50 μg/cm^2). There were 3 replicates for each oil at each concentration tested. Three replicates for the controls (untreated sand and filter paper) were set up. This experiment was repeated twice.

Ten worker termites were added to each Petri dish, and all Petri dishes were covered between observations to eliminate the light. Every 15 minutes for the first hour, termite activity was recorded by summing three scan samples (each reading taken within 3 seconds) in 5 minutes intervals per Petri dish on the number of termites observed on untreated sand/filter paper. After the first hour, termite activity was recorded each hour (three scans in 5 minute intervals) until 6 hr, then counted every 24 hours (three scans in 5 minute intervals) until four weeks. A Chi-Square test indicated that when 23 or more of the 30 termites (mean value of all replicates) were observed on the untreated sand, there was a significant difference in the repellency to termites between the oil-treated group and the control group ($x^2 = 4.59$, $df = 1$, $p = 0.032$) (Anonymous, 1998). Then, the oil was considered repellent. The oil was considered toxic if some or all termites behaved sluggishly, were moribund, or dead. When 29 of the 30 (mean value of all replicates) termites for a given concentration of a tested oil were either dead or moribund, the test was ended.

Tunneling Response, Consumption, and Mortality. Vetiver oil was further tested to evaluate its effect on tunneling response, paper consumption, and mortality of Formosan subterranean termite workers. Three-compartment plastic containers ($18 \times 8 \times 4$ cm) (Pioneer Packaging Co, North Dixon, KY) were used. A small opening (0.3 cm high and 5 cm long) was made at the bottom of each of the two inner walls to permit termite travel between the chamber compartments. Vetiver oil (50 mg) was dissolved in ethanol (10 ml) as the stock solution. Four concentrations (5, 10, 25, and 50 μg/g sand) and one control (0 μg/g sand) were prepared as follows: 500 g of blasting sand for each concentration was mixed with vetiver oil in 25 ml of ethanol containing 2.5 mg, 5 mg, 12.5 mg and 25 mg of vetiver oil in a glass pan and dried in a hood for two hours. The control was treated only with 25 ml ethanol. Then, 100 g of treated sand were added to the middle compartment of the bioassay chamber. One hundred grams of untreated blasting sand were added to one end of each compartment. Four replicates were set up for each treatment. Filter paper (Whatman #1; 2.3 cm dia.) that served as the food source was dried at 70°C for 3 hr and cooled to room temperature for 30 min before weighing. Filter paper and 50 g of untreated sand were placed in the third compartment farthest from the home compartment. Ten ml of distilled water were added to the sand in each compartment just prior to introduction of the termite. Fifty workers and 5 soldiers were placed in the home compartment. The containers were covered with lids and kept in a dark incubator at 25°C. On the 14th day, the experimental units were dismantled, living termites counted, and filter papers cleaned, dried at 70°C for 3 hr, cooled for 30 min, and weighed. The tunnels constructed along the bottom of the chambers in the sand were copied by a scanner for measurements of total tunnel length. A pre-measured string was placed over the curved tunnel paths viewed by the scanner for length measurements. Consumption was calculated as the difference between the weight of filter paper before and after testing. Tunneling response, paper consumption, and mortality of termites were analyzed by ANOVA (Anonymous, 1998). Tukey's studentized range test was used to compare the difference between treatments.

GC-MS Analysis. The major components of the 8 oils investigated were determined by GC-MS through identification of major peaks using the computer database with follow-up confirmation using authentic compounds. The percentage of the major component was calculated by integrating the peaks of the gas chromatographs for each essential oil. Gas chromatography-mass spectrometry (GC-MS) experiments were performed on a Finnigan's GCQ (Trace GC 2000 coupled with Polaris MSD). A silica capillary column DB-5ms (30 m $\times$ 0.25 mm $\times$ 0.25 μm, J&W Scientific, Folsom, CA) was used for separation of the chemicals. The carrier gas was helium (0.8 ml/min). The injection port temperature was at 250°C in splitless mode with 1 μl injection. The initial GC temperature was maintained at 60°C for 1 min, then increased to 150°C at 2.5°C/min and maintained for 15 min, then to 260°C at 5°C/min, and finally maintained at 260°C for 10 min.

The ion-trap mass selective detector was set in full scan mode from m/z 41 to 400.

RESULTS

Repellency. All of the essential oils exhibited some repellency at the lowest concentration tested, i.e., 10 μg/cm^2 (Table 1). Clove bud oil was the most toxic, causing mortality of over 50% at a concentration of 50 μg/ml air in 24 hours. Oil of *Eucalyptus globulus* had the least repellent activity. It lasted for only 4-5 hr at 10 μg/cm^2 and 12 hr at 50 μg/cm^2 and was not toxic. Oil of *Eucalyptus citriadora* was the second weakest repellent, with an effective period of 0.3-1 days. The effective period for lemongrass and geranium was 1-3 days at the concentration of 10 to 50 μg/cm^2. Oils of cedarwood and cassia leaf had better repellent effects. The repelling period was 3-6 days at the concentrations tested. Vetiver oil had the longest activity with a repelling period of 12-24 days.

Tunneling Response, Consumption, and Mortality. Mean consumption of filter paper was decreased as vetiver oil concentration increased ($F = 133.84$, $df = 4, 15$; $P < 0.0001$) (Table 2). Filter paper consumption was not observed when concentrations were $\geq$25 μg/g sand. Termite mortality was not different among

TABLE 1. COMPARISON OF EFFECTIVE PERIOD AND TOXICITY OF 8 ESSENTIAL OILS AGAINST WORKERS OF THE FORMOSAN SUBTERRANEAN TERMITE

Oil	Time beginning the effectiveness[a] (min)	Effective period (days)[b] oil concentration (μg/cm^2) on sand			Mortality
		10	25	50	
Vetiver	<15–60	12	17	24	*c*
Cassia leaf	<15–60	3	4	4	*c*
Clove bud	<15–60	3	30% dead in 2 days and 100% dead in 4 days	50% dead in 1 day and 100% dead in 2 days	yes
Cedarwood	<15–60	3	4	6	*c*
Eucalyptus globulus.	60–120	0	<0.2	<0.3	No
Eucalyptus citrodora.	30–120	<0.5	<0.3	1	No
Lemongrass	<15–30	1	2	3	*c*
Geranium	<15–60	1	2	3	*c*

[a] The range of time included all three concentrations.
[b] Effective period defined as the time when 23 or more of the 30 termites (mean value of all replicates) were observed on the untreated sand. At that time, the oil was considered repellent (see Methods and Materials).
[c] Termites were gathered and sluggishly moved during the effective period.

TABLE 2. FILTER PAPER CONSUMPTION, PERCENT MORTALITY, AND LENGTH OF TUNNELING OF FORMOSAN SUBTERRANEAN TERMITES AFTER A 14-DAY EXPOSURE TO VETIVER OIL

Concentration of Vetiver oil (μg/g sand)	Consumption of filter paper (mg)[a]	% Termite mortality[a]	Tunneling length[a] (cm)
0	64.05 ± 6.85 a	13.50 ± 4.03 a	39.75 ± 3.18 a
5	15.80 ± 8.35 b	21.33 ± 2.08 a	27.87 ± 2.86 ab
10	8.65 ± 5.05 bc	13.00 ± 4.66 a	19.87 ± 4.52 b
25	0.0 ± 0.0 c	13.20 ± 3.78 a	0.00 ± 0.00 c
50	0.0 ± 0.0 c	15.50 ± 9.32 a	0.00 ± 0.00 c

[a]Means ± SE: Means with the same letter were not significantly different using Tukey's studentized range test.

the concentrations of vetiver oil tested ($F = 0.35$, $df = 4, 15$; $P = 0.840$). Vetiver oil decreased termite tunneling activity at concentrations as low as 10 μg/g sand ($F = 39.40$, $df = 4, 15$; $P < 0.0001$). Tunneling was not visible in the middle chamber when the concentration of vetiver oil was ≥ 25 μg/g sand (Table 2).

GC-MS Analysis. Cinnamaldehyde, vetivone, thujopsene, and monoterpenoids (citronellal, citronellol, eugenol, eucalyptol, and citral) were the major components of the tested essential oils (Table 3). Gas chromatography of these semiochemicals indicated that eucalyptol was retained the least (10.41 min), followed by citronellal (16.07 min), citral (20.41 min), citronellol (21.10 min), cinnamaldehyde (22.07 min), eugenol (26.18 min) and thujopsene (30.18 min) (Figure 1). Vetivone (α- and β-) eluted at 55.59 and 54 min., respectively. Therefore, the decreasing order of volatility was: eucalyptol > citronellal > citral ≥ citronellol > cinnamaldehyde > eugenol > thujopsene > α-, β- vetivone.

TABLE 3. THE MAJOR COMPONENTS AND THEIR PERCENTAGES IN ESSENTIAL OILS

Essential oil	Major component	Percentage	Reference[a]
Vetiver	α-, β-vetivone	14	Jain et al., 1982
Cassia leaf	Cinnamaldehyde	81	Lin and Yin, 1995a,b
Clove bud	Eugenol	66	Cornelius et al., 1997
Cedarwood	Thujopsene	34	Adams, 1991
Eucalyptus globules.	Eucalyptol	82	Perrucci, 1995
Eucalyptus citrodora.	Citronellal	71	Cornelius et al., 1997
Lemongrass	Citral	25	Ngoh et al., 1998
Geranium	Citronellol	20	Cornelius et al., 1997

[a]References indicate authors that described major components to have insect-repelling activity.

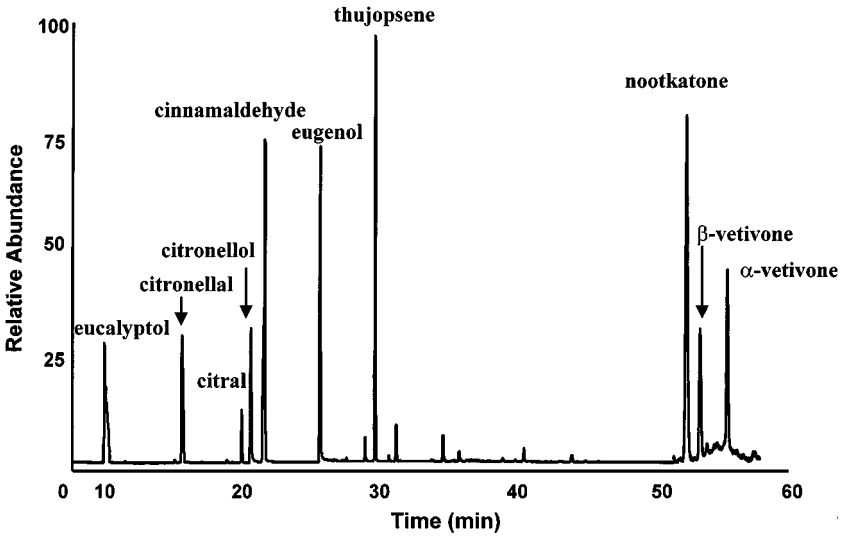


FIG. 1. Gas chromatography of a mixture of nine semiochemicals, those were determined as major components of the 8 essential oils studied.

DISCUSSION

Vetiver oil consists of a complex mixture of more than 300 compounds. Most of these substances belong to the sesquiterpene group of natural products. Among them, α- and β- vetivones are the major components in vetiver oil (St. Pfau and Plattner, 1939). Jain et al. (1982) reported that at least six compounds (α-, β-vetivone, khusimone, zizanal, epizizanal and (+)-(1S, 10R)-1,10-dimethylbicyclo [4,4,0]-dec-6-en- 3-one) were repellent to insects. Recently, we found three other vetiver oil compounds that were repellent to the Formosan subterranean termite including: nootkatone (Zhu et al., 2001), zizanol and bicyclovetivenol (Zhu et al., unpublished). From comparison of the repellent activities of the 8 essential oils (Table 1), we discovered that compound volatility was inversely proportional to repellent effectiveness. Vetivones (α- and β-) were the least volatile and the most effective repellents. The volatility of nootkatone is similar to α- and β- vetivone.

Although thujopsene, eugenol, cinnamaldehyde, citronellal, citral and citronellol have repelling activity against termites (Cornelius et al., 1997, Lin and Yin, 1995b, Adams, 1991), this activity only lasted for a short period of time. We believe that the long-lasting repelling activity of vetiver oil is based on its low volatility.

Essential oils or semiochemicals with low volatility may allow for a longer repelling period. Gas chromatography retention times of such chemicals may be

a useful parameter for developing effective and long-term repellents against the Formosan subterranean termite. Finding new termite-active compounds in essential oils is a direction we are now pursuing.

Acknowledgments—We are grateful to Drs. D.J. Boethel, D. Ring, and M. Stout for a review of this paper. This work was supported by a Specific Cooperative Agreement with the USDA-ARS in New Orleans. This project was approved for publication by the Louisiana State University Agriculture Center and Louisiana Agricultural Experiment Station as manuscript # 00-17-0401.

REFERENCES

ANONYMOUS. 1998. SAS user's guide: Statistics. SAS Institute, Cary, NC.

ADAMS, R. P. 1991. Cedar wood oil-analysis and properties. *Mod. Meth. of Plant Anal.* 12:159–173.

AHMAD, F. B. H., MACKEEN, M. M., ALI, A. M., MASHIRUN, S. R., and YAACOB, M. M. 1995. Repellency of essential oils against the domiciliary cockroach, *Periplaneta americana. Insect Sci. Applic.* 16:391–393.

BULTMAN, J. D., BEAL, R. H., and AMPONG, F. F. K. 1979. Natural resistance of some tropical African woods to *Coptotermes formosanus* Shiraki. *For. Prod. J.* 29:46–51.

CARTER, F. L., GARLO, A. M., and STANELY, J. B. 1978. Termiticidal components of wood extracts: 7-methyljuglone from *Diospyros virginiana. J. Agri. Food Chem.* 26:869–873.

CORNELIUS, M., GRACE, J. K., and YATES III, J. R. 1997. Toxicity of monoterpenoids and other natural products to the Formosan subterranean termite. *J. Econ. Entomol.* 90:320–325.

ERDTMAN, H., and HIROSE, Y. 1962. The chemistry of the natural order Cupressales: 46. The structure of nootkatone. *Acta Chem. Scand.* 16:1311–1314.

FRANZIOS, G., MIROTSON, M., HATZIAPOSTOLOU, E., KRAL, J., SCOURAS, Z. G., and MAVRAGANI-TSIPIDOU, P. 1997. Insecticidal and genotoxic activities of mint essential oils. *J. Agric Food Chem.* 45:2690–2694.

GRACE, J. K., AND YAMAMOTO, R. T. 1994. Natural resistance of Alaska-Cedar, redwood, and teak to Formosan subterranean termites. *Forest Prod. J.* 44:41–45.

IKEDA, T., TAKAHASHI, M., NISHIMOTO, K. 1978. Antitermitic components of Kaya wood, *Torreya nucifera* Sieb. Et Zucc. *Mokuzai Gakkaishi.* 24:262–266.

JAIN, S. C., NOWICKI, S., EISNER, T., and MEINNALD, J. 1982. Insect repellents from vetiver oil: 1. Zizanal and epizizanal. *Tetra. Let.* 23:4639–4642.

LEWIS, D. L., MICHAELS, G. E., HAYS, D. B., CAMPBELL, W., and SMITH, V. 1978. Evaluation of the antitermitic activity of hydroxyquinoline and naphthol derivative formulations using *Reticulitermes* in laboratory and field experiments. *J. Econ. Entomol.* 71:818–821.

LIN, T.-S., and YIN, H.-W. 1995a. Effects of *Litsea cubeba* pres oils on the control of termite *Coptotermes formosanus* Shiraki. *Taiwan For. Res. Inst. New Series* 10:59–63.

LIN, T.-S., and YIN, H.-W. 1995b. The effects of *Cinnamomum* spp. oils on the control of the termite *Coptotermes formosanus* Shiraki. *Taiwan For. Res. Inst. New Series* 10:459–464.

LWANDE, W., NDAKALA, A. L., HASSANALI, L., MOREKA, E., NDUNGU, M., AMIANI, H., GIYU, P. M., MALONZA, M. M., and PUNYUA, D. K. 1999. *Gynandropsis gynadra* essential oil and its constituents as tick (*Rhipicephalus appendiculatus*) repellents. *Phytochemistry* 50:401–405.

NAGNAN, P., and CLEMENT, J. L. 1990. Terpenes from the maritime pine *Pinus pinaster*: toxins for subterranean termites of the genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Biochem. Syst. Ecol.* 18:13–16.

NGOH, S. P., CHOO, L. E., PANG, F. Y., HUANG, Y., KINI, M. R., and HO, S. H. 1998. Insecticidal and repellent properties of nine volatile constituents of essential oils against the American cockroach, *Periplaneta americana* (L.). *Pestic Sci.* 54:261–268.

PERRUCCI, S. 1995. Acaricidal activity of some essential oils and their constituents against *Tyrophagus longior*, a mite of stored food. *J. Food Prod.* 58:560–563.

SAEKI, I., SUMIMOTO, M., and KONDO, T. 1973. The termiticidal substances from the wood *Chamaecyparis pisifera* D. Don. *Holzforschung.* 27:93–96.

SCHEFFRAHN, R. H., HSU, K. C., SU, N. Y., HUFFMAN, J. B., MIDLAND, S. L., and SIMS, J. J. 1988. Allelochemical resistance of bald cypress, *Taxodium distichum*, heartwood to the subterranean termite, *Coptotermes formosanus. J. Chem. Ecol.* 14:765–776.

SHAAYA, E., RAVID, U., PASTER, N., JUVEN, B., ZISMAN, U., and PISSAREV, V. 1991. Fumigant toxicity of essential oils against four major stored-product insects. *J. Chem. Ecol.* 17:499–504.

SHARMA, R. N., TUNGIKAR, V. B., PAWAR, P. V., and VARTAK, P. H. 1994. Vapour toxicity and repellency of some oils and terpenoids to the termite, *Odontotermes brunneus. Insect Sci. Applic.* 15:495–498.

SINGH, D., and SINGH, -A. K. 1991. Repellent and insecticidal properties of essential oils against housefly, *Musca domestica* L. *Insect. Sci. Applic.* 12:487–491.

ST. PFAU, A., and PLATTNER, P. 1939. Etudes sur les matieres vegetales volatiles X. Sur les vetivones, constituents odorants des essences de vetiver. *Helv. Chim. Acta* 22:640

WATANABE, A. K., SHONO, Y., KAKIMIZU, A., MATSUO, N., SATON, A., and NISHIMURA, H. 1993. New mosquito repellent from *Eucalyptus camaldulensis*. *J. Agric. Food Chem.* 41:2164–2166.

ZHU, B. C. R., HENDERSON, G., CHEN, F., MAISTRELLO, L., and LAINE, R. A. (2001). Nootkatone is a repellent for Formosan subterranean termite (*Coptotermes formosanus*). *J. Chem. Ecol.* 27:523–531.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

Zanthoxylum piperitum, AN ASIAN SPICE, INHIBITS FOOD INTAKE IN RATS

GISELA EPPLE,* BRUCE P. BRYANT, IGOR MEZINE, and STANLEY LEWIS

Monell Chemical Senses Center, 3500 Market St., Philadelphia PA 19104

(Received December 19, 2000; accepted April 4, 2001)

Abstract—We investigated the effects of a total extract from *Zanthoxylum piperitum* fruit, of the volatile components of extract, and of a non-volatile fraction containing the major alkylamides of *Zanthoxylum* (NVA) on food intake in rats. In Experiment 1, three groups (A, B, C) of mildly food deprived rats were given one hour long feeding trials during which they were offered choices between an oat-bran wafer paired with vegetable oil and a wafer paired with a *Zanthoxylum* preparation in vegetable oil. Trials were divided into three blocks of two tests each. During Blocks 1 and 3, two wafers coated with vegetable oil were offered to establish baseline consumption. During Block 2, Group A was given choices between wafers coated with oil and wafers coated with total extract. Group B was offered oil coated wafers and wafers coated with the NVA fraction. Group C received two oil coated wafers. One was served on top of a screened dish containing a sample of total extract. In this manner, the rats were exposed to volatile compounds emanating from the extract but could not contact the extract. The second wafer was served on top of a screened sample of oil. Results: In Blocks 1 and 3 trials, the rats fed indiscriminately from both wafers. During Block 2, total extract, the NVA fraction and the volatile compounds from extract all significantly reduced food consumption. In Experiment 2, habituation to the NVA fraction and to the volatile constituents of extract was examined in two additional groups (D, E), using the methods described above. Baseline consumption was tested in Blocks 1 and 3 by offering two oil coated wafers. During Block 2, group D was given 10 trials with oil coated and NVA-fraction coated wafers. Group E was given 10 trials with oil coated wafers paired with the volatile constituents of total extract and with those of oil. Results: Both groups failed to habituate to the *Zanthoxylum* stimuli. Wafers treated with the NVA fraction and wafers paired with the volatile constituents of extract were avoided throughout the 10 test days.

Key Words—Zanthoxylum, secondary plant metabolites, rats, feeding deterrents.

*To whom correspondence should be addressed.

INTRODUCTION

Many plant species produce secondary metabolites such as phenolics, alkaloids and terpenoids in defense against herbivores. These compounds may be toxic, interfere with metabolic processes when ingested, or may act as sensory irritants, causing some form of pain or irritation. Numerous studies have shown that mammalian herbivores avoid eating defended plant parts or, at least, reduce their consumption (Haslam, 1988; Harborne, 1991; McArthur et al., 1991; Palo and Robbins, 1991; Langenheim, 1994; Lawler et al., 1996). Because of this, the potential of secondary plant metabolites as feeding deterrents has received considerable attention as evidenced, for example, by a recent volume on repellents in wildlife management (Mason, 1997). Secondary metabolites, that cause irritation or pain are of particular interest in this respect since animals are unlikely to habituate to these sensations, while they often do so in response to olfactory and gustatory stimuli (Beauchamp, 1997). Thus, non-lethal feeding deterrents based on sensory irritants could be useful for managing herbivore damage in agricultural and forestry settings without danger of non-targeted toxicosis.

Recently, Bryant and Mezine (1999) studied the irritating properties of Szechuan pepper (*Zanthoxylum piperitum*, Rutaceae). This and several other species of *Zanthoxylum*, have been used for centuries as spices in Asian cuisine (Pfänder and Frohne, 1987; Bryant and Mezine, 1999), are employed in traditional Asian medicine (Shibata et al., 1999), and are used as insecticides and insect feeding deterrents (Jacobson and Crosby, 1971; Dube et al., 1990; Bower et al., 1993). Some species contain toxic compounds such as alkaloids, lignanes, furanocoumarin and 8-methoxypsoralen (Pfänder and Frohne, 1987).

In humans, fruits and other parts from *Zanthoxylum piperitum* are strong oral stimuli producing pronounced tingling and cooling sensations (Bryant and Mezine, 1999), which presumably account for its use as a condiment in some Asian cuisines. The extract from the fruit of *Zanthoxylum piperitum* is chemically complex (Yasuda et al., 1982; Pfänder and Frohne, 1987; Kashiwada et al., 1997; Bryant and Mezine, 1999). Many of its components are volatile. These include limonene, α-terpineol, linalool, citral, citronellal, cineol dipentene, and geraniol (Pfänder and Frohne, 1987). Many of these constituents, when delivered in vapor phase, produce ocular irritation in humans (Doty et al., 1978) and stimulate nasal trigeminal nerve fibers in the rat (Bryant and Silver, 2000). In addition to these compounds, a number of non-volatile alkylamides are present (Yasuda et al., 1982; Greger, 1984; Kashiwada et al., 1997).

At least one of the alkylamides, α-HO-sanshool, causes a strong tingling sensation, similar to a mild electric shock, as well as cooling, when applied to the human tongue (Bryant and Mezine, 1999). Using an animal model, the rat, Bryant and Mezine (1999) have shown that HO-α sanshool (2,6,8,10 dodecatetraenoic acid 2-hydroxypropyl amide), induces discharges in trigeminal sensory neurons.

Extracellular recordings from the lingual trigeminal nerve of the rat have shown that HO-α sanshool induces firing in tactile and cool sensitive neurons as well as sensitivity to innocuous stimuli in previously insensitive neurons (Bryant and Mezine, 1999). Moreover, assays of neuronal function that measured induced changes in intracellular calcium, using digital fluorescence imaging of cultured rat trigeminal neurons, showed that the majority of neurons that respond to HO-α sanshool does not belong to the major class of chemically sensitive pain receptive neurons, the polymodal nociceptors. These latter neurons are characterized by their sensitivity to capsaicin, the pungent compound in *Capsicum* peppers that induces heat and pain (Bryant, 1997).

The studies by Bryant and Mezine (1999) suggest that the oral sensations caused by Szechuan pepper depend, at least in part, on neurons other than the trigeminal polymodal nociceptors, which may mediate sensitivity to most irritants (Bryant, 1997). The effects on these neurons would suggest the induction of enhanced and novel oral sensitivity. Given these facts, it is worthwhile to evaluate the effects of *Zanthoxylum*-derived stimuli on food consumption and their potential as feeding deterrents for mammals. Because of the unique oral sensation that HO-α sanshool causes in humans, the alkylamides are of particular interest. The present paper describes studies on an omnivorous rodent, the laboratory rat. Studies with herbivorous rodents (guinea pig, voles) are in progress and will be reported in due course. Taken together, these studies might provide the basis for the development of feeding repellents that target a population of trigeminal neurons not targeted by currently known repellents.

METHODS AND MATERIALS

Preparation of Total Extract. Ripe fruit from *Zanthoxylum piperitum* was obtained from the Morris Arboretum of the University of Pennsylvania. Dried pericarps (5 kg) were extracted twice for 10 hours with ethylacetate (1:4; w : v). The combined extracts were evaporated *in vacuo* to produce a dark brown oil (440 g) that had a strong, characteristic odor for the human nose. We refer to this preparation as the "total extract". This extract constitutes 8.8% of the dry weight of the *Zanthoxylum* fruit.

Preparation of the Non-Volatile Fraction. A fraction containing only the non-volatile alkylamides was prepared. The total extract was re-extracted three times with 1.5 l of a methanol : water mixture (4:1). The upper water-methanol phases were combined and concentrated *in vacuo*. The resulting suspension was extracted four times with 200 ml of methylene chloride. The lower phases were combined, dried over anhydrous sodium sulfate and evaporated, yielding 170 g of a brown oil with a characteristic odor. This material was extracted five times with 100 ml of a mixture of hexane : ethyl acetate : ethanol (40:10:5). The upper organic phases

were filtered through a column (20 × 3 cm) packed with silica gel (40/63 mm, Davisil). Evaporation of the filtrate resulted in 120 g of brown oil with a weak characteristic odor.

The efficacy (>97%) of extraction of the alkylamides was confirmed at each step by analytical reverse-phase high performance liquid chromatography (HPLC). The material (3 × 40 g) was chromatographed using flash chromatography on a silica-gel (Merck 40/63 μ) packed column (50 × 4 cm) with 5% ethanol in hexane as mobile phase. Fractions were collected by volume and analyzed by high performance thin layer chromatography (HPTLC) [Kieselgel F_{254}. Merck; 10% EtOH in hexane]. Fractions containing hydroxy-alkylamides ($R_F = 0.5$) were combined and evaporated *in vacuo* to yield the total alkylamides (25 g) as a light-yellow odorless oil.

The final purification and removal of volatile impurities was achieved by reverse phase preparative HPLC (RP-HPLC). Conditions of RP-HPLC separation were as follows. Column: Zorbax C18 (Varian, Palo Alto) 10 mm, 250 × 22.4 mm; mobile phase: acetonitrile:methanol:water (27:38:35); flow 22 ml/min; sample size 200 mg in 0.5 ml of mobile phase. The eluent containing the hydroxy—alkylamides was concentrated *in vacuo* two times and the compounds were extracted using solid-phase extraction on C18 bonded silica gel (Sigma). Adsorbed compounds were eluted with ethanol, dried over anhydrous sodium sulfate and evaporated *in vacuo* to produce colorless crystals (yield 70%). We refer to this as the non-volatile alkylamide fraction (NVA fraction). The material is stable in ethanol solution (25 mg/ml) at -20°C under nitrogen for three months. For the human subject, the NVA fraction evokes oral sensations that are very similar to those caused by ripe *Zanthoxylum* fruit (Bryant and Epple, personal observation).

Preparation of Feeding Stimuli. For the feeding trials, total extract was diluted with Crisco vegetable oil to yield a 10% solution. In the remainder of the paper, we refer to this as "the extract". The NVA fraction was also diluted with vegetable oil. The concentration in oil was adjusted to be equivalent to the concentration of NVA in a 10% oily solution of total extract.

Subjects. Laboratory rats (*Rattus norvegicus*) who had never experienced *Zanthoxylum* before served as subjects. All animals were adults. The rats were individually housed in polypropylene rat boxes (40 × 20 × 20 cm). Food and water were available *ad libitum*, except during short periods of food deprivation prior to testing (see below).

Test Cages. For the purpose of testing the subjects were transferred to rat boxes identical to their home cages. The boxes contained aspen shavings and a water bottle, but no food. The test cages were oriented on a shelf so that their narrow sides (20 cm) faced the experimenter.

Feeding Experiments. Responses to extract from *Zanthoxylum*, to the NVA fraction, and to volatile compounds emanating from extract were examined by

comparing consumption of oat bran wafers treated with one of these stimuli to consumption of wafers treated with Crisco vegetable oil, the control stimulus. The amounts of wafer consumed during two-choice feeding trials lasting one hour were the dependent variables. All subjects had been habituated to wafers coated with vegetable oil prior to the experiments.

Wafers were prepared from 200 g of Quaker oat bran, 40 g of sugar, 5 ml of molasses, and 200 ml of water. From this mixture, 25 round wafers were cut to a size of 4.5 cm diameter and 5 mm thickness with a cutter. They were punctured with a small standardized tool in order to make them more adsorbent for stimulus fluids. Wafers were then air dried for 24 hr at room temperature. The dried wafers were hard enough so that the rats had to gnaw them rather than bite off large chunks. Wafers for the entire experiment were prepared at one time and were frozen until needed for testing. Immediately before the start of trials the thawed wafers were dipped into the experimental and control stimulus fluids for two minutes.

Stainless steel dishes (5 cm $\times$ 5 cm $\times$ 3 cm deep) were used to serve the wafers. Each dish was covered with a stainless steel lid that had a round hole in the center (diameter 3 cm). The wafers were fastened to the lids with wire, covering the hole so that the rats could not access the inside of the dish. This made it possible to offer oil-coated wafers in close proximity to volatile compounds emanating from extract placed inside the dish (see below).

The subjects were mildly food deprived before testing. All animals were removed from their home cages and placed in individual test cages six hours prior to each feeding trial. They remained without food for these six hours. The rats were then presented with two pre-weighed stimulus wafers and allowed to feed freely for one hour, after which the wafers were removed and the amounts of wafer consumed were determined.

During feeding trials the subjects were given choices between two dishes, each containing a wafer. The dishes were placed in the center of the front and back of the chamber against the cage walls. The front-back position of all stimuli was counterbalanced across subjects and across trials to compensate for possible front-back preferences during feeding.

Statistical Analysis. For each group of rats, significant differences between the amounts of food consumed from the control and the experimental wafers were analyzed by two-way analysis of variance (ANOVA) with repeated measures. Factors in this analysis were wafer-coating and block (see below). Tukey's HSD Method and ANOVA were used to identify *post hoc* differences.

Experiment 1. In this experiment the effects of extract, the NVA fraction and the volatile components of the extract on food consumption were examined. Three groups (A, B, and C) of eight rats (4 males, 4 females) each were used. Each group was given two-choice feeding trials twice a week for a total of six trials. The six trials were divided into three blocks of two trials each. To establish baseline

TABLE 1. SUMMARY OF STIMULUS PRESENTATIONS FOR GROUPS A–E

Group	Treatment Block 1	# of trials	Treatment Block 2	# of trials	Treatment Block 3	# of trials
A	oil vs oil	2	oil vs extract	2	oil vs oil	2
B	oil vs oil	2	oil vs NVA	2	oil vs oil	2
C	oil vs oil	2	oil vs extract volatiles	2	oil vs oil	2
D	oil vs oil	2	oil vs NVA	10	oil vs oil	2
E	oil vs oil	2	oil vs extract volatiles	10	oil vs oil	2

consumption, Block 1 for each group consisted of two trials offering a choice between oil-coated, but otherwise untreated wafers (Table 1).

During Block 2, each group of rats received a different experimental stimulus. For group A, the second block consisted of two trials offering choices between oil coated-wafers and wafers coated with *Zanthoxylum* extract. Group B was given two trials offering choices between oil-coated wafers and wafers coated with the NVA fraction. For group C, Block 2 consisted of two trials with choices between two oil-coated wafers. One wafer was fastened to the lid of a dish containing a disk of filter paper (5 cm diameter) with 200 μl oil, the second wafer was fastened to the lid of a dish containing a filter paper disk with 200 μl extract. The rats were exposed to the volatile compounds emanating from the oil and the extract, but could not contact the filter paper inside the dishes.

For all groups, Block 3 again consisted of choices between two oil-coated wafers. To avoid cross-contamination by volatile compounds emitted by the different *Zanthoxylum* stimuli, the three groups were tested consecutively in a well ventilated room (3.5 $\times$ 3.5 m) containing no other animals (Table 1).

For analysis, individual means of food consumption were calculated. The amounts consumed by each rat from the control wafer were averaged over the two trials representing a block and compared to the average amounts consumed from the experimental wafer. This was done to compensate for potential front-back preferences in feeding location. For trials using two oil coated wafers (Blocks 1 and 3), the amounts consumed in the front of the cage were averaged and compared to the amounts consumed in back of the cage.

Experiment 2. In this experiment, the question whether or not rats will habituate to *Zanthoxylum*, and after repeated exposure will increase consumption of wafers treated with the NVA fraction or with volatile components of the extract was examined.

Two groups (D and E) of *Zanthoxylum*-naive rats served as subjects. Group D contained seven rats (4 males, 3 females), Group E 10 rats (5 males, 5 females). Each group was given three blocks of 2-choice trials. During Block 1, baseline

consumption was established by testing the rats in each group on two different days with choices between two oil-coated wafers (Table 1).

During Block 2, habituation to the NVA fraction or to volatile components of extract was tested. Group D was offered choices between an oil-coated wafer and a NVA-coated wafer twice weekly for a total of 10 trials, i.e., for 5 weeks. Group E was given 10 trials during which choices between two oil-coated wafers were offered twice a week. One wafer was fastened to the lid of a dish containing a disk of filter paper (5 cm diameter) with 200 μl oil, the second one was fastened to the lid of a dish containing a filter paper disk with 200 μl extract. As in Experiment 1, the rats were exposed to the volatile components of the extract and oil, but could not contact the filter paper. During Block 3, the rats in both groups were again given two trials with a choice between two wafers coated with oil (Table 1). As in Experiment 1, both groups were tested consecutively in a well ventilated room.

To illustrate consumption over repeated trials, a preference ratio was calculated for each trial in Block 2 by dividing the amount consumed from the wafer treated with the NVA fraction or the volatile compounds from extract by the total amount consumed from both wafers. For Blocks 1 and 3, the amount consumed in the front of the cage was divided by the total amount eaten. A preference ratio of 0.5 reflects equal acceptance of both wafers offered during a trial.

RESULTS

Experiment 1. For each group of rats, ANOVA revealed differences in the amounts of food consumed from the two wafers available during choice tests, and an interaction between food consumption and Block (Group A: $F = 6.14$, $P = .016$, P for interaction $= .001$; Group B: $F = 19.94$, $P = < .001$, P for interaction $= .01$; Group C: $F = 13.5$, $P = .001$, P for interaction $= .003$). Figures 1–3 show mean amounts consumed from oil-coated control wafers and from wafers coated with the experimental stimuli during the three trial blocks. *Post hoc* comparison showed that during Blocks 1 and 3, animals in all groups consumed similar amounts of food from both dishes, each containing an oil-coated wafer.

During Block 2, Group A exhibited a very strong avoidance of wafers treated with extract (Figure 1). Post hoc analysis revealed that the animals consumed much less of the extract-treated than the oil-treated wafers ($F = 36.5$, $P < .001$). Group B consumed less from NVA-coated wafers than from control wafers ($F = 10.94$, $P = 0.005$) [Figure 2]. Group C avoided wafers presented over the vapors emanating from extract present in the dish below to a similar degree as Group A avoided extract-coated wafers ($F = 47.34$, $P < .001$) [Figure 3]. During Block 3, when given a choice between two oil-coated wavers again, animals in all groups fed indiscriminately from both wafers. Consumption during Block 3 was in the same range as that exhibited during the first block of trials.

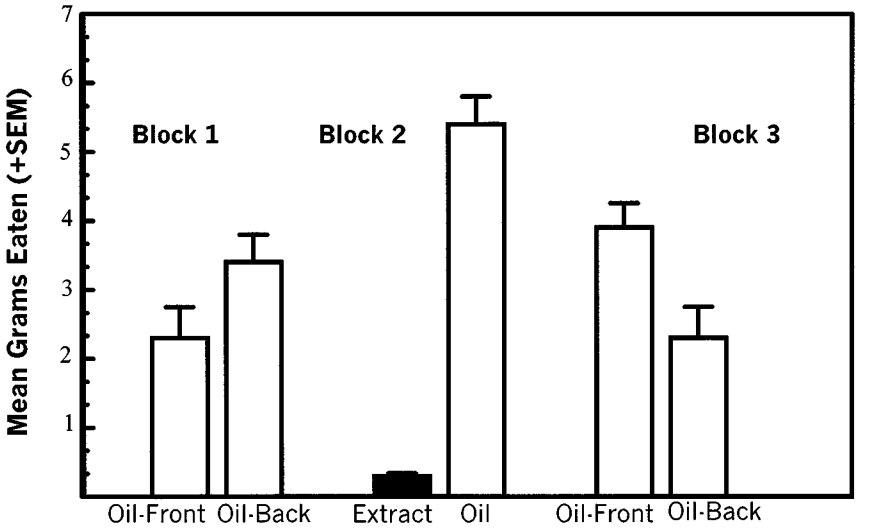


FIG. 1. Exp. 1. Amounts consumed by Group A from wafers coated with oil and wafers coated with extract during trial Blocks 1–3.

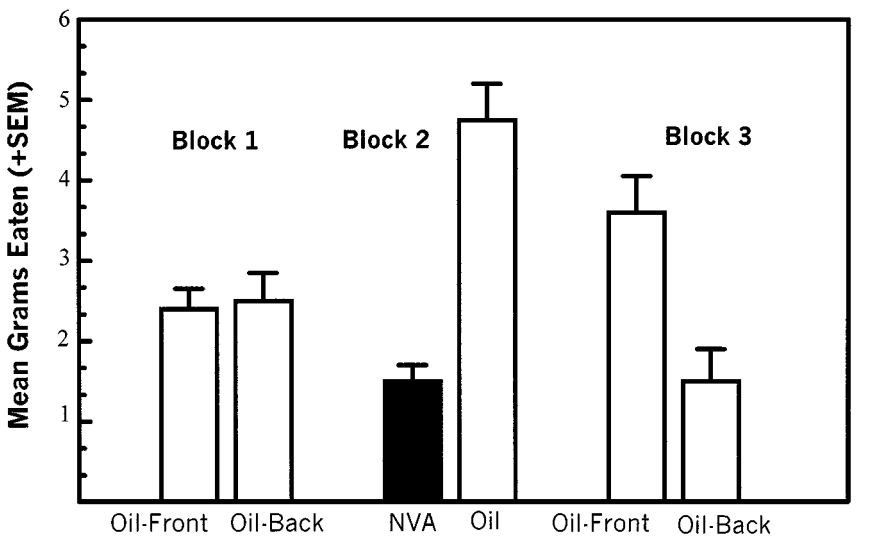


FIG. 2. Exp. 1. Amounts consumed by Group B from wafers coated with oil and wafers coated with the NVA fraction during trial Blocks 1–3.

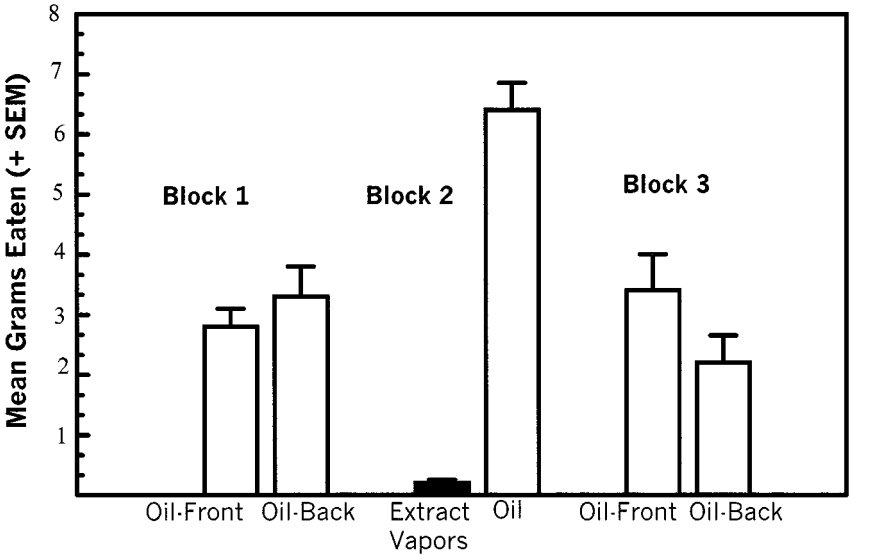


FIG. 3. Exp. 1. Amounts consumed by Group C from wafers served over a sample of oil and wafers served over a sample of extract during trial Blocks 1–3.

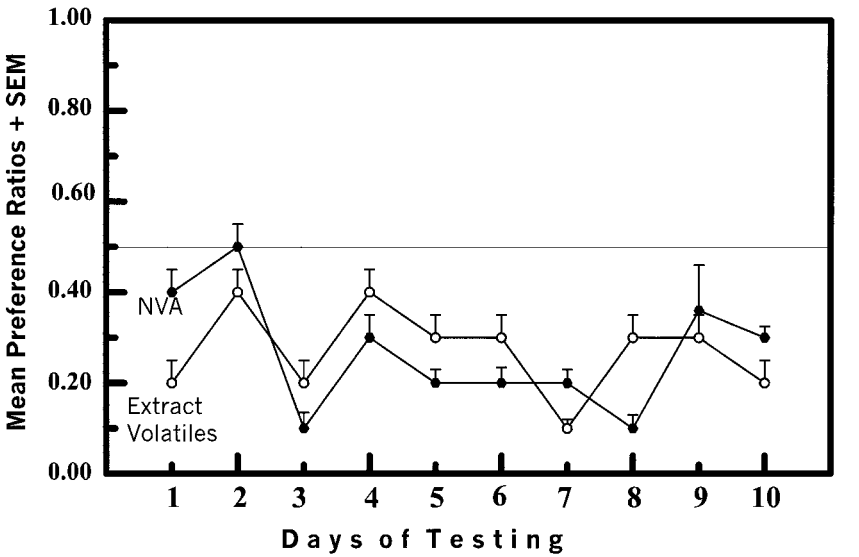


FIG. 4. Exp. 2. Daily preference ratios for trials offering choices between oil-coated wafers and wafers coated with the NVA fraction (Group D) and for trials offering choices between wafers served over a sample of oil and wafers served over a sample of total extract (Group E). The horizontal line marks the ratio of 0.5, reflecting a lack of preference.

For all trial blocks, the total amounts consumed from both dishes during choice tests were similar across blocks and between groups, suggesting that none of the stimuli reduced total intake (Group A: 5.9 ± .2 g; Group B: 5.8 ± .5 g; Group C: 6.0 ± .2 g).

Experiment 2. Figure 4 shows preference ratios obtained by Groups D and E over the 10 days of habituation testing. For both groups two-way ANOVA including trials in all blocks revealed differences in the amounts of food consumed from both dishes and an interaction between food consumption and trial days (Group D: $F = 16.83$, $P = < .001$, P for interaction $= < .001$; Group E: $F = 25.23$, $P = < .001$, P for interaction $= .02$). *Post hoc* 2-way ANOVA on the amounts of food consumed only during Block 2 trials showed an effect of wafer coating, but no interaction between wafer coating and days of testing (Group D: $F = 54.6$, $P = < .001$; Group E: $F = 42.3$, $P = < .001$). NVA coated wafers as well as wafers associated with vapors emanating from *Zanthoxylum* extract were avoided to a similar degree throughout the five weeks of testing. There were no differences in the amounts of food consumed from the two oil coated wafers in Blocks 1 and 3.

DISCUSSION

The results of the present study show that rats avoid food that has been treated with extract and fractions derived from the fruit of Szechuan pepper. In Experiment 1, rats strongly avoided the total extract, which is a complex mixture of volatile and non-volatile constituents (Bryant and Mezine, 1999; Kashiwada et al., 1997; Yasuda et al., 1982). Its volatile constituents alone, when present in close proximity to uncontaminated, palatable food, were as effective as the non-volatile constituents. Indeed, avoidance of wafers treated with the total extract may have largely been based on its volatile components.

Under the present trial conditions, where the rats had a choice between wafers coated with the experimental stimuli and with oil, none of the experimental stimuli affected the total amount of food consumed. They merely shifted consumption from wafers coated with experimental stimuli toward those coated with oil. Thus, *Zanthoxylum* compounds did not cause a generalized food aversion.

Figure 2 shows that the amount of food consumed from NVA treated wafers in Experiment 1 was higher than that consumed from wafers treated with extract and wafers served above a sample of extract. This does not necessarily indicate that the NVA fraction was less aversive to the rats than were extracts and extract vapors. As the result of the chromatographic process employed in its preparation, the NVA fraction does not contain volatile constituents. This was confirmed in behavioral studies with prairie voles. Choice tests, similar to those used in the present study, have shown that the voles avoided uncontaminated wafers associated

with volatile components of extract, but fed indiscriminately from wafers presented on top of dishes containing samples of NVA and wafers presented above samples of oil (Epple et al., unpublish data). Since NVA offers no volatile cues to its irritating effects, the rats probably needed to ingest at least some of the NVA coated wafer before perceiving its quality and responding with avoidance. In humans, there is a 1–2 minute latency between oral stimulation with NVA and the onset of oral irritation (Bryant and Mezine, 1999). In rats, there also is a 1–2 minute delay in the onset of trigeminal lingual nerve responses to oral stimulation with NVA (Bryant and Mezine, 1999). Therefore, in the present experiment, the rats may have ingested a relatively high amount of the treated wafers before they perceived the effect of NVA. The fact that there was no difference between the amounts of NVA treated wafer consumed on day 1 and day 2 of testing, or between preference ratios calculated for each day, also supports the assumption that the animals depended on oral stimulation to assess the aversive quality of NVA.

The results of Experiment 2 show that the avoidance of innocuous wafers associated with extract vapors and of wafers treated with the NVA fraction in Experiment 1 was not due to neophobia. Rats did not habituate to the volatile compounds emanating from *Zanthoxylum* extract or to the NVA fraction. Rather, they exhibited aversion over the entire 5 weeks of testing. Figure 4 shows that the rats tended to eat more of the NVA treated wafers on days 1 and 2 of testing than during the remainder of the trials. This might suggest that the animals learned to sample the wafers very carefully in order to detect the presence of NVA before proceeding to feed from a wafer. However, there was no significant decrease in consumption over time that would support such an assumption. *Post hoc* ANOVA on preference ratios did not reveal differences between days of testing.

It is interesting that the vapors emitted by the extract were so effective in reducing food consumption on a short term basis in Experiment 1, and remained effective in Experiment 2. On the first day of exposure to the volatile constituents of extract during Experiment 1, only one out of the eight rats consumed part of the wafer served on top of the dish containing extract. The remaining seven animals seemed not to have touched the food. Even when the rats were given a chance, during repeated presentation in Experiment 2, to discover that the wafers located above the extract samples were untainted, consumption remained reduced. The animals did not seem to habituate to these volatile compounds. This response is somewhat puzzling since the rats had plenty of opportunity to discover that the wafers were uncontaminated. It is conceivable that the persistant avoidance was not in response to compounds that only stimulated the olfactory system, but in response to irritation or pain mediated by the trigeminal system. Some volatile constituents of the extract are irritants for humans (Doty, 1978) and are known to stimulate nasal trigeminal nerve fibers in rats (Bryant and Silver, 2000). If the vapors emanating from the extract were concentrated enough to cause discomfort,

the rats would be unlikely to habituate to them, even if the food resources associated with the vapors would be more restricted than was the case in the present study. Experiments using anosmic rats and rats whose trigeminal A- and C-fibers have been desensitized with capsaicin could shed light on this question.

Some of the well documented avoidance responses of mammals to volatile constituents from plants may also be due to irritation rather that olfaction alone. Snowshoe hares (*Lepus americanus*) and Townsend voles (*Microtus townsendii*) avoid feeding from sources associated with the scent of pine oil extracted from pulp waste (Bell and Harestad, 1987). Snowshoe hares also avoid feeding on conifer seedlings to which odor dispensers with pinosylvin, a phenolic compound from Alaskan green alder (Clausen et al., 1986) have been attached (Sullivan et al., 1992). The scent of Siberian pine needle oil reduces food caching in pocket gophers (*Geomys bursarius, Thomomys talpoides*) [Epple et al., 1996, 1997]. Woodchucks (*Marmota monax*) avoid feeding on uncontaminated green pepper in the presence of volatile compounds from geranium oil and d-pulegone (Bean et al., 1997). The odors of crushed foliage from lodgepole pine (*Pinus contorta*) and Sitka spruce (*Picea sitchensis*), and of some of their monoterpene constituents, inhibit feeding in red deer calves (*Cervus alephs*) (Elliott and Loudon, 1987). Bell and Harestad (1987) have argued that the monoterpenes in essential oils from conifers have antimicrobial activity, which may interfere with digestive processes in cecal digestors and ruminants, and that such species avoid ingestion of defended conifer parts based on the characteristic odor of their essential oils.

Little seems to be known about which, if any, mammalian species exploit *Zanthoxylum* species as a food resource and what parts of the plants may be avoided. We were not able to find information on this question in the literature. *Zanthoxylum piperitum* trees at the Morris Arboretum, from which we obtained fruit for the present study, appear to be very well defended. The large white-tailed deer (*Odocoileus hemionus*) population at the Arboretum does not damage these trees, although heavy deer damage is incurred by many other trees and shrubs at this site. Intact ripe fruit does not emit an odor since its volatile compounds are encapsulated in oil glands in the pericarp. Any animal chewing the fruit, however, would immediately be exposed to its volatile and non-volatile components. The authors of this paper can attest to the fact that biting down on a single ripe fruit causes very unpleasant and long lasting oral sensations, which they are not eager to experience again. Thus, in summary it appears that the utility of some of the volatile and nonvolatile compounds of Szechuan pepper as feeding deterrents for mammals is worth exploring.

Acknowledgments—This research was supported in part by U.S. Department of Agriculture Cooperative Agreement #12-34-41-0040[CA] between the National Wildlife Research Center and Monell Chemical Senses Center and the Pennsylvania Department of Agriculture. We thank the Morris Arboretum of the University of Pennsylvania for the *Zanthoxylum piperitum* fruit.

REFERENCES

BEAN, N. J., KORFF, W., and MASON, J. R. 1997. Repellancy of plant, natural products, and predator odors to woodchucks, pp. 139–146, *in* J. R. Mason (ed.). Repellents in Wildlife Management. USDA National Wildlife Res. Center, Fort Collins, CO.

BEAUCHAMP, G. K. 1997. Chemical signals and repellancy: Problems and prognosis, pp. 1–10, *in* J. R. Mason (ed.). Repellents in Wildlife Management. USDA National Wildlife Res. Center, Fort Collins, CO.

BELL, C. M., and HARESTAD, A. S. 1987. Efficacy of pine oil as repellent to wildlife. *J. Chem. Ecol.* 13:1409–1417.

BOWERS, W. S., ORTEGO, F., YOU, X., and EVANS, P. H. 1993. Insect repellents from the Chinese prickly ash *Zanthoxylum buganeum. J. Nat. Prod.* 56:935–938.

BRYANT, B. 1997. Peripheral trigeminal neural processes involved in repellency, pp. 19–25, *in* J. R. Mason (ed.). Repellents in Wildlife Management. USDA National Wildlife Res. Center, Fort Collins, CO.

BRYANT, B. P., and MEZINE, I. 1999. Alkylamides that produce tingling paresthesia activate tactile and thermal trigeminal neurons. *Brain Res.* 842:452–460.

BRYANT, B. P., and SILVER, W. L. 2000. Chemesthesis: The common chemical sense, pp. 73–100, *in* T. E. Finger, W. L. Silver, and D. Restreppo (eds.). The Neurobiology of Taste and Smell. Wiley-Liss, N.Y.

CLAUSEN, T. P., REICHARDT, P. B., and BRYANT, J. P. 1986. Pinosylvin and pinosylvin methyl ether as feeding deterrents in green alder. *J. Chem. Ecol.* 12:2117–2131.

DOTY, R. L., BRUGGER, W. E., JURS, P. C., ORNDORFF, M. A., SNYDER, P. J., and LOWRY, L. D. 1978. Intranasal trigeminal stimulation from odorous volatiles: Psychometric responses from anosmic and normal humans. *Psychol. Behav.* 20:175–185.

DUBE, S., KUMAR, A., and TRIPATHI, S. C. 1990. Antifungal and insect-repellent activity of essential oil of *Zanthoxylum alatum. Ann. Botany* 65:457–459.

ELLIOTT, S., and LOUDON, A. 1987. Effects of monoterpene odors on food selection by red deer calves (*Cervus elaphus). J. Chem. Ecol.* 13:1343–1349.

EPPLE, G., NIBLICK, H., LEWIS, S., NOLTE, D. L., CAMPBELL, D. L., and MASON, J. R. 1996. Pine needle oil causes avoidance behavior in the pocket gopher *Geomys bursarius. J. Chem. Ecol.* 22:1013–1025.

EPPLE, G., NOLTE, D. L., MASON, J. R., ARONOV, E., and WAGER-PAGÉ, S. 1997. Behavioral responses to pine needle oil in the northern pocket gopher, pp. 157–167, *in* J. R. Mason (ed.). Repellents in Wildlife Management. National Wildlife Research Center, Fort Collins, CO.

GREGER, H. 1984. Alkamides: Structural relationships, distribution and biological activity. *Planta Medica* 1984:366–375.

HARBORNE, J. B. 1991. The chemical basis of plant defense, pp. 45–59, *in* R. T. Palo and C. T. Robbins (eds.). Plant Defenses Against Mammalian Herbivory. CRC Press Inc., Boca Raton.

HASLAM, E. 1988. Plant polyphenols (syn. vegetable tannins) and chemical defense—a reappraisal. *J. Chem. Ecol.* 14:1789–1805.

JACOBSON, M. and CROSBY, D. G. (eds.). 1971. Naturally Occuring Insecticides. Dekker, NY.

KASHIWADA, Y., Ito, C., KATAGIRI, H., MASE, I., KOMATSU, K., NAMBA, T., and IKESHIRO, Y. 1997. Amides of the fruit of *Zanthoxylum* spp. *Phytochemistry* 44:1125–1127.

LANGENHEIM, J. H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol.* 20:1223–1279.

LAWLER, I. R., FOLEY, W. J., ESCHLER, B. M., PASS, D. M., and HANDASYDE, K. 1996. Intraspecific variation in *Eucalyptus* secondary metabolites determined food intake by folivorous marsupials. *Oecologia* 116:160–169.

MASON, J. R. (ed.). 1997. Repellents in Wildlife Management. USDA National Wildlife Res. Center, Fort Collins, CO.

MCARTHUR, C., HAGERMAN, A. E., and ROBBINS, C. T. 1991. Physiological strategies of mammalian herbivores against plant defenses, pp. 104–114, *in* R. T. Palo and C. T. Robbins (eds.). Plant Defense against Mammalian Herbivory. CRC Press Inc., Boca Raton.

PALO, R. T., and ROBBINS, C. T. (eds.). 1991. Plant Defense against Mammalian Herbivory. CRC Press Inc., Boca Raton.

PFÄNDER, H. J., and FROHNE, D. 1987. Szechuan-Pfeffer. Die Früchte von Zanthoxylum piperitum DC. (Rutaceae). *Dtsch. Apoth. Ztg.* 127:2381–2384.

SHIBATA, C., SASAKI, I., NAITO, H., UENO, T., and MATSUNO, S. 1999. The herbal medicine Dai-Kenchu-Tou stimulates upper gut motility through cholinergic and 5-hydroxytryptamine 3 receptors in concious dogs. *Surgery* 126:918–924.

SULLIVAN, T. P., CRUMP, D. R., WIESER, H., and DIXON, E. A. 1992. Influence of the plant antifeedant, pinosylvin, on suppression of feeding by snowshoe hares. *J. Chem. Ecol.* 18:1151–1164.

YASUDA, I., TAKEYA, K., and ITOKAWA, H. 1982. Distribution of unsaturated aliphatic acid amides in Japanese *Zanthoxylum* species. *Phytochemistry* 21:1295–1298.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

ARE CHEMICAL ALARM CUES CONSERVED WITHIN SALMONID FISHES?

REEHAN S. MIRZA* and DOUGLAS P. CHIVERS

Department of Biology
112 Science Place, University of Saskatchewan
Saskatoon, SK S7N 5E2, Canada

(Received January 26, 2001; accepted April 11, 2001)

Abstract—A wide diversity of fishes possess chemical alarm signalling systems. However, it is not known whether the specific chemicals that act as alarm signals are conserved within most taxonomic groups. In this study we tested whether cross-species responses to chemical alarm signals occurred within salmonid fishes. In separate laboratory experiments, we exposed brook charr (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss*) to chemical alarm signals from each of the three salmonid species and from swordtails (*Xiphophorus helleri*). In each case, the test species responded with appropriate antipredator behavior to all three salmonids alarm cues, but did not react to swordtail cues. These data suggest that chemical alarm cues are partially conserved within the Family Salmonidae. For each species tested, the intensity of the response was stronger to conspecific alarm cues, than to heterospecific alarm cues, indicating that salmonids could distinguish between chemical cues of conspecifics versus heterospecifics. These results suggest that the chemical(s) that act as the alarm cues may be: 1) identical and that there may be other chemical(s) that allow the test fish to distinguish between conspecifics and heterospecifics, or 2) that the cues that act as signals are not identical, but are similar enough to be recognized.

Key Words—Brook charr, rainbow trout, brown trout, alarm signals, antipredator behavior, Salmonidae.

INTRODUCTION

Chemical cues that serve as alarm signals have been reported in a wide variety of fishes. These chemical cues are released by prey upon detecting or being captured by a predator (Chivers and Smith, 1998). Chemical alarm signals function to 'warn'

*To whom correspondence should be addressed. email: mirza@skyway.usask.ca

0098-0331/01/0800-1641$19.50/0

nearby conspecifics of potential predators in the vicinity. Individuals warned by alarm signals may gain a survival benefit by avoiding the predator or by evading the predator during an encounter (Mathis and Smith, 1993a; Wisenden et al., 1999; Mirza and Chivers, unpublished data).

Chemical alarm signals may be particularly important when visual cues are limited, such as at night, in highly structured habitats or in areas of high turbidity (Smith, 1992). Furthermore, chemical signals function well in aquatic media because a large number of compounds can dissolve in water, giving a large number of potential chemical signals to be detected (Kleerekoper, 1969; Hara, 1994). Although controversy exists concerning the function of chemical alarm signals (Magurran et al., 1996; Smith, 1997; Brown and Godin, 1999), there exists considerable evidence for the antipredator function of alarm signals for both signal senders and receivers (Smith, 1992; Chivers and Smith, 1998). Thus, chemical signals provide reliable information to assess predation risk (Kats and Dill, 1998).

Chemical cues that are released when captured by a predator are referred to as damage-released alarm signals (Chivers and Smith, 1998). Damage-released alarm signals are found within a wide diversity of fishes and have been best characterized in the Superorder Ostariophysi (Schutz, 1956; Smith, 1992). Ostariophysans comprise 64% of all freshwater fishes, and includes minnows, suckers, loaches, characins, and catfishes (Nelson, 1994). Recently, other researchers have reported the presence of damage-released alarm signals in other groups of fishes, including gobies (Smith, 1989; Smith et al., 1991), poeciliids (Reed, 1969; García et al., 1992, Nordell, 1998; Brown and Godin, 1999; Mirza et al., unpublished data), sticklebacks (Mathis and Smith, 1993b; Brown and Godin, 1997), darters (Smith, 1979, 1982; Commens and Mathis, 1999), sculpins (Hugie et al., 1991, Chivers et al., 2000), cichlids (Wisenden and Sargent, 1997) and salmonids (Brown and Smith, 1997; Berejikian et al., 1999, Mirza and Chivers, 2000).

With the exception of research on Ostariophysan fishes, we know very little about the nature of chemicals that act as alarm signals for most species. The data of Schutz (1956) suggests that alarm signals are probably conserved within Ostariophysan fishes. He found that Ostariophysans responded to alarm signals from other Ostariophysans. Brown et al. (2000) recently showed that a nitrogen oxide functional group probably acts as the chief molecular trigger in the Ostariophysan alarm signalling system. The molecular trigger appears to be conserved within the Superorder. Whether or not alarm signals are conserved within other groups of fishes remains unknown. It is common for closely related species to show cross-species responses to alarm cues, however, it is unclear whether these cross-species responses occur because the species use the same chemical(s) in their signalling systems or whether the species have learned to recognize each other's alarm signals (Smith, 1982, Brown and Godin, 1997). Chivers and Smith (1994) and Chivers et al. (1995) have established that sympatric members of the same prey guild can learn to recognize each other's alarm signals.

In this study, we examined cross-species reactions to chemical alarm cues within the Family Salmonidae (Superorder Proacanthopterygii). In three separate laboratory experiments, we exposed brook charr (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss*) to chemical alarm cues from each of the three salmonids, plus chemical cues of swordtails (*Xiphophorus helleri*, Superorder Acanthopterygii). Swordtails represent a generalized injured fish cue since they are allopatric with all three salmonid species and phylogenetically distant from salmonids. Both rainbow trout and brook charr have been shown to possess chemical alarm signalling systems (Brown and Smith, 1997; Mirza and Chivers, 2000).

METHODS AND MATERIALS

Fish Maintenance. Juvenile brook charr, brown trout and rainbow trout were obtained from the provincial hatchery in Ft. Qu'Appelle, Saskatchewan, Canada in May of 2000. The three species of salmonids were kept in separate 350-L artificial stream tanks at 14°C on a 14 : 10 L : D photoperiod. Fresh dechlorinated water was introduced into each holding tank at a rate of 1 l/min. Fish were fed daily on a combination of commercial trout pellets and frozen brine shrimp, *Artemia* spp.

Brook Charr Responses to Alarm Cues. In this experiment, we exposed juvenile brook charr to one of four treatments: 1) brook charr skin extract, 2) brown trout skin extract, 3) rainbow trout skin extract or 4) swordtail skin extract. Each charr received only one of the four treatments, and the order of testing was randomized. A total of 90 charr were tested ($N = 20$ for each salmonid treatment, $N = 30$ for the swordtail treatment). Previous work had shown that brook charr do not respond to swordtail skin extract (Mirza and Chivers, 2000); however, the responses to swordtail skin extract were more variable than predicted, thus, we increased our sample size.

Each test chamber was set up as a gravity flow-through system (modified from Petranka et al., 1987). Water flowed into one of two 60 l header tanks, then spilled over into a 113 l test chamber (measuring 54 × 44 × 36 cm) and finally down the drain at a rate of 1 l/min. A single plastic tube ran into one of the two header tanks for introduction of chemical stimuli. Each test chamber was subdivided into two compartments by an opaque barrier with screen mesh at either end. The barrier was 12 cm from the long wall of the tank. This created an observation area of 54 × 32 × 36 cm. Behind the barrier, two small powerheads were used to create a circular flow in the tank. Each test chamber contained a single centrally located shelter object constructed from a ceramic tile (15 × 8 cm) with three glass legs (7 cm). A single plastic tube was situated at the right hand side of the chamber for introduction of food. A grid of 9 × 9 cm squares was constructed on the front of

the barrier to quantify movement of fish in the tank. A horizontal line divided the tank into upper and lower halves (Figure 1).

Skin extracts were obtained from donor fishes that were euthanized with a single blow to the head in accordance to guidelines set by the Canadian Council on Animal Care. For each set of trials, a fillet of skin was removed from both sides of each fish (approximately 2.5 cm^2) and placed in 50 ml of chilled glass-distilled water. Skin was homogenized and then filtered through filter floss, to remove the larger particles, then diluted in distilled water to make a final volume of 250 ml. Extracts were kept on ice and used approximately one hour after preparation. Each fish was exposed to a concentration of 0.5 cm^2 of skin. We used 4 brook charr (mean $\pm$ SD standard length $= 5.1 \pm 0.25$ cm), 4 brown trout (5.1 ± 0.26 cm), 4 rainbow trout (5.8 ± 0.26 cm) and 6 swordtails (4.5 ± 0.16 cm) as donors for skin extracts.

Fish were allowed to acclimate for 24 hr prior to trials. Each trial was 18 min in length and consisted of an 8-min pre- and 8-min post-stimulus period, with a 2-min stimulus introduction period between the pre- and post-stimulus periods. Dye trials indicated that it took approximately 1.5 min for the stimulus to enter the test chamber from the header tank. At the beginning of both the pre- and post stimuli periods, approximately 5 ml of thawed brine shrimp was introduced into the tank. The food was added to stimulate activity and would only remain in the water column for a few minutes. Charr were observed to forage predominantly in the water column, rather than pick food off the bottom. The water flow was switched over to the second header tank after the pre-stimulus period to allow introduction of the chemical stimulus.

During both the pre- and post-stimulus periods we recorded the number of line crosses, the amount of time spent under shelter, amount of time in the lower half of the tank (water-column position), number of feeding bites and latency to first feeding bite. A line cross was recorded when 75% of the fish's body crossed from one grid square to another. We also recorded whether the fish was motionless (stationary on the bottom or in the water column for a minimum of 30 sec). Changes between the pre-stimulus and post-stimulus periods were calculated and the difference in changes among treatments was analysed using a MANOVA followed by one-way ANOVAs on significant variables (Zar, 1996). Post hoc Tukey tests were used to determine which pairs of treatments differed for significant response variables. Frequency of motionlessness was compared using a *Chi*-square analysis. All statistics were calculated using SPSS 9.0.

Brown Trout Responses to Alarm Cues. In this experiment, we exposed brown trout to chemical alarm cues from each species of salmonid as well as swordtails. The experimental set-up procedures and statistical analysis were identical to that outlined for brook charr. There were 20 replicates of each treatment. Skin extracts were prepared from 4 juvenile brown trout (mean $\pm$ SD standard length $= 5.58 \pm 0.26$ cm), 4 brook charr (5.23 ± 0.17 cm), 4 rainbow trout (6.65 ± 0.31 cm) and 4 swordtails (4.55 ± 0.26 cm).

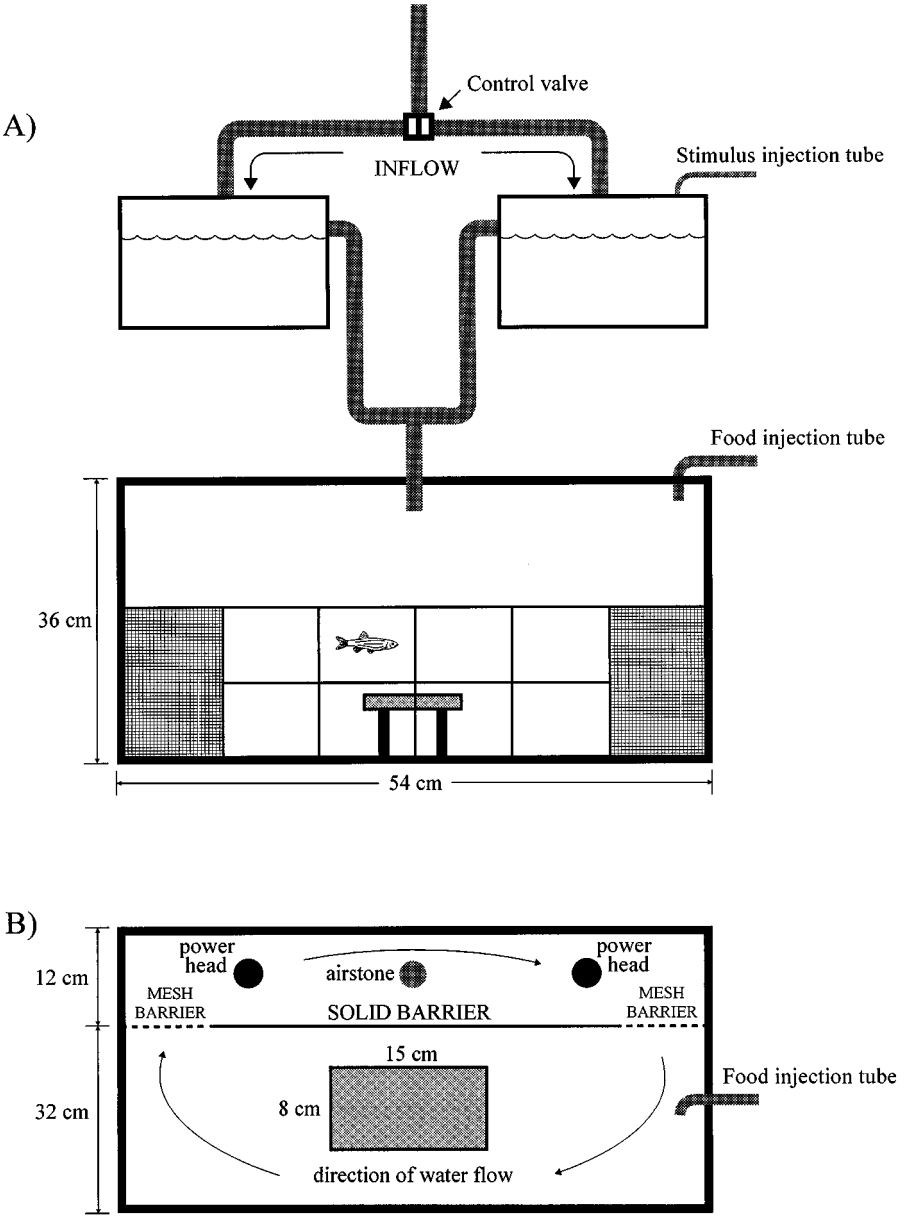


FIG. 1. Schematic diagram of the gravitational flow-through apparatus. A) front view, B) top view.

Rainbow Trout Responses to Alarm Cues. In this experiment, we exposed rainbow trout to chemical alarm cues from each species of salmonid as well as swordtails. The experimental set-up, procedures, and statistical analysis were identical to that outlined for brook charr. There were 20 replicates for each treatment. Skin extracts were prepared from 4 juvenile rainbow trout (mean $\pm$ SD standard length $= 7.60 \pm 0.29$ cm), 4 brook charr (5.83 ± 0.27 cm), 4 brown trout (6.57 ± 0.33 cm) and 4 swordtails (4.95 ± 0.39 cm).

RESULTS

Brook Charr Responses to Alarm Cues. MANOVA results reveal there was an overall difference among treatments (Table 1). Subsequent ANOVAs showed that there was a significant difference in changes in the number of line crosses, number of feeding strikes, and latency to first feeding strike, however, there were no significant differences among treatments for changes in position in the water column or shelter use (Table 1). Post hoc comparisons reveal that brook charr reduced activity more in response to all three salmonid skin extracts, than to swordtail skin. Moreover, there was less activity by brook charr exposed to brook charr extract than those exposed to brown trout extract (Figure 2). Brook charr reduced foraging in response to the salmonid extracts compared to swordtail skin extract, however, the only statistical difference was observed between rainbow trout skin extract and swordtail skin extract (Figure 2). The latency to first feeding strike was greater when brook charr were exposed to either conspecific or brown trout skin extract (Figure 2). However, there was no difference in response between swordtail and rainbow trout cues in terms of latency to first feeding strike.

TABLE 1. MANOVA AND ANOVA RESULTS FOR RESPONSES OF BROOK CHARR TO BROOK CHARR SKIN EXTRACT, RAINBOW TROUT SKIN EXTRACT, BROWN TROUT SKIN EXTRACT AND SWORDTAIL SKIN EXTRACT

	F	*df*	*P*
MANOVA			
Treatment	3.34	15,226	< 0.001
ANOVA			
Change in position in water column	2.13	3,86	0.102
Change in shelter use	0.44	3,86	0.728
Change in number of line crosses	11.93	3,86	< 0.001
Change in number of feeding strikes	3.31	3,86	0.024
Change in latency to first feeding strike	5.34	3,86	0.002

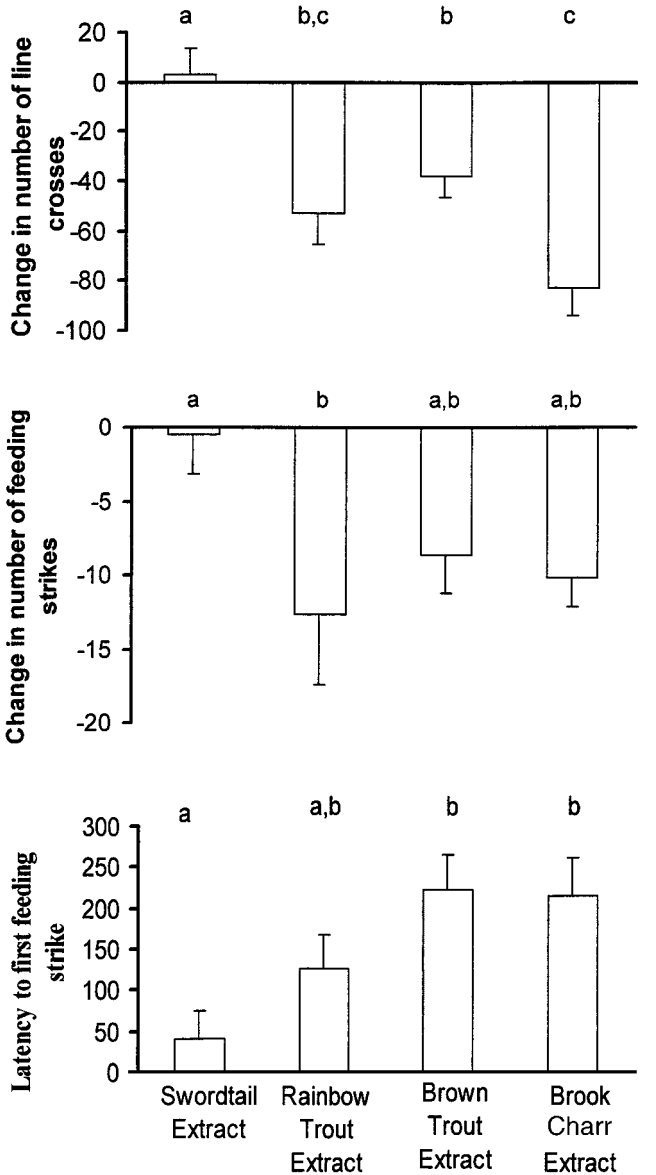


FIG. 2. Mean (+SE) change in number of line crosses, number of feeding strikes, and latency to first feeding strike for brook charr exposed to either brook charr skin extract, brown trout skin extract, rainbow trout skin extract, or swordtail skin extract. Different letters over bars denote significant differences at $P < 0.05$.

Brook charr were motionless in 12 out of 20 trials when exposed to brook charr skin extract compared to 6 out of 20 trials when exposed to brown trout skin extract, 1 out of 20 trials when exposed to rainbow trout skin extract, and 0 out of 30 trials when exposed to swordtail skin extract. Since no brook charr were motionless when exposed to swordtail skin extract, we excluded this data from the analysis to avoid having cells with frequencies of less than 5. We constructed a 2×2 contingency table comparing frequency of motionless to conspecific (brook charr) skin extract versus a combined heterospecific (brown trout and rainbow trout) skin extract. Brook charr were motionless more in response to conspecific skin extract than heterospecific skin extract ($X^2 = 11.13$, $df = 1$, $P = 0.001$).

Responses of Brown Trout to Alarm Cues. MANOVA revealed that there was an overall difference among treatments (Table 2). Subsequent ANOVAs showed that there was a difference in changes in the number of line crosses, number of feeding strikes, and latency to first feeding strike among treatments, however, there were no differences among treatments for changes in position in water column or shelter use (Table 2). Post hoc comparisons reveal that brown trout decreased activity more in response to all three salmonid skin extracts, than to swordtail skin extract. Moreover, there was less activity when brown trout were exposed to brown trout skin extract than to rainbow trout skin extract (Figure 3). Brown trout reduced the number of feeding strikes in response to all three trout skin extracts compared to swordtail skin extract (Figure 3). The latency to first feeding strike by brown trout was greater when exposed to brown trout skin extract compared to swordtail skin extract (Figure 3).

Brown trout were motionless in 13 out of 20 trials when exposed to brown trout skin extract compared to 5 out of 20 trials when exposed to brook charr skin extract, 8 out of 20 trials when exposed to rainbow trout skin extract, and 0 out of 20 when exposed to swordtail skin extract. Using the same basis for the *Chi*-square

TABLE 2. MANOVA AND ANOVA RESULTS FOR RESPONSES OF BROWN TROUT TO BROOK CHARR SKIN EXTRACT, RAINBOW TROUT SKIN EXTRACT, BROWN TROUT SKIN EXTRACT AND SWORDTAIL SKIN EXTRACT

	F	*df*	*P*
MANOVA			
Treatment	3.73	15,199	< 0.001
ANOVA			
Change in position in water column	0.37	3,76	0.770
Change in shelter use	1.98	3,76	0.124
Change in number of line crosses	12.48	3,76	< 0.001
Change in number of feeding strikes	8.84	3,76	< 0.001
Change in latency to first feeding strike	3.00	3,76	0.036

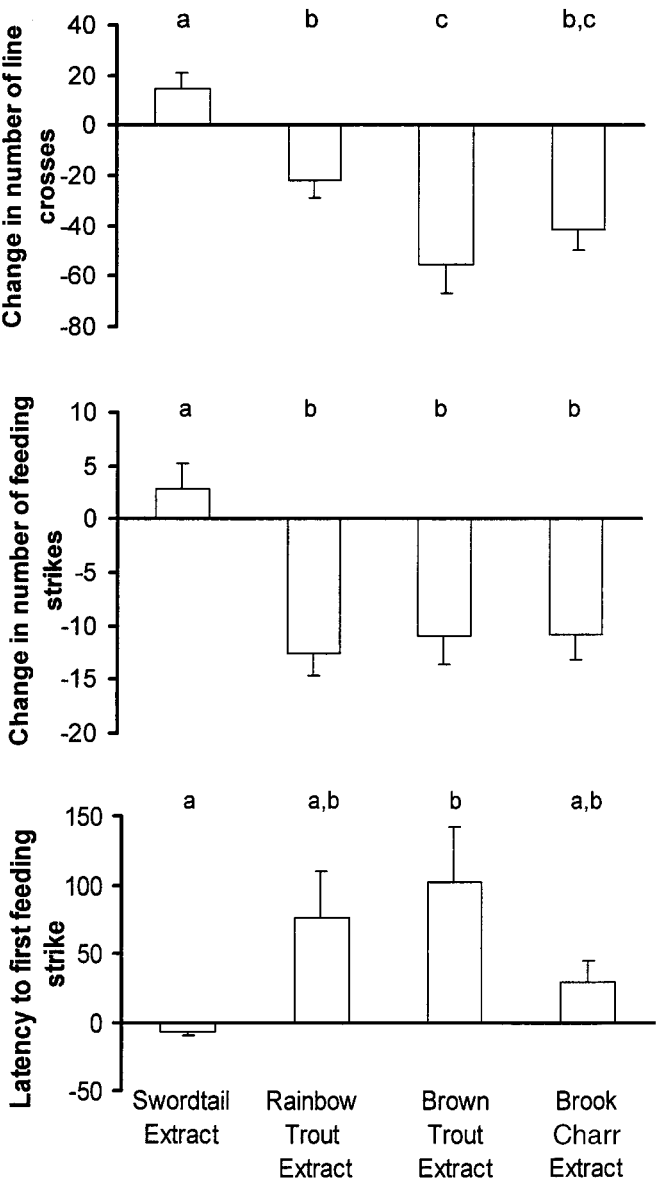


FIG. 3. Mean (+SE) change in number of line crosses, number of feeding strikes, and latency to first feeding strike of brown trout exposed to either brook charr skin extract, brown trout skin extract, rainbow trout skin extract or swordtail skin extract. Different letters over bars denote significant differences at $P < 0.05$.

analysis as in the brook charr experiment, we constructed a 2×2 contingency table comparing conspecific (brown trout) skin extract versus a combined heterospecific (brook charr and rainbow trout) skin extract. More trout were motionless when exposed to conspecific skin extract than heterospecific skin extract ($X^2 = 5.74$, $df = 1$, $P = 0.017$).

Responses of Rainbow Trout to Alarm Cues. MANOVA revealed that there was an overall difference among treatments (Table 3). Subsequent ANOVAs showed that there was a difference in changes in the number of line crosses, number of feeding strikes, and latency to first feeding strike among treatments, however, there were no differences among treatments for changes in position in the water column or shelter use (Table 3). Post hoc multiple comparisons reveal that rainbow trout decreased activity more in response to all three salmonid skin extracts, than swordtail skin extract. Moreover, there was less activity by rainbow trout exposed to conspecific skin extract than brook charr skin extract (Figure 4). Rainbow trout reduced the number of feeding strikes in response to all three salmonid skin extracts compared to swordtail skin extract (Figure 4). The latency to first feeding strike of rainbow trout was greater when exposed to rainbow trout skin and brown trout skin extract compared to swordtail skin extract (Figure 4). Moreover, rainbow trout responded more strongly to conspecific skin extract than to brook charr skin extract in terms of latency to first feeding. There was no difference between the swordtail and brook charr treatments in terms of latency to first feeding strike.

Rainbow trout were motionless in 13 out of 20 trials when exposed to rainbow trout skin extract compared to 10 out of 20 trials when exposed to brown trout skin extract, 4 out of 20 trials when exposed to brook charr skin, and 0 out of 20 trials when exposed to swordtail skin extract. We constructed a 2×2 contingency table comparing frequency of motionless of conspecific (rainbow trout) alarm cues to heterospecific (brook charr and brown trout) alarm cues. Motionless

TABLE 3. MANOVA AND ANOVA RESULTS FOR RESPONSES OF RAINBOW TROUT TO BROOK CHARR SKIN EXTRACT, RAINBOW TROUT SKIN EXTRACT, BROWN TROUT SKIN EXTRACT AND SWORDTAIL SKIN EXTRACT

	F	*df*	*P*
MANOVA			
Treatment	6.53	15,199	<0.001
ANOVA			
Change in position in water column	1.41	3,76	0.245
Change in shelter use	1.98	3,76	0.124
Change in number of line crosses	30.45	3,76	<0.001
Change in number of feeding strikes	12.46	3,76	<0.001
Change in latency to first feeding strike	13.13	3,76	<0.001

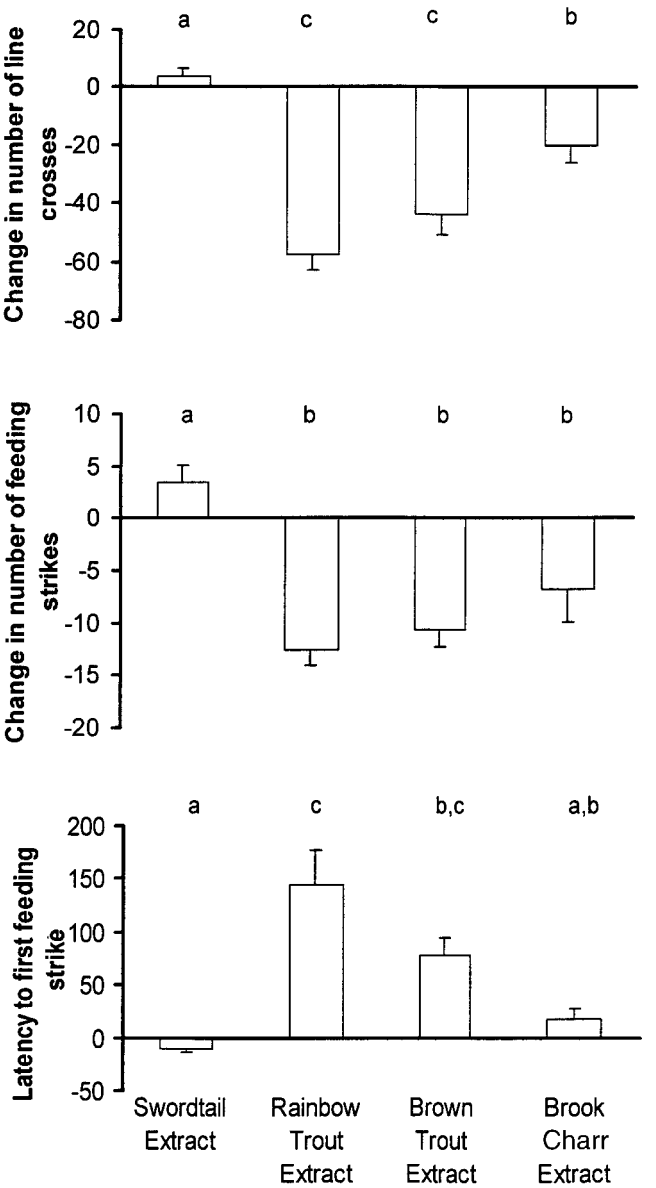


FIG. 4. Mean (+SE) change in number of line crosses, number of feeding strikes, and latency to first feeding strike of rainbow trout exposed to either brook charr skin extract, brown trout skin extract, rainbow trout skin extract or swordtail skin extract. Different letters over bars denote significant differences at $P < 0.05$.

behavior was exhibited more in response to conspecific alarm cues than heterospecific alarm cues ($X^2 = 4.85$, $df = 1$, $P = 0.028$).

DISCUSSION

The results of the present study and those of three recent studies (Brown and Smith, 1997, Berejikian et al., 1999; Mirza and Chivers, 2000) indicate that it is common for salmonid fishes use chemical alarm cues to mediate their risk of predation. Our results confirm previous studies showing alarm responses in rainbow trout (Brown and Smith, 1997) and brook charr (Mirza and Chivers, 2000). Moreover, we are the first to report the presence of a response to damage-released conspecific alarm cues in brown trout. Berejikian et al. (1999) demonstrated an alarm system in chinook salmon (*Oncorhynchus tshawytscha*). Each of the species tested to date has shown a clear response to damage-released alarm cues. Even though only 4 of 66 salmonids have been tested, the experiments have been completed on 3 different genera (*Salmo*, *Salvelinus* and *Oncorhynchus*). Consequently, we suggest that alarm systems are widespread among salmonid fishes. In each of our experiments, salmonids decreased activity and reduced foraging. By decreasing activity, salmonids can make themselves harder to detect by predators (Lima and Dill, 1990; Chivers and Smith, 1998).

Our study documents that each of the species of salmonids tested responds to each other's alarm cues, but does not respond to cues from injured swordtails. This is the first report of cross-species responses to alarm cues by salmonid fishes. Numerous other studies have tested for cross-species responses in other groups of fishes. For example, cross-species responses have been documented among gobies (Smith et al., 1991), darters (Smith, 1982, Commens and Mathis, 1999), stickleback (Mathis and Smith, 1993b; Brown and Godin, 1997), poeciliids (Mirza et al., unpublished data) and Ostariophysans (Schutz, 1956; Brown et. al., 2000).

Cross-species responses to alarm cues may reflect that the chemical(s) that serve as alarm cues are conserved within the taxonomic group. Brown et al. (2000) documented that a nitrogen oxide functional group is conserved as the chief molecular trigger of Ostariophysan alarm signals. If alarm cues are conserved in salmonids, we should observe cross-species responses even when the receivers have no experience with the cue (i.e., the first time the prey is exposed to the cue). If the alarm signals are not conserved, then we should not observe cross-species responses when the prey are first exposed to the cue. Even though cross-species responses are common in a variety of different fishes, researchers have not addressed whether the cues are conserved, because they have not ruled out the possibility that cross-species responses could be learned. Chivers and Smith (1994) and Chivers et al. (1995) have shown that species that are members of

the same prey guild (i.e., those species that occur in the same habitat and share predators) can learn to recognize each other's alarm cues.

In this study, we observed cross-species responses among salmonids that have no prior experience with each other. This indicates that the salmonid alarm cues are at least partially conserved. However, we also found evidence that each species could differentiate conspecific signals from those of heterospecifics. For all three species, we observed that test animals exhibited significantly more freezing and less movement in response to conspecific cues than to cues of heterospecifics. The fact that salmonids can recognize all heterospecific cues and also can distinguish between conspecifics and heterospecifics indicates that the chemical(s) that act as the alarm cue may be similar, but not identical. Alternatively, the chemical(s) that act as the alarm cue may be identical and there may be another chemical(s) present that allows the test fish to distinguish between conspecifics and heterospecifics. Additional work should address the nature of the chemicals that act as alarm cues in salmonid fishes.

Our results show that juvenile salmonids that detect chemical alarm cues released by other juvenile salmonids will obtain an antipredator advantage. They decrease activity and reduce foraging in response to cues of other injured heterospecifics. This type of cross-species reaction makes intuitive sense. If a particular predator were to capture a juvenile salmonid, then chances are the same predator would also be a threat to each of the other species. However, as the salmonids grow, they may come to occupy different ecological niches and have different predators. Whether adult salmonid fishes use alarm cues in the same manner as juveniles remain unknown. Further research should address this issue.

Acknowledgments—We would like to thank Jerry Banks and the Ft. Qu'Appelle hatchery for supplying the fishes for this study. This study was funded by grants to D. P. Chivers from NSERC of Canada and the University of Saskatchewan. The research described was approved by the University Committee on Animal Use and Supply at the University of Saskatchewan (Animal care protocol # 19920077).

REFERENCES

BEREJIKIAN, B. A., SMITH, R. J. F., TEZAK, E. P., SCHRODER, S. L., and KNUDSEN, C. M. 1999. Chemical alarm signals and complex hatchery rearing habitats affect antipredator behavior and survival of chinook salmon (*Oncorhynchus tshawytscha*) juveniles. *Can. J. Fish. Aquat. Sci.* 56:830–838.

BROWN, G. E., and GODIN, J.-G. J. 1997. Anti-predator responses to conspecific and heterospecific skin extracts by threespine sticklebacks: Alarm pheromones revisited. *Behaviour* 134:1123–1134.

BROWN, G. E., and GODIN, J.-G. J. 1999. Chemical alarm signals in wild Trinidadian guppies (*Poecilia reticulata*). *Can. J. Zool.* 77:562–570.

BROWN, G. E., and SMITH, R. J. F. 1997. Conspecific skin extracts elicit anti-predator responses in juvenile rainbow trout (*Oncoryhnchus mykiss*). *Can. J. Zool.* 75:1916–1922.

BROWN, G. E., ADRIAN, J. C., SMYTH, E., LEFT, H., and BRENNAN, S. 2000. Ostariophysan alarm pheromones: laboratory and field tests of the functional significance of nitrogen oxides. *J. Chem. Ecol.* 26:139–154.

CHIVERS, D. P., and SMITH, R. J. F. 1994. Intra- and interspecific avoidance of areas marked with skin extract from brook sticklebacks (*Culaea inconstans*) in a natural habitat. *J. Chem. Ecol.* 20:1517–1524.

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Ècoscience* 5:338–352.

CHIVERS, D. P., PUTTLITZ, M. H., and BLAUSTEIN, A. R. 2000. Chemical alarm signaling by reticulate sculpins, *Cottus perplexus. Env. Biol. Fish.* 57:347–352.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, R. J. F. 1995. The role of experience in the response of fathead minnows (*Pimephales promelas*) to skin extract of Iowa darters (*Etheostoma exile*). *Behaviour* 132:665–674.

COMMENS, A. M., and MATHIS, A. 1999. Alarm pheromones of rainbow darters: Responses to skin extracts of conspecifics and congeners. *J. Fish Biol.* 55:1359–1362.

GARCÍA, C., ROLAN-ALVÁREZ, E., and SÁNCHEZ, L. 1992. Alarm reaction and alert state in *Gambusia affinis* (Pisces, Poecilidae) in response to chemical stimuli from injured conspecifics. *J. Ethol.* 10:41–46.

HARA, T. J. 1994. The diversity of chemical stimulation in fish olfaction and gustation. *Rev. Fish Biol. Fish.* 4:1–35.

HUGIE, D. M., THURINGER, P. L., and SMITH, R. J. F. 1991. The response of the tidepool sculpin, *Oligocottus maculosus*, to chemical stimuli from injured conspecifics, alarm signalling in cottidae (Pisces). *Ethology* 89:322–334.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Ècoscience* 5:361–394.

KLEEREKOPER, H. A. 1969. Olfaction in Fishes. Indiana University Press, Bloomington.

LIMA, S. L., and DILL, L. M. 1990. Behavioural decisions made under the risk of predation: A review and prospectus. *Can. J. Zool.* 68:619–640.

MAGURRAN, A. E., IRVING, P. W., and HENDERSON, P. A. 1996. Is there a fish alarm pheromone? A wild study and critique. *Proc. Roy. Soc. Lond.* 263:1551–1556.

MATHIS, A., and SMITH, R. J. F. 1993a. Chemical alarm signals increase the survival time of fathead minnows (*Pimephales promelas*) during encounters with northern pike (*Esox lucius*). *Behav. Ecol.* 4:260–265.

MATHIS, A., and SMITH, R. J. F. 1993b. Intraspecific and cross-superorder responses to chemical alarm signals by brook stickleback. *Ecology* 74:2395–2404.

MIRZA, R. S., and CHIVERS, D. P. 2000. Predator-recognition training enhances survival of brook trout: Evidence from laboratory and field enclosure studies. *Can. J. Zool.* 78:2198–2208.

NELSON, J. S. 1994. Fishes of the World. 3rd edn. John Wiley and Sons, New York.

NORDELL, S. E. 1998. The response of female guppies, *Poecilia reticulata*, to chemical stimuli from injured conspecifics. *Env. Biol. Fish.* 51:331–338.

PETRANKA, J. W., KATS, L. B., and SIH, A. 1987. Predator-prey interactions among fish and larval amphibians: use of chemical cues to detect predatory fish. *Anim. Behav.* 35:420–425.

REED, J. R. 1969. Alarm substances and fright reactions in some fishes from the southeastern United States. *Trans. Amer. Fish. Soc.* 4:664–668.

SCHUTZ, F. 1956. Vergleichende Untersuchungen über die Schrekreaktion bei Fischen und deren Verbreitung. Z. *vergl. Physiol.* 38:84–135.

SMITH, R. J. F. 1979. Alarm reaction of Iowa and johnny daters (*Etheostoma*, Percidae, Pisces) to chemicals from injured conspecifics. *Can. J. Zool.* 57:1278–1282.

SMITH, R. J. F. 1982. Reaction of *Percina nigrofasciata*, *Ammocrypta beani* and *Etheostoma swaini* (Percidae, Pisces) to conspecific and intergeneric skin extracts. *Can. J. Zool.* 60:1067–1072.

SMITH, R. J. F. 1989. The response of *Asterropteryx semipunctatus* and *Gnatholepis anjerensis* (Pisces, Gobiidae) to chemical stimuli from injured conspecifics, an alarm response in gobies. *Ethology* 81:279–290.

SMITH, R. J. F. 1992. Alarm signals in fishes. *Rev. Fish Biol. Fish.* 2:33–63.

SMITH, R. J. F. 1997. Does one result trump all others? A response to Magurran, Irving and Henderson. *Proc. Roy. Soc. Lond.* 264:445–450.

SMITH, R. J. F., LAWRENCE, B. J., and SMITH, M. J. 1991. Cross-reaction to skin extract between two gobies, *Asterropteryx semipunctatus* and *Brachygobius sabanus. J. Chem. Ecol.* 17:2253–2259.

WISENDEN, B. D., and SARGENT, R. C. 1997. Antipredator behaviour and suppressed aggression by convict cichlids in response to injury-released chemical cues of conspecifics but not to those of an allopatric heterospecific. *Ethology* 103:283–291.

WISENDEN, B. D., CLINE, A., and SPARKES, T. C. 1999. Survival benefit to antipredator behaviour in the amphipod *Gammarus minus* (Crustacea: Amphipoda) in response to injury-released chemical cues from conspecifics and heterospecifics. *Ethology* 105:407–414.

ZAR, J. H. 1996. Biostatistical Analysis, 4th edn. Prentice-Hall Inc., New York.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

ATTACK CONE AVOIDANCE DURING PREDATOR INSPECTION VISITS BY WILD FINESCALE DACE (*Phoxinus neogaeus*): THE EFFECTS OF PREDATOR DIET

GRANT E. BROWN,* JUSTIN L. GOLUB, and DESIREE L. PLATA

Department of Biological Sciences
Science and Engineering Center
Union College, Schenectady, New York, 12308

(Received January 16, 2001; accepted April 16, 2001)

Abstract—When confronted by potential predators, many prey fishes engage in predator inspection behavior. Previous authors have argued that by selectively avoiding the predator's head during an inspection visit (attack cone avoidance), individual inspectors may reduce their local risk of predation. In field trials, we investigated the effects of predator diet cues on the presence of 'attack cone avoidance' during predator inspection visits. Wild, free-ranging finescale dace (*Phoxinus neogaeus*) were exposed to the combined cues of a model predator and a distilled water control or the odor of a yellow perch (*Perca flavescens*) fed dace (with alarm pheromone), swordtail (*Xiphophorus helleri*) (lacking Ostariophysan alarm pheromone), or perch that were food deprived for four days. Finescale dace modified their predator inspection behavior following exposure to the odor of a perch fed dace (fewer dace present, reduced frequency of inspections, and an increased per capita inspection rate) compared to those exposed to the odor of a perch fed swordtails, perch that were food deprived, or a distilled water control. In addition, dace inspected the tail region more often only when the model predator was paired with the odor of a perch fed dace. In all other treatments, dace inspected the head region of the model predator more often. These data suggest that attack cone avoidance of inspecting prey fishes may be more likely to occur in high-risk situations, such as in the presence of conspecific alarm pheromones in the diet of potential predators.

Key Words—Predator inspection, Ostariophysan fishes, alarm pheromones, predator diet, anti-predator behavior.

*Address for correspondence: Department of Biology, Concordia University, 1455 de Maisonneuve Blvd. W., Montreal, Quebec, H3G 1M8 Canada.

INTRODUCTION

Predator inspection behavior, characterized by the saltatory approach towards and withdrawal from a potential predator, either alone or in groups (Dugatkin and Godin, 1992a; Pitcher, 1992), is a common phenomenon among many prey taxa, including fishes. Predator inspection is an inherently risky behavioral pattern, as it involves the approach towards a potentially risky predator. In order for this behavioral pattern to have evolved and be maintained within prey populations, there must be significant benefits associated with predator inspection.

Several benefits associated with predator inspection in fishes have been demonstrated, including visual alarm signaling (Smith and Smith, 1989; Murphy and Pitcher, 1997; Brown et al., 1999), predator deterrence (Magurran, 1990; Godin and Davis, 1995 a,b; Brown et al., 1999), and the preferential mate selection of inspecting males by females (Godin and Dugatkin, 1996). One of the potentially most important benefits associated with predator inspection is the ability to acquire information regarding local predation risk (Dugatkin and Godin, 1992a; Pitcher, 1992; Brown and Godin, 1999a). By inspecting potential predators, prey can assess local predation risk (Licht, 1989; Murphy and Pitcher, 1997) or acquire the recognition of a novel predator (Brown and Godin, 1999a).

Predator inspection behavior appears to involve assessment of both visual and chemical information (Licht, 1989; Murphy and Pitcher, 1997; Brown et al., 2000). Visual cues, such as the posture and behavior of potential predators, can provide reliable information to inspecting prey regarding the likelihood of an attack by predators (Licht 1989; Murphy and Pitcher, 1997). Chemical cues, such as a predator's dietary odor, can also provide reliable cues regarding a predator's past foraging preferences and/or hunger status (Mathis and Smith, 1993; Smith, 1997; Brown and Godin, 1999a). A likely source of chemical information in Ostariophysan prey fishes is conspecific alarm pheromones in the diet of potential predators (Mathis and Smith, 1993; Brown and Godin, 1999a).

Many fishes of the superorder Ostariophysi (approximately 65% of all freshwater species; Nelson, 1994) possess chemical alarm signals or alarm pheromones (Smith, 1992; Chivers and Smith, 1998). This chemical is released into the water column following mechanical damage to the skin. Conspecifics and some sympatric heterospecifics respond with dramatic, short-term increases in antipredator behavior (Smith, 1992; Chivers and Smith, 1998). Alarm pheromones can chemically label a predator as dangerous, and inspecting individuals may modify their predator inspection behavior in the presence of conspecific alarm pheromones in the diet of potential predators. Glowlight tetras (*Hemigrammus erythrozonus*), inspected in smaller numbers, remained further from the predator and took longer to initiate inspection visits when the predator had been fed tetras (with an alarm pheromone) than when either swordtails (lacking Ostariophysan

alarm pheromones) or food deprived (Brown and Godin, 1999a; Brown et al., 2000). Similar results have been shown for finescale dace (*Phoxinus neogaeus*; Brown and Cowan, 2000).

Dugatkin and Godin (1992b) have demonstrated that wild Trinidadian guppies typically inspect the trunk and tail region of a model predator more often than the head region. This 'attack cone avoidance' (George, 1960) is argued to reduce the individual's risk associated with approaching a potential predator. However, there may be costs associated with this avoidance, such as loss of information about the posture and/or behavior of the predator (Licht, 1989; Murphy and Pitcher, 1997). Also, if predator inspection functions in attack deterrence (Godin and Davis, 1995 a,b; Brown et al., 1999), inspection visits that are directed away from the head would dilute the effectiveness of this benefit.

We conducted this study to determine the effects of predator dietary cues on the predator inspection behavior, particularly attack cone avoidance, of free ranging finescale dace towards a model predator. Previous studies looking at attack cone avoidance (i.e., Dugatkin and Godin, 1992b) have not considered chemical cues associated with the predator's diet. Thus, it is unknown if attack cone avoidance during predator inspection visits is likewise affected by conspecific alarm pheromones in the diet of potential predators. We predict that attack cone avoidance should only be present under high risk situations, such as in the presence of dietary cues containing conspecific alarm pheromones.

METHODS AND MATERIALS

Field trials were conducted at Lock 7 Kill, a small tributary stream to the Mohawk River, near Schenectady NY. This small, spring-fed stream is devoid of predator fishes and is dominated by finescale dace. Pearl dace (*Margariscus margarita*), red-bellied dace (*Phoxinus eos*), and emerald shiners (*Notropis atherinoides*) were also present in the stream at lower frequencies (personal observations). Observations were conducted in a series of pools, separated by runs and riffle sections.

Stimulus Preparation. We collected predator odor from two adult yellow perch (14.7 and 15.2 cm S.L.). We fed the perch two finescale dace (with alarm pheromone) or two green swordtails per day for five consecutive days, or food deprived them for a period of 96 hr prior to stimulus collection. Swordtails were used as a control diet, as they lack Ostariophysan alarm pheromones and are native to Central America (Axelrod and Vorderwinkler, 1983) and hence do not co-occur with finescale dace. Following the final feeding (or the final day of food deprivation), we transferred the perch to 5-l tanks which contained an airstone but were unfiltered. We left the perch in the stimulus collection tanks for a period of

36 hr, at which time we returned them to their holding tanks. The stimulus water from the two perch fed the same diets was mixed, and we collected and filtered 1 l and froze it in 50 ml aliquots at -20°C until needed. As a control, we also froze 50 ml aliquots of distilled water. The order of stimulus collection was random, and perch were fed *ad libitum*, twice daily with brine shrimp (*Artemia francisca*) for 7 days between feedings for specific diets.

Experimental Protocol. Observations were conducted in a series of 10 pools along a 600-m stretch of the stream. Pools were separated by at least 40 m, had similar mixed cobble and fine-cobble substrates, and an average ($\pm$S.E.) surface area of 6.8 ± 1.2 m^2. Each pool was tested once for each chemical stimulus, and the order of treatments was randomized with the proviso that no pool could be tested twice for the same treatment. We allowed at least 24 hr between trials for each pool. During each trial, we conducted a five min prestimulus and a five min poststimulus injection observation period.

From a vantage point on the bank of the stream, we submerged a model predator, suspended from a 2-m fiberglass rod by monofilament nylon lines, into a pool and held it stationary, about 5 cm above the substrate. The model predator consisted of an 18 cm long fishing lure, painted to resemble an esocid predator. We positioned the model near the center of the pool, perpendicular to the current and away from any obstructions, to remove any bias associated with individual dace approaching from downstream versus upstream. A 3-m length of airline tubing, which was attached to a rubber stopper located under the model, was used to introduce the chemicals remotely. We tested a total of four chemical stimuli: distilled water (DW), the odor of perch fed dace (DACE), perch fed swordtails (SWT), or the odor of a perch that had been food deprived (FD). We injected 50 ml of stimuli into the tubing and flushed it through with stream water at a rate of approximately 10 ml/min.

During both pre- and poststimulus observation periods, we recorded: 1) the number of finescale dace within a 50 cm radius of the model, 2) the number of predator inspection visits, 3) the size of each inspecting group, and 4) the location of the inspection visit (head = anterior half of model; tail = posterior half of model). In addition, we recorded the occurrence of dashing behavior, defined as the rapid, apparently disoriented swimming of at least one dace. Prior to each trial, we established visual landmarks in each pool to facilitate recording the number of dace present within a 50 cm radius of the model.

Statistical Analysis. For all behavioral measures, except the frequency of occurrence of dashing, we calculated the difference between the pre- and poststimulus injection observation periods and compared these difference scores among diet treatments by using one-way ANOVA's. Planned contrasts were made with Fisher's PLSD tests. Frequency of occurrence of dashing behavior was compared among the treatment groups by using a χ^2 analysis.

RESULTS

We observed a significant effect of predator diet on the number of dace within a 50 cm radius of the predator model ($F_{(3,36)} = 32.54$, $P < 0.0001$; Figure 1A). Planned contrasts revealed that there was a reduction in the number of dace present following the injection of DACE versus the remaining diet treatments (Figure 1A). In addition, there was an increase in the frequency of dashing behavior during the poststimulus observation periods when the model was paired with the odor of a perch fed dace versus distilled water, food deprived perch, or perch fed swordtails (DACE = 80%, DW = 0%, FD = 30%, SWT = 0%, $\chi^2_3 = 21.44$, $P < 0.0001$).

We found an effect of predator diet on the overall frequency of predator inspection vists as well ($F_{(3,36)} = 8.38$, $P < 0.001$; Figure 1B). Again, planned contrasts revealed that this overall effect was due to a reduction in the number of inspections following the injection of DACE versus DW, FD, or SWT (Figure 1B). When we calculated a per capita rate of inspection, we found an overall effect ($F_{(3,36)} = 3.39$, $P < 0.03$; Figure 1C), with dace increasing their rates of inspection following the injection of DACE and FD (Figure 1C).

We also observed an effect of predator diet on the change in the proportion of inspections directed towards the tail ($F_{(3,36)} = 20.76$, $P < 0.0001$; Figure 2A) and proportion of inspections towards the tail by singletons ($F_{(3,36)} = 4.45$, $P < 0.01$; Figure 2B). Following the injection of DACE, we observed an increase in the proportion of inspections towards the tail versus the remaining diet treatments (Figure 2A, Table 1). In addition, we observed an increase in the proportion of inspections towards the tail by singletons following the injection of DACE and FD (versus DW and SWT; Figure 2B, Table 1).

TABLE 1. MEAN (±S.E.) PROPORTION OF TOTAL INSPECTIONS AND PROPORTION OF INSPECTIONS BY SINGLETONS DIRECTED TOWARDS TAIL REGION OF THE MODEL PREDATOR FOR THE PRE- AND POSTSTIMULUS INJECTION OBSERVATIONS IN EACH OF THE DIET TREATMENTS. SEE FIGURE 2 AND TEXT FOR DETAILS.

		Proportion inpsections directed towards tail	Proportion of singletons directed towards tail
DW	Pre	0.34 ± 0.03	0.42 ± 0.11
	Post	0.36 ± 0.06	0.45 ± 0.05
DACE	Pre	0.31 ± 0.05	0.38 ± 0.04
	Post	0.75 ± 0.09	0.76 ± 0.07
FD	Pre	0.30 ± 0.03	0.44 ± 0.09
	Post	0.54 ± 0.03	0.72 ± 0.06
SWT	Pre	0.25 ± 0.03	0.31 ± 0.06
	Post	0.23 ± 0.05	0.29 ± 0.06

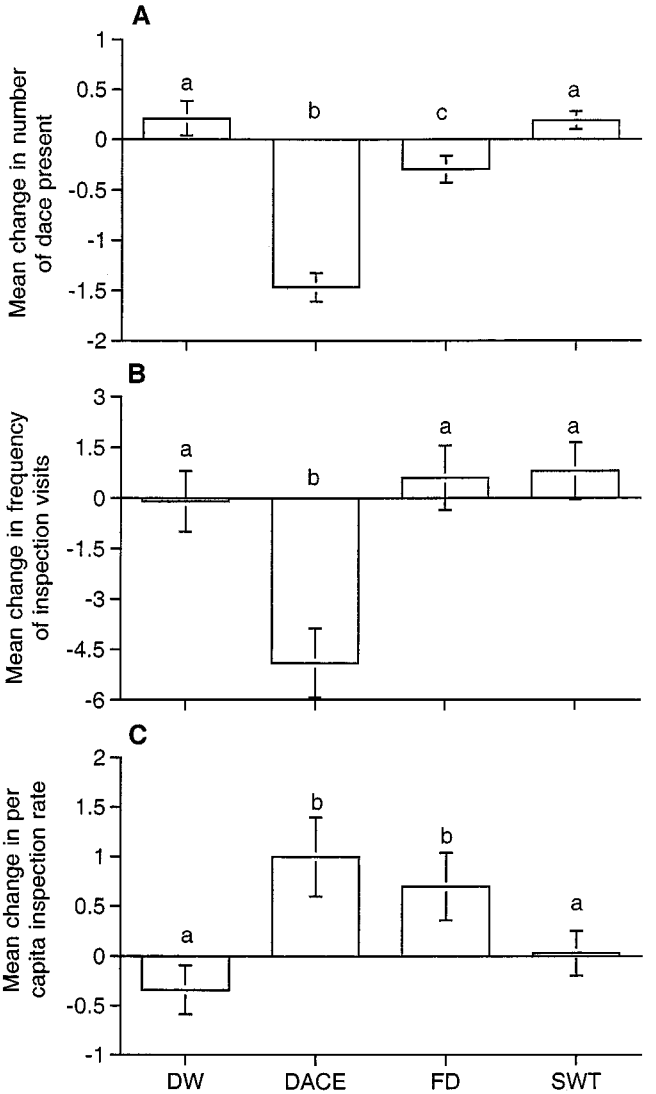


FIG. 1. Mean (±S.E.) change in the number of individuals present within a 50 cm radius (A), frequency of predator inspection visits (B), and per capita inspection rate by finescale dace exposed to a model predator (C) paired with distilled water (DW), the odor of perch fed dace (DACE), food deprived for 96 hours (FD) or fed a diet of swordtails (SWT). Different letters denote significant differences ($P < 0.05$) based on Fisher's PLSD.

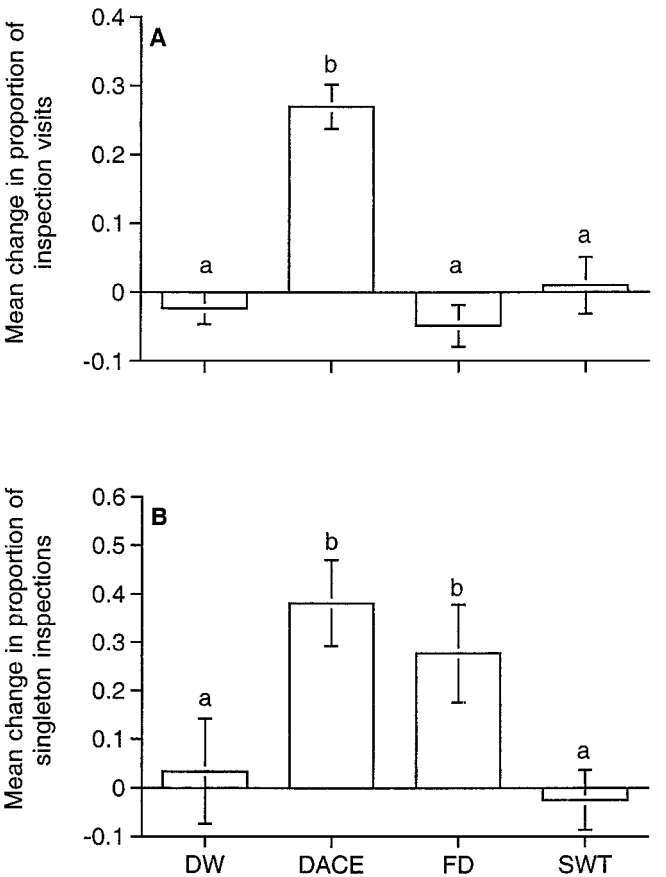


FIG. 2. Mean (±S.E.) change in the proportion of inspection visits (A) and change in proportion of inspections by singletons (B) directed towards the tail of the model predator paired with dietary cues. Treatment abbreviations as in Figure 1. Different letters denote significant differences ($P < 0.05$) based on Fisher's PLSD.

DISCUSSION

Our data demonstrate that wild finescale dace alter the form of predator inspection behavior based on the availability of predator dietary cues. Dace exhibited a significant increase in predator avoidance (fewer individuals present within 50 cm of the model and increased occurrence of dashing) when the model predator was paired with the odor of a perch fed dace. Similarly, dace reduced their frequency of predator inspection visits when exposed to the model paired with dace-fed perch

odor. However, we observed an increase in the per capita rate of inspection by dace exposed to the odor of dace-fed perch or food-deprived perch, suggesting that those individuals remaining inspected at a higher rate.

In the presence of dietary alarm pheromone cues, dace inspected the tail region more often and directed more initial inspections towards the tail. In contrast, Dugatkin and Godin (1992b) demonstrated that Trinidadian guppies (which possess an alarm pheromone; Nordell, 1998; Brown and Godin, 1999b) inspect the head of a model predator less often than the trunk or tail region, and those that do inspect the head, do so more often as singletons. This 'attack cone avoidance' (sensu George, 1960) has been suggested as a mechanism by which inspectors can gain information regarding local risk of predation while reducing their individual risk of mortality. Our results, however, demonstrate that in the absence of dietary cues containing conspecific alarm pheromones, inspectors directed a greater proportion of their inspections towards the head of the predator. Brown and Schwarzbauer (in press) have shown that under laboratory conditions, glowlight tetras (Superorder Ostariophysi) similarly modified their inspection behavior. Tetras directed a greater proportion of inspection visits to the tail region of a live cichlid predator (*Cichlasoma octofasciatum*), when the predator had been fed tetras (with alarm pheromone) versus swordtails (lacking Ostariophysan alarm pheromone) or food deprived.

This apparent conflict may be due to one of two (or both) differences between previous studies and ours. Initially, prior studies (i.e., Dugatkin and Godin, 1992b) were conducted on populations exposed to high levels of predation risk, and hence they were likely experienced with a variety of predators. Prey populations, which are under moderate to heavy predation pressure, may inspect more cautiously than those under lower predation risk (Magurran and Seghers, 1994). Secondly, previous studies examining attack cone avoidance have not included chemical cues. Our results demonstrate that dietary cues are an important source of information regarding local predation risk. Attack cone avoidance may occur only after an inspecting prey has determined that local predation risk is high based on the presence or absence of specific chemical cues. Diet-related chemical signals are honest indicators that the predator has previously selected conspecifics as food items or is hungry and, therefore, motivated to forage.

Dace also exhibited a graded response in singleton inspections, with the greatest proportion of solitary inspections towards a dace-fed predator and the lowest proportion towards a swordtail-fed predator. Dugatkin and Godin (1992b) argued that an increase in the proportion of singleton inspections indicates an increased perceived risk of predation. The observation that the proportion of solitary inspectors towards the food-deprived perch was intermediate to the dace-fed and swordtail-fed perch odor treatments suggests that dace are perceiving a food-deprived predator as a greater risk than a predator fed a neutral diet, but not as risky as predators fed a diet containing an alarm pheromone.

Inspecting prey, upon encountering a potential predator, may benefit from initially sampling chemical information and basing decisions regarding subsequent inspection behavior on the presence or absence of conspecific alarm pheromone. If the predator is 'chemically labeled' as dangerous (i.e., has been eating prey with an alarm pheromone; sensu Mathis and Smith, 1993), then high-level antipredator measures such as attack cone avoidance should be utilized. If no chemical labels are detected, the inspecting prey may more closely assess visual cues. By doing so, prey individuals may be able to obtain significant benefits associated with inspection visits while reducing their immediate risk of predation.

Acknowledgments—The authors wish to thank Manuel Leal, Carl George, and Isabelle Désormeaux for helpful comments on previous versions of this manuscript. All work reported within was conducted in accordance with Animal Research Protocol No. 2-27-98 approved on 27 February 1998 by the Institutional Animal Care and Use Committee of Union College.

REFERENCES

AXELROD, H. R., and VORDERWINKLER, W. 1983. Encyclopedia of Tropical Fishes. T.F.H. Publications, Neptune City, NJ.

BROWN, G. E., and COWAN, J. 2000. Foraging trade-offs and predator inspection in an Ostariophysan fish: Switching from chemical to visual cues. *Behaviour* 137:181–196.

BROWN, G. E., and GODIN, J.-G. J. 1999a. Who dares, learns: Chemical inspection behaviour and acquired predator recognition in a Characin fish. *Anim. Behav.* 57:475–481.

BROWN, G. E., and GODIN, J.-G. J. 1999b. Chemical alarm signals in Trinidadian guppies: Laboratory and field evidence. *Can. J. Zool.* 77:562–570.

BROWN, G. E., GODIN, J.-G. J., and PEDERSEN, J. 1999. Fin flicking behaviour: A visual anti-predator alarm signal in a Characin fish (*Hemigrammus erythrozonus*). *Anim. Behav.* 59:469–476.

BROWN, G. E., PAIGE, J. A., and GODIN, J.-G. J. 2000. Chemically-mediated predator inspection behaviour in the absence of predator visual cues by a characin fish, *Hemigrammus erythrozonus*. *Anim. Behav.* 60:315–321.

BROWN, G. E., AND SCHWARZBAUER, E. M. Chemical predator inspection and attack cone avoidance in a characin fish: The effects of predator diet. *Behaviour* (in press).

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Écoscience* 5:338–352.

DUGATKIN, L. A., and GODIN, J.-G. J. 1992a. Prey approaching predators: A cost-benefit perspective. *Ann. Zool. Fennici* 29:233–252.

DUGATKIN, L. A., and GODIN, J.-G. J. 1992b. Predator inspection, shoaling and foraging under predation hazard in the Trinidadian guppy, *Poecilia reticulata*. *Environ. Biol. Fish.* 34:265–276.

GEORGE, C. J. W. 1960. Behavioral interactions of the pickerel (*Esox niger* and *Esox americanus*) and the mosquitofish (*Gambusia patruelis*). PhD thesis, Harvard University.

GODIN, J.-G. J., and DAVIS, S. A. 1995a. Who dares, benefits: Predator approach behaviour in the guppy (*Poecilia reticulata*) deters predator pursuit. *Proc. R. Soc. Lond., B.* 259:193–200.

GODIN, J.-G. J., and DAVIS, S. A. 1995b. Boldness and predator deterrence: A reply to Milinski and Boltshauser. *Proc. R. Soc. Lond., B.* 262:107–112.

GODIN, J.-G. J., and DUGATKIN, L. A. 1996. Female mating preference for bold males in the guppy, *Poecilia reticulata*. *Proc. Nat. Acad. Sci., U.S.A.* 93:10262–10267.

LICHT, T. 1989. Discriminating between hungry and satiated predators: The response of guppies (*Poecilia reticulata*) from high and low predation sites. *Ethology* 82:238–243.

MAGURRAN, A. E. 1990. The adaptive significance of schooling as an anti-predator defence in fish. *Ann. Zool. Fennici* 27:51–66.

MAGURRAN, A. E., and SEGHERS, B. H. 1994. Predator inspection behaviour covaries with schooling tendency amongst wild guppy, *Poecilia reticulata*, populations in Trinidad. *Behaviour* 128:121–134.

MATHIS, A., and SMITH, R. J. F. 1993. Chemical labeling of northern pike (*Esox lucius*) by the alarm pheromone of fathead minnows (*Pimephales promelas*). *J. Chem. Ecol.* 19:1967–1979.

MURPHY, K. E., and PITCHER, T. J. 1997. Predator attack motivation influences the inspection behaviour of European minnows. *J. Fish Biol.* 50:407–417.

NELSON, J. S. 1994. Fishes of the World. 3rd ed. John Wiley and Sons, New York.

NORDELL, S. E. 1998. The response of female guppies, *Poecilia reticulata*, to chemical stimuli from injured conspecifics. *Environ. Biol. Fish.* 51:331–338.

PITCHER, T. J. 1992. Who dares, wins: The function and evolution of predator inspection behaviour in Shoaling fish. *Neth. J. Zool.* 42:371–391.

SMITH, R. J. F. 1992. Alarm signals in fishes. *R. Fish Biol. Fish.* 2:33–63.

SMITH, R. J. F. 1997. Avoiding and deterring predators. in Behavioural Ecology of Teleost Fishes (Ed. by J.-G. J. Godin), Oxford: Oxford University Press.

SMITH, R. J. F., and SMITH, M. J. 1989. Predator-recognition behaviour in two species of gobiid fishes, *Asterropteryx semipunctatus* and *Gnatholepis anjerensis*. *Ethology* 83:19–30.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

AVOIDANCE RESPONSE OF JUVENILE PACIFIC TREEFROGS TO CHEMICAL CUES OF INTRODUCED PREDATORY BULLFROGS

DOUGLAS P. CHIVERS,[1,*] ERICA L. WILDY,[2] JOSEPH M. KIESECKER,[3] and ANDREW R. BLAUSTEIN[4]

[1]*Department of Biology, University of Saskatchewan, Saskatoon*
[2]*Department of Biological Sciences, California State University, Hayward*
[3]*Department of Biology, Pennsylvania State University, University Park*
[4]*Department of Zoology, Oregon State University, Corvallis*

(Received December 5, 2000; accepted April 7, 2001)

Abstract—Bullfrogs (*Rana catesbeiana*), native to eastern North America, were introduced into Oregon in the 1930's. Bullfrogs are highly efficient predators that are known to eat a variety of prey including other amphibians. In laboratory experiments, we investigated whether juvenile Pacific treefrogs (*Hyla regilla*) recognize adult bullfrogs as a predatory threat. The ability of prey animals to acquire recognition of an introduced predator has important implications for survival of the prey. We found that treefrogs from a population that co-occurred with bullfrogs showed a strong avoidance of chemical cues of bullfrogs. In contrast, treefrogs from a population that did not co-occur with bullfrogs, did not respond to the bullfrog cues. Additional experiments showed that both populations of treefrogs use chemical cues to mediate predation risk. Treefrogs from both populations avoided chemical alarm cues from injured conspecifics.

Key Words—Predator recognition, introduced predators, chemical cues, alarm signals, Pacific treefrogs, bullfrogs, *Hyla regilla*, *Rana catesbeiana*.

INTRODUCTION

The failure of a prey animal to recognize and respond to a predator increases the probability that it will be captured during an encounter with a predator. As a result, there should be strong selective pressure for prey to identify potential predators (Sih, 1987; Lima and Dill, 1990). Studying the responses of prey

*To whom correspondence should be addressed. e-mail: Doug.Chivers@usask.ca

0098-0331/01/0800-1667$19.50/0

to recently introduced predators provides an ideal system for testing the acquisition of predator recognition by prey populations (Kiesecker and Blaustein, 1997a).

Previous studies have demonstrated that in populations where larval amphibians have a long evolutionary history with a particular predator, they often recognize and respond to the predator without any prior experience. For example, Kats et al. (1988) demonstrated that larval amphibians, collected as eggs from a pond with predatory fish, responded to chemical cues of the fish predators. Similar results have been reported by Sih and Kats (1994). Kiesecker and Blaustein (1997a) documented that larval red-legged frogs (*Rana aurora*), collected as eggs from populations that co-occur with predatory bullfrogs (*Rana catesbeiana*), exhibit an antipredator response to the bullfrogs. In this case the response occurs even through red-legged frogs and bullfrogs do not share a long evolutionary history; they have co-occurred for less than 70 years (Nussbaum et al., 1983).

The first objective of this study was to examine the responses of Pacific treefrogs (*Hyla regilla*) from west central Oregon to chemical cues of introduced adult bullfrogs. We tested two different populations of treefrogs, one of which is syntopic with bullfrogs and one that is allotopic from bullfrogs. Bullfrogs were introduced into Oregon in the 1930's (Nussbaum et al., 1983). The adults are predators that are known to feed on a variety of invertebrate and vertebrate prey including other amphibians (Bury and Whelan, 1986; Werner et al., 1995; Kiesecker and Blaustein, 1997a, 1998). In our study, we tested the response of post-metamorphic (hereafter juvenile) treefrogs. Although studies of larval amphibians to chemical cues of predators are widespread (e.g., Kiesecker et al., 1996; Kats and Dill, 1998), few studies have examined responses of post-metamorphic amphibians to predators (but see Flowers and Graves, 1997; Chivers et al., 1999). It is unknown whether chemosensory responses of post-metamorphic anurans are widespread.

A second objective of our study was to examine the responses of juvenile Pacific treefrogs from both populations to chemical alarm signals released from injured conspecifics. A wide variety of larval amphibians, including those of the Pacific treefrogs (Adams and Claeson, 1998), exhibit an avoidance response to chemical alarm cues (see review by Chivers and Smith, 1998). However, to our knowledge only one study (Chivers et al., 1999) has documented responses of post-metamorphic anurans to chemical alarm cues. In that study, western toads (*Bufo boreas*, Family Bufonidae) and red-legged frogs (Family Ranidae) responded to cues of injured conspecifics while those of Cascades frogs (*Rana cascadae*) did not. The absence of a response in juvenile Cascades frogs is particularly noteworthy because tadpoles are thought to respond to cues of injured conspecifics (Hews and Blaustein, 1985). Our study is the first to test whether metamorphic frogs from the Family Hylidae respond to cues of injured conspecifics.

MATERIALS AND METHODS

We tested juvenile treefrogs from each of two populations for a response to both cues of injured conspecifics and cues of introduced bullfrogs. Recently metamorphosed treefrogs from the first population were collected in the Willamette Valley at the E. E. Wilson Wildlife Refuge (16 km north of Corvallis, Benton County, Oregon). Bullfrogs are common in the Willamette Valley. The specific pond where the treefrogs were collected contains a breeding population of bullfrogs (pers. obs.). Treefrogs from the second population were collected from a pond in the Oregon Cascade Mountains (89 km east of Albany, Linn County, Oregon). Bullfrogs are absent from this location. The closest bullfrog population is approximately 25 km away from this site.

Treefrogs were collected in the summer and fall of 1996 and transported to Oregon State University for testing. We maintained the treefrogs individually in Petri dishes (150 mm diameter $\times$ 25 mm high) lined with damp paper towels. The frogs were kept on a 14 hr light:10 hr dark photoperiod at approximately 16°C for a minimum of two weeks prior to testing. Treefrogs were fed *ad libitum* with crickets.

Responses of Treefrogs to Cues of Bullfrogs. In these experiments we tested the avoidance response of individual treefrogs exposed to chemical cues from: 1) predatory adult bullfrogs and 2) non-predatory adult conspecifics. Testing the response of treefrogs for a response to non-predatory treefrogs was done as a control to determine whether an avoidance of the predator stimulus could be simply a general response to any amphibian cue. Experiments on the two populations were completed separately.

Bullfrogs spend considerable periods of time sitting on the banks of ponds, lakes, slow moving streams, and rivers. As a result they are in a good position to encounter treefrogs that are undergoing metamorphosis and leaving the water. Many amphibians, including treefrogs, may spend several days at the water/land interface before moving to land. Bullfrogs that spend time at the water's edge may also be in a good position to prey on adult treefrogs that return to breed. To prepare the bullfrog cue we placed a single bullfrog into a container that was filled with 3 l of dechlorinated tap water. The bullfrog was removed after 24 hr at which time the experiments began. We used two different bullfrogs (mass = 27.6 and 33.3 g) as alternative donors in our experiments. We prepared the treefrog stimulus in the same manner as the bullfrog stimulus. However, to prepare the treefrog stimulus we added either 7 or 8 treefrogs to the stimulus collection chamber to match the mass of the particular bullfrog that we used. Prior to collecting stimuli, the bullfrogs and treefrogs were maintained on a diet of crickets for at least 2 wk.

For each trial we lined half of a rectangular plastic container (32 $\times$ 18 $\times$ 8 cm) with paper towel that was moistened with dechlorinated tap water (control side). The treatment side was lined with paper towel that contained 5 ml of the bullfrog

or treefrog cue. The two paper towel substrates in each container were separated by approximately 2 cm to reduce contamination of chemicals between the sides. After the appropriate stimuli were added to each side of the test containers, we used a spray bottle containing dechlorinated tap water to saturate the paper towels. This ensured that any observed avoidance of the control or treatment sides was not attributable to differences in moisture level.

At the start of each trial, we introduced a single test animal into the center of the test container. Every 30 min for 2.5 hr we recorded whether the test animal was located on the control or treatment side of the container. In the event the treefrog was positioned across the middle of the container, we used the position of the snout to assign location. We rotated the containers 180° every half hour during the experiment to control for the possibility of a bias in the treefrogs' orientation in the room. Our observation schedule followed the design of Chivers et al. (1999). We made observations only every half hour because juvenile anurans will not move if they are disturbed by an observer.

For the Cascade Mountain population we tested 30 frogs in the control treatment and 30 in the experimental treatment. Our sample size for the Willamette Valley population was reduced to 27 and 25 in the bullfrog (experimental) and treefrog (control) treatments respectively. Individual treefrogs were used in only one test. For each trial, we summed the number of times each animal was located on the treatment side of the container out of a possible 5 observations (one observation every 30 min for 150 min = 5 observations). For both of the treatments, we compared whether treefrogs spent significantly less time than expected on the treatment side of the containers using a Wilcoxon Signed Rank test (Siegel and Castellan, 1988). Several researchers (e.g., Lutterschmidt et al., 1994; Chivers et al., 1996a, 1997, 1999) have used a similar bioassay for testing responses of terrestrial amphibians to chemical cues.

Responses of Treefrogs to Cues of Injured Conspecifics. In these experiments we tested the avoidance response of individual treefrogs exposed to chemical stimuli from: (1) injured juvenile conspecifics, and (2) non-injured juvenile conspecifics. Experiments on the two populations were completed separately. Testing the response of treefrogs to non-injured conspecifics was done as a control to determine whether any avoidance of the injured stimulus was a general response to any conspecific stimulus (Chivers et al., 1996a, 1997).

Treefrogs are subject to predation by many different predators, including frogs, snakes, birds and mammals (Nussbaum et al., 1983). The amount of alarm cues released onto the ground and surrounding vegetation during a predation event must be highly variable. In designing this experiment, we followed the methodology of Chivers et al. (1999) to prepare the injured treefrog stimulus. We dispatched ten animals by decapitation and removed their viscera, spinal column and legs. The tissue (approximately 4.5 g, which contained mostly skin, but also some muscle tissue) was homogenized with 150 ml of dechlorinated tap water in a blender. The

homogenate was filtered through a 0.5 mm mesh net. We used 5 ml of injured treefrog stimulus per trial. The stimulus was used within 30 min of preparation. For a source of chemical stimuli from non-injured treefrogs, we used moistened paper towels that had housed a single conspecific for 48 hr.

For each trial, we lined half of a rectangular plastic container (32 × 18 × 8 cm) with a paper towel that was moistened with dechlorinated tap water (control side). The treatment side was lined with a paper towel that contained cues from injured treefrogs or cues from non-injured treefrogs. We used the same experimental protocol and statistical analyses as in the previous experiments. For both populations of treefrogs, we tested the response of 30 individuals to cues of injured conspecifics and 30 individuals to cues of non-injured conspecifics. Individual treefrogs were used in only one test.

RESULTS

Treefrogs from both the Willamette Valley and Cascade Mountain populations showed an avoidance response to cues from injured conspecifics (Willamette Valley: $Z = 3.94$, $P < 0.001$; Cascade Mountain: $Z = 3.87$, $P < 0.001$; Figure 1), but not cues of non-injured conspecifics (Willamette Valley: $Z = 0.39$, $P = 0.70$; Cascade Mountain: $Z = 1.13$, $P = 0.26$; Figure 1).

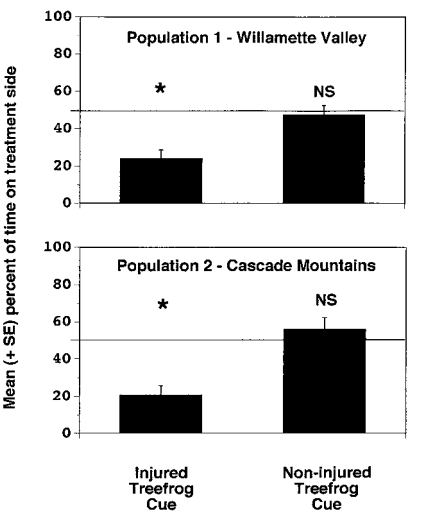


FIG. 1. Mean (+SE) percent time juvenile treefrogs from the Willamette Valley and Cascade Mountain populations spent on the treatment side of test chambers containing cues of injured conspecifics or non-injured conspecifics (NS indicates $P > 0.25$, * indicates $P < 0.001$).

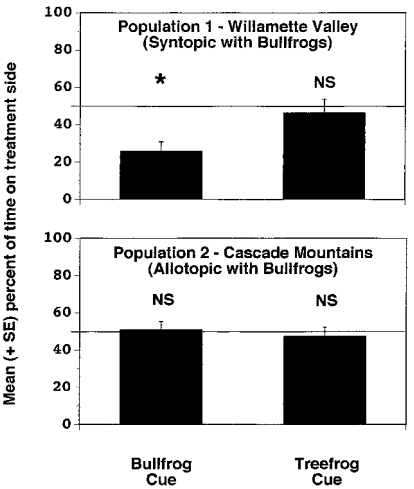


FIG. 2. Mean (+SE) percent time juvenile treefrogs from the Willamette Valley and Cascade Mountain populations spent on the treatment side of test chambers containing cues of predatory bullfrogs or non-predatory treefrogs (NS indicates $P > 0.50$, * indicates $P < 0.001$).

Treefrogs that co-occurred with bullfrogs in the Willamette Valley showed an avoidance response to cues of bullfrogs ($Z = 3.52$, $P < 0.001$, Figure 2), but did not show an avoidance response to cues of treefrogs ($Z = 0.58$, $P = 0.56$, Figure 2). In contrast, treefrogs from the Cascade Mountains did not show an avoidance response to cues of either bullfrogs ($Z = 0.02$, $P > 0.95$, Figure 2), or treefrogs ($Z = 0.57$, $P = 0.57$, Figure 2).

DISCUSSION

Our results show that juvenile treefrogs from two different populations use chemical cues to mediate predation risk. We observed avoidance responses of chemical alarm cues by treefrogs from both populations and avoidance of the bullfrog cues by treefrogs from the population syntopic with bullfrogs.

Numerous species of larval amphibians are known to exhibit antipredator behavior to chemical alarm cues (review Chivers and Smith, 1998). However, the responses of post-metamorphic anurans are less well known. Our results with Pacific treefrogs (Family Hylidae) show that the response to alarm cues is retained after metamorphosis. In our study, juvenile treefrogs from two distantly separated populations both exhibited avoidance of cues from injured conspecifics. Future studies should determine the amount of alarm cues released during predation events

and determine the threshold levels of responses exhibited by prey animals. This information would allow us to determine the scope of potential responses under natural conditions.

We did not have access to heterospecific frogs at the time of our study and hence could not test whether the avoidance of treefrogs to injured conspecific cues was a specific response to injured conspecifics or was a general response to injured amphibian cues. The avoidance of injured cues did not represent avoidance of a general amphibian cue. Treefrogs did not avoid cues of intact treefrogs. From a predation perspective it is of little importance if the avoidance of injured cues represents avoidance of injured conspecifics specifically or avoidance of injured amphibians in general. The important point is that by avoiding cues of injured conspecifics, treefrogs will decrease their probability of being captured. Chivers et al. (1999) used a similar approach to examine responses of juvenile red-legged frogs to alarm cues.

In our study, we observed that juvenile Pacific treefrogs from a population that co-occurred with bullfrogs exhibited an avoidance response to chemical cues of bullfrogs. The other population of treefrogs that had no contact with bullfrogs did not avoid the bullfrog cues. Inter-population differences in antipredator responses, whereby individuals from populations in which predators are common exhibit stronger antipredator responses than individuals from populations that experience lower levels of predation, have been reported in a variety of taxa (e.g., spiders: Riechert and Hedrick, 1990; salamanders: Ducey and Broodie, 1991; fishes: Seghers, 1974). However, few studies have documented population differences based on responses to chemical cues (but see Mathis et al., 1993). Our results most likely reflect that individuals from one population did not recognize the bullfrog cues, while individuals from the other population did recognize the bullfrog cues. However, our results could also reflect a population difference in the concentration threshold needed to elicit a response.

Population differences in antipredator responses may result from learning by prey in the high predation population (Mathis et al., 1993; Chivers and Smith, 1994). Several recent studies have documented that naïve prey animals can learn the identity of unknown predators by detecting conspecific alarm cues in the diet of the predator (Mathis and Smith, 1993; Chivers et al., 1996b). Population differences in anti-predator responses may also be genetically determined (Kiesecker and Blaustein, 1997a). The importance of genetics should increase if the predator and prey have a long evolutionary history together. Genetic changes can occur rapidly under natural conditions. For example, Reznick et al. (1990) documented changes in genetically determined life history traits in 30–60 generations. Pacific treefrogs and bullfrogs have co-occurred together in Oregon for only about 70 years (Nussbaum et al., 1983). For our experiments, we collected juvenile treefrogs.

Consequently, we do not know whether treefrogs from the population syntopic with bullfrogs have to learn that bullfrogs are a threat or whether this recognition is genetically determined. Future studies should differentiate these alternatives. We should also examine many populations of treefrogs with different densities of bullfrogs in order to determine whether responses vary with the intensity of predation or other site specific characteristics.

Many amphibian populations have been declining and undergoing range reductions over the last few decades (Blaustein and Wake, 1990; Stebbins and Cohen, 1995). The reasons for these declines are complex (Blaustein and Wake, 1995). Some studies have documented the decline of native frogs following the introduction of bullfrogs (Moyle, 1973; Kiesecker and Blaustein, 1998; Kupferburg, 1997). Pacific treefrogs appear to be one species that is not declining (Kiesecker and Blaustein, 1997a,b). This may be in part due to its success in recognizing and avoiding introduced predators such as bullfrogs. Responses of other amphibians to introduced bullfrogs remains mostly unknown (but see Kiesecker and Blaustein, 1997a). The success of treefrogs in regions now occupied by bullfrogs may stem from other factors besides their ability to recognize bullfrogs as predators. For example, treefrogs breed in a variety of habitats, including temporary ponds, where bullfrogs do not occur (Nussbaum et al., 1983).

The effects of introduced predators on native species are complex (Kiesecker and Blaustein 1997a, 1998). The ability of prey to recognize an introduced predator should minimize the prey's risk of capture. However, recognition of the predator does not by itself imply that there will not be significant predator effects. For example, Kiesecker and Blaustein (1997a) showed that red-legged frogs shift their microhabitat use in the presence of adult bullfrogs, and that this has a substantial cost in terms of decreasing growth rates and altering metamorphic characteristics, including time to and size at metamorphosis.

Acknowledgments—We thank Reehan Mirza and Lisa Belden for providing helpful comments on an earlier version of this manuscript. Funding was provided by the University of Saskatchewan, Oregon State University, Pennsylvania State University, NSERC of Canada and the National Science Foundation (Grant number DEB-9423333).

REFERENCES

ADAMS, M. J., and CLAESON, S. 1998. Field response of tadpoles to conspecific and heterospecific alarm. *Ethology* 104:955–962.

BLAUSTEIN, A. R., and WAKE, D. B. 1990. Declining amphibian populations: A global phenomenon? *Trends Ecol. Evol.* 5:203–204.

BLAUSTEIN, A. R., and WAKE, D. B. 1995. The puzzle of declining amphibian populations. *Sci. Am.* 272:52–57.

BURY, R. B., and WHELAN, J. A. 1986. Ecology and management of the bullfrog. U.S. Fish and Wildlife Service, Resource Publication 155:1–24.

CHIVERS, D. P., and SMITH, R. J. F. 1994. The role of experience and chemical alarm signalling in predator recognition by fathead minnows, *Pimephales promelas. J. Fish. Biol.* 44:273–285.

CHIVERS, D. P., KIESECKER, J. M., ANDERSON, M. T., WILDY, E. L., and BLAUSTEIN, A. R. 1996. Avoidance response of a terrestrial salamander (*Ambystoma macrodactylum*) to chemical alarm cues. *J. Chem. Ecol.* 22:1709–1716.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, R. J. F. 1996. Damselfly larvae learn to recognize predators from chemical cues in the predator's diet. *Anim. Behav.* 52:315–320.

CHIVERS, D. P., KIESECKER, J. M., WILDY, E. L., ANDERSON, M. T., and BLAUSTEIN, A. R. 1997. Chemical alarm signalling in terrestrial salamanders: Inter and intraspecific responses. *Ethology* 103:599–613.

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Écoscience* 5:338–352.

CHIVERS, D. P., KIESECKER, J. M., WILDY, E. L., BELDEN, L. K., KATS, L. B., and BLAUSTEIN, A. R. 1999. Avoidance response of post-metamorphic anurans to cues of injured conspecifics and predators. *J. Herp.* 33:472–476.

DUCEY, P. K., and BRODIE, E. D. Jr. 1991. Evolution of antipredator behaviour: Individual and population variation in a neotropical salamander. *Herpetologica* 47:89–95.

FLOWERS, M. A., and GRAVES, B. M. 1997. Juvenile toads avoid chemical cues from snake predators. *Anim. Behav.* 53:641–646.

HEWS, D. K., and BLAUSTEIN, A. R. 1985. An investigation of the alarm response in *Bufo boreas* and *Rana cascadae* tadpoles. *Behav. Neural Biol.* 43:47–57.

KATS, L. B., PETRANKA, J. W., and SIH, A. 1988. Anti-predator defenses and the persistence of amphibian larvae with fishes. *Ecology* 69:1865–1870.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals: *Écoscience* 5:361–394.

KIESECKER, J. M., CHIVERS, D. P., and BLAUSTEIN, A. R. 1996. The use of chemical cues in predator recognition by western toad (*Bufo boreas*) tadpoles. *Anim. Behav.* 52:1237–1245.

KIESECKER, J. M., and BLAUSTEIN, A. R. 1997a. Population differences in responses of red-legged frog tadpoles (*Rana aurora*) to introduced bullfrogs. *Ecology* 78:1752–1760.

KIESECKER, J. M., and BLAUSTEIN, A. R. 1997b. Egg laying behavior influences pathogenic infection of amphibian embryos. *Con. Biol.* 11:214–220.

KIESECKER, J. M., and BLAUSTEIN, A. R. 1998. Effects of introduced bullfrogs and smallmouth bass on the microhabitat use, growth and survival of native red-legged frogs. *Con. Biol.* 12:776–787.

KUPFERBERG, S. J. 1997. Bullfrog (*Rana catesbeiana*) invasion of a California river: The role of larval competition. *Ecology* 78:1736–1751.

LIMA, S. L., and DILL, L. M. 1990. Behavioral decisions made under the risk of predation: A review and prospectus. *Can. J. Zool.* 68:619–640.

LUTTERSCHMIDT, W. I., MARVIN, G. A., and HUTCHINSON, V. H. 1994. Alarm responses by a plethodontid salamander (*Desmognathus ochrophaeus*): Conspecific and heterospecific "Schreckstoff". *J. Chem. Ecol.* 20:2751–2759.

MATHIS, A., and SMITH, R. J. F. 1993. Fathead minnows, *Pimephales promelas*, learn to recognize northern pike, *Esox lucius*, as predators on the basis of chemical stimuli from minnows in the pike's diet. *Anim. Behav.* 46:645–656.

MATHIS, A., CHIVERS, D. P., and SMITH, R. J. F. 1993. Population differences in responses of fathead minnows (*Pimephales promelas*) to visual and chemical stimuli from predators. *Ethology* 93: 31–40.

MOYLE, P. B. 1973. Effects of introduced bullfrogs, *Rana catesbeiana*, on the native frogs of the San Joaquin Valley, California. *Copeia* 1973:18–22.

NUSSBAUM, R. A., BRODIE, E. D. JR., and STORM, R. M. 1983. Amphibians and reptiles of the Pacific Northwest. Univ. Idaho Press, Moscow, Idaho.

REZNICK, D. A., BRYGA, H., and ENDLER, J. A. 1990. Experimentally induced life-history evolution in a natural population. *Nature* 346:357–359.

RIECHERT, S. E., and HEDRICK, A. V. 1990. Levels of predation and genetically based antipredator behaviours in the spider, *Agelenopsis aperta, Anim. Behav.* 40:679–687.

SEGHERS,. B. H. 1974. Schooling behavior in the guppy (*Poecilia reticulata*): An evolutionary response to predation. *Evolution* 28:486–489.

SIH, A. 1987. Predator and prey lifestyles: An evolutionary and ecological overview, pp. 203–224, *in* Kerfoot, W. C. and SIH, A. (eds.). Predation: Direct and indirect impacts on aquatic communities. Univ. Press of New England, Hanover.

SIH, A., and KATS, L. B. 1994. Age, experience, and the response of streamside salamander hatchlings to chemical cues from predatory sunfish. *Ethology* 96:253–259.

SIEGEL, S., and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral Sciences, 2nd ed. McGraw-Hill, New York.

STEBBINS, R. C., and COHEN, N. W. 1995. A natural history of amphibians. Princeton Univ. Press, Princeton, New Jersey.

WERNER, E. E., WELLBORN, G. A., and MCPEEK, M. A. 1995. Diet composition in post metamorphic bullfrogs and green frogs: Implications for interspecific predation and competition. *J. Herp.* 29:600–607.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

CHEMICAL COMPOSITION OF PRECLOACAL SECRETIONS OF *Liolaemus* LIZARDS

CARLOS A. ESCOBAR, ANTONIETA LABRA, and HERMANN M. NIEMEYER*

Departamento de Ciencias Ecológicas
Facultad de Ciencias, Universidad de Chile
Casilla 653, Santiago, Chile

(Received October 24, 2000; accepted April 16, 2001)

Abstract—Interspecific chemical variation of precloacal pore secretions of *Liolaemus* lizards was characterized in 20 species, and intraspecific chemical variation was characterized using nine individuals of *L. bellii*. The latitude (Chile, 18° to 33° South latitude) and altitude (100 to 4350 m.a.s.l.) of the capture sites were recorded, as well as the number of precloacal pores of each lizard. Secretions were analyzed by GC-MS. A total of 49 compounds were found distributed among the 20 species of *Liolaemus*. Different chemical patterns occurred at intra- and interspecific levels. Compounds belonged to three main families: *n*-alkanes, long chain carboxylic acids, and steroids. Cholesterol and five carboxylic acids (tetradecanoic, hexadecanoic, hexadecenoic, octadecanoic, and *Z*-9-octadecenoic) appeared in all species. The number of precloacal pores correlated positively with altitude and negatively with latitude, suggesting that lizards produce more secretions under harsh environments.

Key Words—Chilean lizards, Tropiduridae, *Liolaemus*, precloacal pores, holocrine glands.

INTRODUCTION

Chemical communication is a well-documented phenomenon in different lizard genera (for reviews, see Mason, 1992; Font, 1996; Cooper, 1998). Pheromones are important for territory marking (e.g., Alberts, 1992), discrimination of familiar from unfamiliar individuals (e.g., Cooper, 1996), self-recognition (Graves and Halpern, 1991), and sexual recognition (Cooper et al., 1996). Although *Liolaemus* is a lizard genus with more than 150 species (Etheridge, 1995), little is known

*To whom correspondence should be addressed.

0098-0331/01/0800-1677$19.50/0

about the pheromones and chemical communication of its species. Chemical self-recognition and conspecific discrimination have been shown in *L. tenuis* (Labra and Niemeyer, 1999) and *L. bellii* (Labra et al., 2001). Potential sites of semiochemical production are the cloaca and the precloacal pores, the latter located in the external border of the cloacal shield and present mainly in males in the genus *Liolaemus* (Donoso-Barros, 1966). Given that precloacal pores have been described as producing secretions in lizards (Chauhan, 1986a, 1986b), and amphisbaenians (Antoniazzi et al., 1993, 1994), and that these secretions have pheromonal properties (Cooper et al., 1994; López et al., 1997), it is likely that precloacal secretions in *Liolaemus* also are involved in chemical communication. In fact, *Liolaemus* males are often seen dragging their cloaca over the substrate (Labra, personal observations), as has been observed in amphisbaenians (Chauhan, 1986b), a behavior allowing the release of secretions.

Proteins and lipids are the main compounds involved in chemical communication of squamates, although it is not clear which particular chemical compounds (or combination of them) are most relevant in this process. Although inter- and intraspecific variation in the protein content of femoral glands are claimed to be responsible for specific, self, and sex chemical recognition in different lizard species (Alberts, 1991; Alberts et al., 1993), most evidence points to lipids in pheromonal secretions as the main compounds involved in communication. The lipid content in the secretion of femoral glands (Alberts et al., 1992) and skin secretions (Mason and Gutzke, 1990) has been extensively studied, and *n*-alkanes, long chain carboxylic acids, and cholesterol and its derivatives have been found (Alberts, 1990; Weldon et al., 1990; Alberts et al., 1992). Bull et al. (1999) proposed that discrimination of individuals is determined by a complex relationship among different lipidic compounds found in feces, and Cooper and Garstka (1987) found that neutral lipid fractions of urodaeal glands secretions had pheronomal properties.

We report herein a characterization of the lipidic fraction of precloacal secretions of 20 *Liolaemus* species from different localities of Chile and from nine individuals of *L. bellii*, in order to evaluate the chemical patterns at the species and individual levels. The potential effect of environmental factors on the chemical composition of secretions was evaluated.

METHODS AND MATERIALS

Sample Collection. Liolaemus males were captured between October 1999 and February 2000, in northern and central Chile (Table 1). To collect secretions, males were placed backwards and pores were pressed gently with forceps. The yellow-reddish greasy secretion of each individual was dissolved in 400 μl purified *n*-hexane (see below), and placed individually in glass ampoules, which were

TABLE 1. MEAN NUMBER OF PRECLOACAL PORES (NP) OF 20 *Liolaemus* SPECIES, STANDARD ERRORS (SE), RANGE OF NUMBER OF PORES RECORDED, AND SAMPLE SIZE (*N*). CAPTURE SITES OF THE SPECIES INCLUDE THE ALTITUDE (M.A.S.L.) AND THE LATITUDE/LONGITUDE (°S/°W)

	Pores				Capture sites	
Liolaemus	np	SE	Range	*N*	Altitude	Latitude/Longitude
alticolor	2.75	0.48	2–4	4	4350	18°10'; 69°25'
bellii	2.00	0.14	1–3	20	2300	33°20'; 70°19'
bisignatus	2.33	0.33	2–3	3	710	26°08'; 70°35'
chiliensis	2.40	0.51	1–4	5	800	33°28'; 70°32'
constanzae	2.57	0.14	2–3	14	2250	23°46'; 68°14'
dorbigni	5.00		5	1	4250	22°36'; 68°03'
eleodori	5.16	0.40	4–7	6	3670	27°04'; 69°10'
fabiani	3.25	0.25	3–4	4	2450	23°23'; 68°21'
fitzgeraldi	2.40	0.16	2–3	10	2900	32°50'; 70°08'
fuscus	2.00	0.00	2	4	950	33°35'; 70°28'
hellmichi	2.66	0.33	2–3	3	100	23°32'; 70°21'
jamesi	5.73	0.12	5–6	15	4350	18°10'; 69°25'
lemniscatus	2.25	0.16	1–4	20	950	33°35'; 70°28'
monticola	2.11	0.08	2–3	18	1850	33°46', 70°15'
nigroroseus	2.14	0.14	2–3	7	2300	23°02'; 68°04'
nigroviridis	3.10	0.39	2–5	8	2300	33°20'; 70°19'
nitidus	1.62	0.18	1–2	8	950	33°35'; 70°28'
ornatus	6.64	0.38	5–10	14	3710	19°15'; 68°43'
platei	2.25	0.25	2–3	4	200	27°03'; 70°51'
tenuis	2.57	0.11	2–3	19	600	34°03'; 70°35'

sealed and kept at −18°C until use. After extraction of the secretion, lizards were released at their sites of capture. The chemical characterization of the secretion of each species was performed after mixing secretions from three randomly selected individuals.

Solvent Purification. The *n*-hexane (Merck, chromatography grade) used for dissolving the precloacal secretions was previously purified by stirring over H_2SO_4 overnight, decanting, and stirring over K_2CO_3 for two hours. The solution was filtered, dried ($MgSO_4$), and redistilled before use. The glass ampoules used to keep secretions were heated to 150°C for four days before use.

GC-MS Analysis of Secretion Samples. The chemical characterization of the secretion of each species started with a pool of 150 μg of secretion, 50 μg from each individual (except in the case of *L. dorbigni*, where 50 μg were used from the single individual available). An aliquot (ca. 20 μl) of the mixture dissolved in *n*-hexane, containing 50 μg of the mixed secretion was submitted to the derivatization procedure (see below). The chemical characterization of the secretion of each individual

of *L. bellii* was performed by submitting directly 50 μg of each individual's secretion dissolved in *n*-hexane (ca. 20 μl), to the derivatization procedure.

Derivatization was performed by treating 50 μg of the secretion in v-vials with excess (5 μl) of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), and heating the closed vial to 90°C for 60 min. Thereafter, vials were cooled on ice, and excess MSTFA eliminated under a stream of dry nitrogen. The solution was concentrated to 2 μl and injected in the GC-MS.

Analyses were performed in a capillary column (Ultra 2, 25m $\times$ 0.2 mm ID) directly coupled to a mass detector with an integrated data system (GC model HP-5890, MD model HP-5972). Ionization by electron impact (70 eV) was carried out at 280°C. The GC oven was programmed to remain at 50°C for 10 min, then to increase up to 280°C at a rate of 5°C/min, and to remain at 280° for 45 min. The presence or absence of a given compound in the chromatographic profile of each *Liolaemus* species or individual of *L. bellii*, was determined by coinjection of commercial standards and comparison of retention times and mass spectra. GC peaks of coinjected compounds were considered coincident if retention times did not differ by more than $\pm$ 0.03 min. When standards were not available, spectra were compared with a library database by a reverse search technique, which verifies that main peaks in the reference spectrum are present in the unknown spectrum (Pesyna et al., 1976). Spectra were considered coincident if the similarity index was higher than 95% (Pesyna et al., 1976). Alternatively, mass fragmentation patterns were used to determine tentative chemical structures.

Data Analysis. Pearson product-moment correlations (Zar, 1984) were used to test relationships between normalized chromatographic areas of the different compounds in different species and environmental variables (altitude and latitude of capture site), and lizard characteristics (mean number of precloacal pores, snout-vent length, and weight of individuals, and mean weight of precloacal pore secretions). Analysis of similitude of the chemical composition of the secretions using normalized chromatographic areas, were performed among species and among individuals of *L. bellii*. Clusters of species and individuals of *L. bellii* were estimated by using unweighted pair-group of arithmetic average (UPGMA) as a linkage rule (Manly, 1994). The use of the comparative method has been recommended for interspecific comparisons (Martins and Hansen, 1996). However, since the phylogeny hypothesis proposed by Shultte et al. (2000) included only 11 of the 20 *Liolaemus* species in the present study, and those 11 species did not encompass a wide range of environments, a phylogenetic analysis was not performed.

RESULTS

GC-MS Analysis. The mean total mass of the secretions was 1.02 mg (SE = 0.25; N = 58, corresponding to the secretions that were analyzed by

GC-MS). The part soluble in *n*-hexane constituted approximately 67.5% (SE = 2.6; $N = 20$) of the total mass of the secretion. Eighteen compounds were found in the nine individuals of *L. bellii* studied (Table 2). Three different groups of compounds were found: *n*-alkanes, representing 5.8% (SE = 1.0; $N = 9$); long chain carboxylic acids, representing 48.7% (SE = 5.5; $N = 9$), and steroids, representing 45.5% (SE = 5.9; $N = 9$). Tricosane, tetradecanoic acid, hexadecanoic acid, hexadecenoic acid, octadecanoic acid, *Z*-9-octadecenoic acid, eicosanoic acid, and cholesterol, were present in all individuals analyzed. Among the major compounds found in the secretions of *L. bellii* are hexadecanoic and *Z*-9-octadecenoic acids, and cholesterol. Compounds found in *L. bellii* were also present in the other species. From the 20 *Liolaemus* species studied, a total of 49 compounds were identified (Table 3), which belong to the same three categories described for *L. bellii*: *n*-alkanes, representing 18.6% (SE = 4.0; $N = 20$);

TABLE 2. CHEMICAL COMPOSITION OF THE PRECLOACAL SECRETIONS OF NINE INDIVIDUALS OF *Liolaemus bellii*

			Normalized chomatographic areas (%)[a]								
No.	Compound	Im[b]	1	2	3	4	5	6	7	8	9
	n-Alkanes										
1	tricosane	A	1.1	1.5	3.3	1.5	3.5	1.5	1.5	1.1	1.0
2	tetracosane	A	nd	6.0	2.5	3.7	nd	nd	3.7	6.4	4.3
3	pentacosane	A	1.7	5.7	1.4	0.7	nd	0.7	0.7	nd	1.6
	Carboxylic acids										
4	2-hydroxy-propanoic	B	5.1	2.3	0.6	1.1	2.7	2.9	nd	4.8	3.8
5	hexanoic	B	3.6	nd	nd	nd	nd	2.0	nd	3.4	0.4
6	dodecanoic	B	0.8	0.5	0.5	0.5	1.1	0.5	nd	0.8	0.2
7	tetradecanoic	B	2.5	1.3	1.1	1.8	2.0	1.4	1.8	2.4	2.1
8	hexadecanoic	A	11.8	13.7	14.0	8.6	15.8	6.7	8.6	11.3	11.1
9	hexadecenoic	C	3.2	5.4	2.0	1.0	2.3	1.8	1.0	3.0	2.7
10	heptadecanoic	B	0.8	2.6	2.0	nd	1.1	nd	nd	0.8	0.6
11	octadecanoic	A	5.1	7.5	6.2	2.8	10.3	2.9	2.8	4.9	4.5
12	*Z*-9-octadecenoic	A	5.7	24.7	36.9	15.1	36.4	15.4	15.1	5.4	7.4
13	eicosanoic	B	3.0	5.7	5.4	3.6	6.0	3.7	3.6	2.9	2.3
14	docosanoic	A	4.6	4.8	1.4	nd	1.6	nd	nd	4.4	3.3
15	butanedioic	B	1.4	nd	nd	nd	nd	nd	nd	1.4	0.3
	Steroids										
16	cholesterol	A	6.7	12.5	18.2	55.9	8.6	56.8	56.0	38.1	44.5
17	cholestan-3-ol	C	40.0	5.9	nd	3.7	8.6	3.7	3.6	6.3	6.2
18	cholest-4-en-3-ol	C	2.9	nd	4.5	nd	nd	nd	1.6	2.6	3.7

[a] Normalized chromatographic areas expressed as percent of total detected compounds. nd = compound not detected.

[b] Identification method (Im) codes: A = comparison with authentic compounds; B = GC-MS comparison between the recorded and library mass spectra with similarity index higher than 95%; C = fragmentation patterns in the mass spectra.

TABLE 3. CHEMICAL COMPOSITION OF PRECLOACAL SECRETIONS OF TWENTY *Liolaemus* SPECIES

No.	Compound	Normalized chromatographic areas (%)[a,b]																			
		Im[c]	alt	bel	bis	chi	con	dor	ele	fab	fit	fus	hel	jam	lem	mon	nig	nir	nit	orn	pla	ten
	n- Alkanes																					
1	decane	A	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.0	nd	nd	nd	nd	nd	nd
2	undecane	A	nd	nd	nd	nd	nd	nd	0.1	nd	nd	nd	nd	nd	nd	0.3	1.1	nd	0.4	nd	nd	nd
3	dodecane	A	nd	nd	nd	nd	nd	nd	0.2	nd	nd	34.1	nd	nd	nd	0.5	2.5	1.2	nd	0.3	nd	1.4
4	tridecane	A	0.2	nd	nd	nd	0.5	0.3	0.7	nd	nd	nd	1.7	nd	4.1	1.6	3.3	1.0	nd	1.2	nd	1.1
5	tetradecane	A	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.1	nd	nd	2.1	nd	nd	nd	2.6	nd	nd
6	pentadecane	A	nd	nd	0.3	0.1	0.8	nd	nd	nd	nd	nd	2.4	nd	1.2	1.7	1.8	0.8	0.6	1.5	3.0	1.2
7	hexadecane	A	nd	nd	nd	nd	1.1	nd	2.2	nd	nd	nd	4.1	nd	2.1	nd	3.4	1.4	nd	2.9	nd	1.4
8	heptadecane	A	nd	nd	nd	nd	2.3	2.4	4.8	nd	11.6	5.1	8.8	nd	3.2	5.9	6.4	2.4	nd	nd	nd	2.1
9	octadecane	A	nd	nd	nd	nd	nd	nd	6.5	nd	nd	nd	14.8	3.1	4.6	10.7	9.3	3.1	nd	nd	nd	1.6
10	nonadecane	A	nd	nd	nd	nd	nd	nd	3.5	nd	nd	nd	7.9	1.4	2.1	5.8	6.1	nd	nd	5.6	nd	nd
11	eicosane	A	nd	nd	1.7	nd	nd	nd	2.7	nd	nd	nd	7.5	nd	2.3	nd	nd	1.8	nd	6.4	0.5	0.7
12	heneicosane	A	nd	nd	0.8	nd	2.1	0.8	1.6	nd	nd	nd	4.8	0.9	nd	3.0	nd	nd	nd	4.1	1 nd	nd
13	docosane	A	nd	nd	nd	nd	2.9	1.2	1.3	nd	nd	nd	nd	1.5	18.9	nd	2.9	1.9	nd	5.1	nd	nd
14	tricosane	A	nd	1.1	0.8	1.3	2.1	0.7	1.2	nd	4.1	1.8	nd	1.5	1.6	nd	2.1	1.3	nd	3.5	1.9	2.7
15	tetracosane	A	nd	0.9	nd	nd	1.7	nd	0.7	nd	nd	nd	2.8	0.9	nd	nd	nd	nd	nd	1.5	nd	nd
16	pentacosane	A	nd	1.7	0.7	nd	1.9	nd	1.2	nd	nd	1.8	nd	nd	4.2	nd	0.5	1.2	nd	1.9	nd	nd
	Carboxylic acids																					
17	2-hydroxy-propanoic	B	nd	5.1	nd	nd	nd	4.6	9.2	7.7	5.0	nd	nd	nd	6.3	0.2	nd	6.7	0.2	0.5	12.8	2.5
18	hexanoic	B	nd	3.6	nd	nd	nd	0.4	nd	0.4	5.1	nd	nd	nd	nd	nd	nd	0.4	nd	0.2	nd	0.8
19	octanoic	B	0.3	nd	nd	nd	0.4	nd	nd	0.4	nd	nd	nd	nd	nd	nd	0.7	0.5	nd	nd	nd	1.0
20	nonanoic	B	0.2	nd	nd	nd	0.3	nd	nd	0.7	nd	nd	nd	nd	nd	nd	nd	0.4	nd	nd	nd	0.7

21	decanoic	B	0.2	nd	nd	nd	0.3	nd	nd	1.1	nd	nd	nd	nd	0.3	nd	nd	nd	nd	nd	nd	1.1
22	dodecanoic	B	1.0	0.8	nd	nd	2.6	0.8	1.0	nd	nd	nd	3.2	1.6	1.9	8.1	4.5	2.4	nd	5.8	nd	2.6
23	tetradecanoic	B	4.5	2.5	0.5	1.7	8.2	4.3	3.5	3.4	8.8	3.9	6.6	9.0	4.0	4.3	11.9	7.5	3.8	8.6	3.3	4.9
24	tetradecenoic	C	0.3	nd	nd	nd	1.5	nd	0.4	0.5	nd	nd	nd	nd	nd	nd	nd	0.9	nd	nd	nd	nd
25	tetradecanoic, methyl ester	B	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2.1	nd	nd	nd
26	hexadecanoic	A	41.9	11.9	13	32.6	29.2	14.3	2.4	9.9	32.1	23.7	5.3	1.8	10.6	3.8	19.0	24.5	14.0	17.2	26.0	21.1
27	hexadecanoic, methyl ester	B	nd	nd	0.2	nd	nd	nd	3.8	5.3	nd	nd	nd	1.4	1.4	nd	nd	8.5	15.9	nd	19.2	nd
28	hexadecenoic	C	16.6	3.2	7.6	4.9	9.1	4.1	3.0	3.9	5.8	0.2	1.1	4.7	3.7	4.5	7.9	8.1	3.6	11.5	0.9	5.8
29	heptadecanoic	B	0.9	0.8	nd	1.0	1.7	0.7	0.5	nd	1.6	nd	0.6	0.1	0.6	1.0	nd	1.4	nd	2.8	nd	0.8
30	heptadecenoic	C	1.5	nd	nd	nd	nd	nd	0.7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
31	octadecanoic	A	7.8	5.1	0.8	9.0	8.6	4.3	4.8	3.5	12.5	10.3	2.8	0.7	1.7	6.2	2.4	5.5	6.2	4.3	10.9	6.0
32	octadecanoic, methyl ester	B	nd	nd	nd	nd	nd	nd	1.2	2.5	nd	nd	nd	nd	4.3	nd	nd	3.1	10.3	nd	3.6	nd
33	z-9-octadecenoic	A	12.5	5.7	2.1	7.5	12.6	5.6	5.0	5.4	12.8	2.9	1.7	0.2	2.9	11.9	3.9	12.2	6.9	9.4	4.5	17.8
34	eicosanoic	B	0.4	3.0	6.4	12.1	2.8	3.3	4.5	11.3	nd	10.3	1.8	0.7	5.5	3.3	2.6	nd	2.3	nd	nd	4.9
35	eicosanoic, methyl ester	B	nd	nd	nd	nd	nd	nd	1.4	6.4	nd	nd	nd	nd	nd	nd	nd	0.4	8.7	nd	2.2	nd
36	docosanoic	A	10.5	4.6	4.7	4.8	1.9	nd	6.0	nd	nd	4.8	1.3	0.7	3.4	4.3	0.7	nd	nd	nd	nd	5.2
37	docosanoic, methyl ester	B	nd	nd	nd	nd	nd	nd	nd	9.0	nd	nd	nd	nd	nd	nd	nd	nd	6.2	nd	nd	nd
38	tetracosanoic	A	nd	nd	nd	2.8	nd	nd	nd	nd	nd	nd	nd	0.4	nd	nd	nd	nd	nd	nd	nd	nd
39	tetracosanoic, methyl ester	B	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.9	nd	nd	nd
40	hexacosanoic	B	nd	nd	nd	0.7	nd	nd	1.4	nd	nd	nd	nd	0.1	nd	nd	nd	nd	nd	nd	nd	nd
41	hexacosanoic, methyl ester	B	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.6	nd	nd	nd
42	butanedioic	B	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	07	nd	nd
43	octadecadienoic, methyl ester	C	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.2	nd	nd	nd	nd	nd	nd	nd

(*Continued*)

TABLE 3. CONTINUED

No.	Compound	Im[c]	Normalized chromatographic areas (%)[a,b] alt	bel	bis	chi	con	dor	ele	fab	fit	fus	hel	jam	lem	mon	nig	nir	nit	orn	pla	ten
	Steroids																					
44	cholesterol	A	0.9	6.7	52.98	2.6	4.1	50.6	24.3	28.7	0.7	1.1	17.5	9.4	3.8	20.0	6.7	1.3	15.4	1.7	11.0	0.8
45	cholest-4-en-3-one	C	0.2	nd	nd	nd	nd	0.1	nd	nd	nd	nd	nd	59.8	nd	nd	nd	nd	nd	0.7	nd	nd
46	β-sitosterol	A	0.2	nd	nd	nd	1.4	1.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
47	cholestan-3-ol	C	nd	40.2	nd	13.6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
48	3,5-dihydroxy-cholestane	C	nd	nd	nd	5.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
49	cholest-4-en-3-ol	C	nd	2.8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	11.8

[a] Normalized chromatographic areas expressed as percent of total detected compounds; mass spectra with similarity index higher than 95%; C = fragmentation patterns in the mass spectra.

[b] *Liolaemus* species: alt = *L. alticolor*; bel = *L. bellii*; bis = *L. bisignatus*; chi = *L. chiliensis*; con = *L. constanzae*; dor = *L. dorbigni*; ele = *L. eleodori*; fab = *L. fabiani*; fit = *L. fitzgeraldi*; fus = *L. fuscus*; hel = *L. hellmichi*; jam = *L. jamesi*; lem = *L. lemniscatus*; mon = *L. monticola*; nig = *L. nigroviridis*; nir = *L. nigroroseus*; nit = *L. nitidus*; orn = *L. ornatus*; pla = *L. platei* and ten = *L. tenuis*.

[c] Identification methods (Im): A = comparison with authentic compounds; B = GC-MS comparison between recorded and library. C = Fragmentation patterns in the mass spectra (as trimethylsilyl derivatives if not otherwise stated): tetradecenoic acid, ms: m/z 55 (60), 73 (100), 81 (17), 117 (55), 145 (21), 166 (9), 208 (5), 283 (35), 298 (M^+, 4); hexadecenoic acid, ms: m/z 55 (50), 73 (100), 117 (63), 129 (49), 185 (6), 194 (10), 236 (8), 267 (2), 311 (53), 326 (M^+,8); heptadecenoic acid, ms: m/z 55 (40), 75 (100), 84 (14), 96 (20), 117 (60), 129 (31), 145 (17), 221 (7), 250 (4), 281 (10), 325 (25), 340 (M+, 3); octadecadienoic acid methyl ester, ms: m/z 55 (64), 57 (100), 81 (83), 95 (56), 109 (30), 121 (22), 135 (19), 150 (21), 164 (10), 220 (10), 262 (8), 294 (M^+,33); cholest-4-en-3-one, ms: m/z 55 (52), 69 (37), 95 (39), 124 (100), 147 (28), 229 (39), 271 (22), 298 (23), 342 (16), 384 (M+, 47); cholestan-3-ol, ms: m/z 55 (67), 81 (58), 95 (60), 121 (30), 149 (28), 165 (63), 194 (8), 215 (70), 233 (80), 248 (23), 262 (15), 331 (5), 355 (22), 373 (27), 388 (M^+, 100); dihydroxy-cholestane, ms: m/z 73 (69), 95 (50), 107 (37), 121 (31), 129 (100), 145 (28), 213 (14), 247 (14), 255 (11), 329 (65), 353 (28), 368 (42), 458 (M^+,19); cholest-4-en-3-ol, ms: m/z 55 (67), 78 (65), 95 (53), 107 (60), 119 (40), 133 (18), 147 (42), 161 (25), 173 (13), 213 (35), 229 (30), 255 (69), 273 (19), 353 (7), 371 (28), 386 (M^+,100).

long chain carboxylic acids, representing 61.5% (SE = 4.8; $N = 20$), and steroids, representing 19.9% (SE = 4.6; $N = 20$). All the species shared six compounds: cholesterol and five carboxylic acids (tetradecanoic, hexadecanoic, hexadecenoic, octadecanoic, and Z-9-octadecenoic). Hexadecanoic acid was the main compound of the carboxylic acid fraction, and cholesterol was the major steroidal compound.

Environmental Relationships. The number of precloacal pores (np) negatively correlated with the latitude ($r = -0.61$; $P = 0.004$) and positively correlated with the altitude ($r = 0.67$; $P = 0.001$) of the collection site. Different correlations were attempted among the normalized chromatographic areas of the different compounds and environmental or specific lizard characteristics. Nevertheless, the only significant correlations found were between the normalized chromatographic area of hexadecenoic acid and altitude ($r = 0.49$; $P = 0.028$), and latitude ($r = -0.44$; $P = 0.050$) of the collection site. Furthermore, for each species, the volatility of the secretion was estimated by using a melting point index (MPI) defined as the sum of the products between the normalized chromatographic areas and the melting point of each compound present in the secretion. No significant correlations were found between MPI and the environmental variables tested.

Similitude Analysis. The similitude analysis (UPGMA) performed with nine individuals of *L. bellii* (Figure 1A) shows the existence of variation in chemical composition at the individual level. A similitude analysis was performed with all 20 species together (Figure 1B). The relationship between the species could not be related to their phylogeny (Schulte et al., 2000).

DISCUSSION

Pheromone research in squamates is a relatively recent endeavor. The most precise knowledge of pheromonal compounds come from studies in snakes, where the nature of the active compounds has been elucidated in some cases (Mason, 1992, 1999; Mason et al., 1989). For lizards, most studies have dealt with the description of components that are part of secretions that are claimed to have pheromonal properties, rather than with the nature of the active compounds (Chauhan, 1986b; Alberts, 1990; Mason and Gutzke, 1990; Weldon et al., 1990; Alberts et al., 1992).

In all the *Liolaemus* species studied herein, the families of chemical components in the lipidic fractions of the precloacal secretions are similar to those reported for other lizards species, i.e., *n*-alkanes, carboxylic acid, and steroids (Chauhan, 1986b; Mason and Gutzke, 1990; Alberts et al., 1992). In *L. bellii*, the similitude analysis (Figure 1A) indicates that there are no two individuals with secretions having the same chemical composition. These differences among individuals may be important for self-recognition.

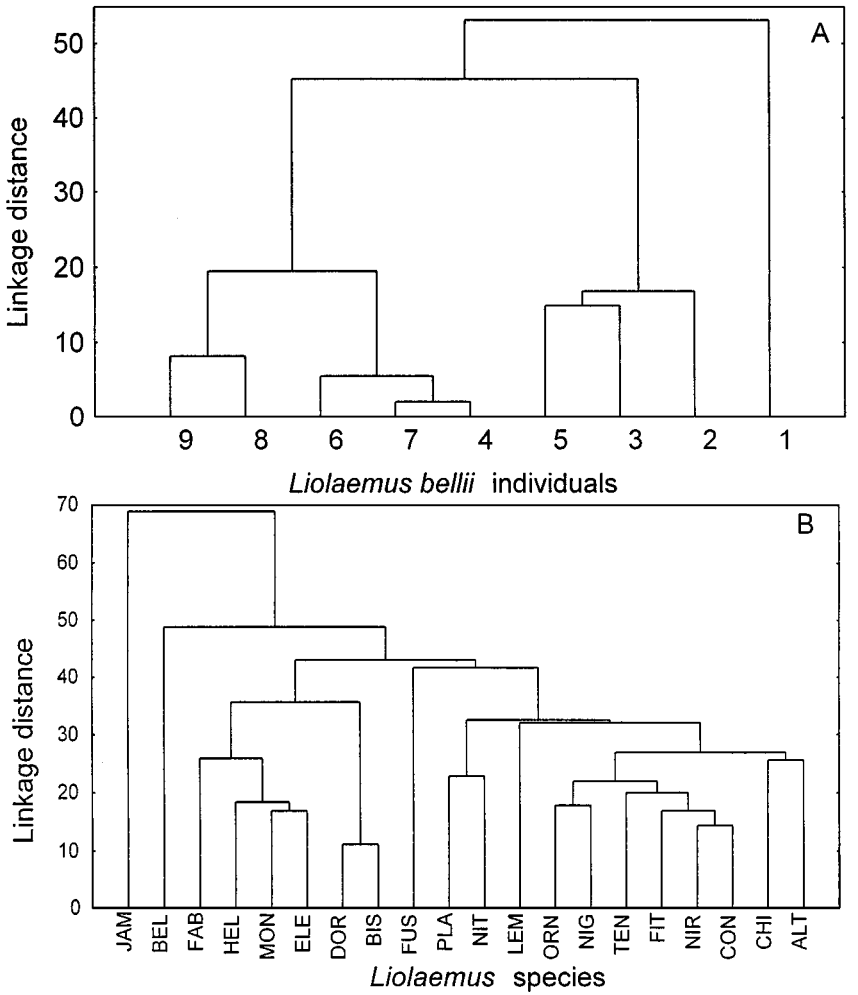


FIG. 1. Similitude analysis (UPGMA) of the normalized chromatographic areas of the different compounds found in the secretions of nine individuals of *Liolaemus bellii* (A) and in the secretions of 20 *Liolaemus species* (B). See Table 3 for meaning of abbreviations in Figure 1B.

The secretions of precloacal pores in *Liolaemus* contained a series of *n*-alkanes. In earlier studies with snake skin, these compounds were associated with sample contamination (Ahern and Downing, 1974), although it is now accepted that *n*-alkanes are components of saurian skin secretions (Weldon and Bagnall, 1987; Mason, 1992). Most of the *n*-alkanes of *Liolaemus* had an odd

number of carbon atoms. Since hydrocarbons of biogenetic origin generally consist of a greater proportion of odd over even number of carbon atoms (Weldon et al., 1990), *n*-alkanes in our samples are likely to be of biogenetic origin rather than a product of contamination. Nevertheless, the exact function of *n*-alkanes in *Liolaemus*, as well as in other lizard species (Mason and Gutzke, 1990), remains unknown.

Long chain carboxylic acids are common constituents of lizards skin and femoral glands (Mason and Gutzke, 1990; Alberts et al., 1992), and now they are reported in precloacal secretions of *Liolaemus* lizards. Carboxylic acids in *Liolaemus* belong to two categories: those common to all species analyzed (i.e., tetradecanoic, hexadecanoic, hexadecenoic, octadecanoic, and Z-9-octadecenoic), and those that are randomly distributed and present in minor amounts across the species (heptadecanoic, eicosanoic, and docosanoic). As previously suggested for snakes (Burken et al., 1985), compounds shared by different species may have a common function in all of them. Therefore, the five ubiquitous carboxylic acids may provide information related to genus status of the lizards (i.e., *Liolaemus*), while the randomly distributed carboxylic acids may be involved in specific discrimination, or species-specific functions.

The ubiquitous and most abundant carboxylic acid in *Liolaemus* secretions was hexadecanoic acid, also found in femoral secretions of *Iguana iguana* in breeding and nonbreeding seasons (Alberts et al., 1992). However, *Liolaemus* secretions showed differences with respect to previous records in squamates. In fact, hexanoic and nonanoic acids are reported for first time in squamate secretions. In addition, an important difference of *Liolaemus* secretions is the presence of compounds of lower molecular mass than compounds in other squamate species. Thus, in the leopard gecko, *n*-alkanes ranged from C_{18} to C_{32} and carboxylic acids from *n*-C_{16} to *n*-C_{18} (Mason, 1990); in *Iguana iguana*, carboxylic acids ranged from *n*-C_{14} to *n*-C_{26} (Alberts, 1992) and in the Florida indigo snake, carboxylic acids ranged from *n*-C_{12} to *n*-C_{18} (Ahern and Downing, 1974). In contrast, *Liolaemus* *n*-alkanes ranged from C_{10} to C_{25} and carboxylic acids from *n*-C_3 to *n*-C_{26}. Additional comparative research will be necessary to explain the biological implications of the presence of more volatile compounds in *Liolaemus* than in other squamates.

In squamates, precloacal (Chauhan, 1986a; Antoniazzi et al., 1993; Jared et al., 1999) and femoral glands (Weldon et al., 1990) have been described as exocrine organs producing a holocrine secretion (Alberts, 1990), which is constituted, among others, by long chain carboxylic acids (myristic, palmitic, stearic, and oleic acids), compounds normally present in internal tissues (Nicolaides, 1974). The presence of these carboxylic acids in the precloacal secretions of *Liolaemus* suggests the holocrine nature of the gland.

Cholesterol is of frequent occurrence in secretions involved in chemical communication, having been found in lizard skin (Weldon and Bagnall, 1987; Mason and Gutzke, 1990), femoral glands (Alberts et al., 1992) and now in precloacal pores in *Liolaemus* species. It has been suggested that steroids identified in lizards,

although not being sex steroids, may still give information about the sex of the individual (Mason and Gutzke, 1990). Since *Liolaemus* precloacal secretions analyzed were only from males, it may be speculated that the function of cholesterol derivatives is to provide information about the condition of the male, for example its dominance status or genetic quality (e.g., Martin and López, 2000).

It may be hypothesized that lizards deposit on the substrate a given "effective" amount of pheromones, which allows for efficient communication. However, these "effective" amounts may depend on the interplay of environmental or climatic conditions, and the volatility and chemical characteristics of the semiochemical. Thus, it would be expected that under conditions of high temperature, wind, and low atmospheric pressure, such as occur at higher altitudes and lower latitudes among the collecting sites studied, lizards would be equipped to produce higher quantities of secretion, or compounds in the secretions would be less volatile and chemically more stable. For *Liolaemus*, the main strategy seems to be the first one, given the significant relationships between number of precloacal pores with the altitude and latitude. Interestingly, the most volatile and one of the most chemically reactive members of the carboxylic acid family showed a negative correlation with latitude, and a positive one with altitude. However, the lack of correlation, between melting point index and both altitude and latitude, indicates that environmental conditions did not impose major changes in the pattern of chemical characteristics of the secretions.

Acknowledgments—This work was supported by the International Foundation for Science (IFS) through grant F 2933-1 awarded to AL and F 2934-1 awarded to CAE; FONDECYT through a postdoctoral fellowship awarded to AL; the Presidential Chair in Sciences awarded to HMN, and the International Program in the Chemical Sciences (IPICS). This work was part of the activities of the Center for Advanced Studies in Ecology and Research in Biodiversity funded by the Millennium Scientific Iniciative (P099-103F ICM). The authors thank the Servicio Agrícola y Ganadero (SAG), for lizard capture permit No. 992 (April, 1998) and No. 3112 (October 1999), and Corporación Nacional Forestal (CONAF) for permission No. 03 (December 1999), to capture lizards in protected areas.

REFERENCES

AHERN, D. G., and DOWNING, D. T. 1974. Skin lipids of the Florida indigo snake. *Lipids* 9:8–14.

ALBERTS, A. C. 1990. Chemical properties of femoral gland secretions in the desert iguana, *Dipsosaurus dorsalis. J. Chem. Ecol.* 16:13–25.

ALBERTS, A. C. 1991. Phylogenetic and adaptive variation in lizard femoral gland secretions. feromonal self-recognition in desert iguanas. *Copeia* 1991:69–79.

ALBERTS, A. C. 1992. Pheromonal self-recognition in desert iguanas. *Copeia* 1992:229–232.

ALBERTS, A. C., PHILLIPS, J. A., and WERNER, D. I. 1993. Sources of intraspecific variability in the protein composition of lizard femoral gland secretions. *Copeia* 1993:775–781.

ALBERTS, A. C., SHARP, T. R., WERNER, D. I., and WELDON, P. J. 1992. Seasonal variation of lipids in femoral gland secretions of male green iguanas (*Iguana iguana*). *J. Chem. Ecol.* 18:703–712.

ANTONIAZZI, M. M., JARED, C., PELLEGRINI, C. M. R., and MACHA, N. 1993. Epidermal glands in Squamata: Morphology and histochemistry of the precloacal glands in *Amphisbaena alba* (Amphisbaenia). *Zoomorphology (Berlin)* 113:199–203.

ANTONIAZZI, M. M., JARED, C., and JUNQUEIRA, L. C. U. 1994. Epidermal glands in Squamata: fine structure of pre-cloacal glands in *Amphisbaena alba* (Amphisbaenia, Amphisbaenidae). *J. Morphol.* 221:101–109.

BULL, M. C., GRIFFIN, C. L., and PERKINS, V. 1999. Some properties of a pheromone allowing individual recognition, from the scats of an Australian lizard, *Egernia striolata. Acta Ethologica* 2:35–42.

BURKEN, R. R., WERTZ, P. W., and DOWNING, T. D. 1985. A survey of polar and nonpolar lipids extracted from snake skin. *Biochem. Physiol.* 81B:315–318.

CHAUHAN, N. B. 1986a. Histological and structural observations on pre-anal glands of the gekkonid lizard, *Hemidacttylus flaviviridis. J. Anat.* 144:93–98.

CHAUHAN, N. B. 1986b. A preliminary report on the lipid components of pre-anal gland secretion of lizards *Hemidactylus flaviviridis* and *Uromastix hardwickii. J. Anim. Morphol. Physiol.* 33:73–76.

COOPER, W. E. 1996. Chemosensory recognition of familiar and unfamiliar conspecifics by the scincid lizard *Eumeces laticeps. Ethology* 102:454–464

COOPER, W. E. 1998. Evaluation of the swap and related tests as a bioassay for assessing responses by squamata reptiles to chemical stimuli. *J. Chem. Ecol.* 24:841–866.

COOPER, W. E., and GARSTKA, W. R. 1987. Lingual responses to chemical fractions of urodaeal glandular pheromones of the skink *Eumeces laticeps. J. Exp. Zool.* 242:249–253.

COOPER, W. E. J., LÓPEZ, P., and SALVADOR, A. 1994. Pheromones detection in amphisbaenian. *Anim. Behav.* 47:1401–1411.

COOPER, JR., W. E., VAN WYK, J. H., and MOUTON, P. LE F. N. 1996. Pheromonal detection and sex discrimination of conspecific substrate deposits by the rock-dwelling cordylid lizard, *Cordylus cordylus. Copeia* 1996:839–845.

DONOSO-BARROS, R. 1966. Reptiles de Chile. Editorial Universitaria, Universidad de Chile, Santiago, Chile. 458 pp.

ETHERIGDE, R. 1995. Redescription of *Ctenoblepharys adspersa* Tschudi, 1845, and the taxonomy of Liolaeminae (Reptilia: Squamata: Tropiduridae). *Amer. Mus. Novitates* 3142:1–34.

FONT, E. 1996. Los sentidos químicos de los reptiles. Un enfoque etológico, pp. 197–259, *in* Colmenares, F. (ed.). *Etología, Psicología Comparada y Comportamiento Animal*. Madrid: Síntesis Psicológica. Editorial Síntesis S. A.

GRAVES, B. M., and HALPERN , M. 1991. Discrimination of self from conspecific chemical cues in *Tiliqua scincoides* (Sauria: Scincidae). *J. Herpetol.* 25:125–126.

JARED, C., ANTONIAZZI, M. M., SILVA, J. R. M. C., and FREYMULLER, E. 1999. Epidermal glands in squamata: Microscopical examination of precloacal glands in *Amphisbaena alba* (Amphisbaenia, Amphisbaenidae). *J. Morphol.* 241:197–206.

LABRA, A., and NIEMEYER, H. M. 1999. Intraspecific chemical recognition in the lizard *Liolaemus tenuis. J. Chem. Ecol.* 25:1799–1811.

LABRA, A., BELTRÁN, S., and NIEMEYER, H. M. 2001. Chemical exploratory behavior in the lizard *Liolaemus bellii. J. Herpetol.* (in press)

LÓPEZ, P., SALVADOR, A., and COOPER, JR., W. E. 1997. Discrimination of self from other males by chemosensory cues in the amphisbaenian *Blanus cinereus*. *J. Comp. Psych*. 111:105–109.

MANLY, B. F. J. 1994. Multivariate Statistical Methods. A Primer. Chapman & Hall, London.

MARTÍN, J., and LÓPEZ, P. 2000. Chemoreception, symmetry and mate choice in lizards. *Proc. R. Soc. Lond.* B267:1265–1269.

MARTINS, E. P., and HANSEN, T. F. 1996. The statistical analysis of interspecific data: a review and evaluation of phylogenetic comparative method, pp. 22–75, *in* E. Martins (ed.). *Phylogenies and the Comparative Method in Animal Behavior.* Oxford University Press, Oxford.

MASON, R. T. 1992. Reptilian pheromones, pp. 114–228, *in* C. Gans, and D. Crews (eds.). *Hormones, Brain and Behavior. Biology of Reptilia*, Vol 18 E. The University Chicago Press, Chicago.

MASON, R. T. 1999. Integrated pest management: The case for pheromonal control of habu (*Trimeresurus flavoviridis*) and brown tree snakes (*Boiga irregularis*), pp. 196–205, *in* G. H. Rodda, Y. Sawai, D. Chiszar, and H. Tanaka (eds.). *Problem Snake Management: the Habu and the Brown Tree Snake*. Cornell University Press.

MASON, R. T., FALES, H. M., JONES, T. H., PANNELL, L. K., CHINN, J. W., and CREWS, D. 1989. Sex pheromones in snakes. *Science* 245:290–293.

MASON, R. T., and GUTZKE, W. H. N. 1990. Sex recognition in the leopard gecko, *Eublepharis macularius* (Sauria: Gekkonidae) possible mediation by skin-derived semiochemicals. *J. Chem. Ecol.* 16:27–36.

NICOLAIDES, N. 1974. Skin lipids: their biochemical uniqueness. Science 186:19–26.

PESYNA, G. M., VENKATARAGHAVAN, R., DAYRINGER, H. E., and MCLAFFERTY, F. W. 1976. Probability based matching system using a large collection of reference mass spectra. *Anal. Chem*. 48:1362–1368.

SCHULTE, J. A., MACEY, J. R., ESPINOZA, R. E., and LARSON, A. 2000. Phylogenetic relationships in the iguanid lizard genus *Liolaemus*: multiple origins of viviparous reproduction and evidence for recurring Andean vicariance and dispersal. *Biol. J. Linnean Soc*. 69:75–102.

WELDON, P. J., and BANGALL, D. 1987. A survey of polar and nonpolar skin lipids from lizards by thin-layer chromatography. *Comp. Biochem. Physiol*. 87B:345–349.

WELDON, P. J., DUNN, B. S., MCDANIEL, C. A., and WERNER, D. I. 1990. Lipids in the femoral gland secretions of the green iguana (*Iguana iguana*). *Comp. Biochem. Physiol*. 95B:541–543.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice-Hall International, Englewood Cliffs, New Jersey, 718 pp.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

ALLELOCHEMICALS IN WHEAT (*Triticum aestivum* L.): PRODUCTION AND EXUDATION OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE

HANWEN WU,[1,5]* TERRY HAIG,[2,5] JAMES PRATLEY,[1,5] DEIRDRE LEMERLE,[4,5] and MIN AN[3]

[1]*Farrer Centre for Conservation Farming, hwu@csu.edu.au*

[2]*School of Science and Technology, and*

[3]*Environmental and Analytical Laboratories, Charles Sturt University, PO Box 588, Wagga Wagga NSW 2678, Australia*

[4]*NSW Agriculture, Wagga Agricultural Institute, Wagga Wagga, NSW 2650, Australia*

[5]*Cooperative Research Centre for Weed Management Systems*

(Received August 10, 2000; accepted April 9, 2001)

Abstract—An analytical technique employing gas chromatography and tandem mass spectrometry (GC/MS/MS) was employed to systematically screen fifty-eight wheat accessions for their differential production of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) from three consecutive sources, i.e., the shoots, roots, and in the associated agar growth medium (collected as root exudates) of 17-day-old wheat seedlings. DIMBOA content differed significantly in the shoots, roots, or in the agar growth medium between accessions. DIMBOA accumulated differentially within the plant, with roots containing more DIMBOA than the shoots. Only 19% of accessions were able to exude DIMBOA from living roots into their growth medium, indicating the exudation of DIMBOA is accession-specific. DIMBOA level in root tissues is expected to be high when a high level of DIMBOA content is detected in the shoots. Wheat seedlings did not release detectable amounts of DIMBOA when the DIMBOA level was low in the root tissues. The valuable genetic material with high levels of DIMBOA in the shoots or roots identified in the present research could be used to breed for wheat cultivars with elevated allelopathic activity.

Key Words—Wheat (*Triticum aestivum* L.), allelochemicals, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, root exudates; weed suppression.

*To whom correspondence should be addressed.

INTRODUCTION

Cyclic hydroxamic acids (Hx), a novel class of alkaloids, have been claimed as natural defense agents against bacteria, fungi and insects (Bohidar et al., 1986; Niemeyer, 1988a; Leszczynski et al., 1995; Figueroa et al., 1999; Wilkes et al., 1999). Hx are also associated with allelopathic activity for weed suppression (Pérez, 1990; Blum et al., 1992). DIMBOA and its decomposition product 6-methoxy-benzoxazolin-2-one (MBOA) inhibited root growth of wild oats (*Avena fatua*) by 50% at a concentration of 0.7 and 0.5 mM, respectively. MBOA also inhibited seed germination of *A. fatua* (Pérez, 1990). Blum et al. (1992) found that MBOA was more potent than its precursor (DIMBOA) and inhibited germination, radicle and hypocotyl length of *Trifolium incarnatum* and *Ipomoea hederacea*.

The hydroxamic acids have been found in a wide range of Gramineae, including wheat (*Triticum aestivum*) (Niemeyer, 1988a,b). The most abundant of these acids in wheat is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Bohidar et al., 1986). In the search for wheat accessions with a high level of DIMBOA for integrated pest management, intensive research has been undertaken to determine the differential production of DIMBOA in shoots/leaves of *Triticum* spp. In a direct screening of 55 accessions of 17 wheat progenitors, Niemeyer (1988b) found that DIMBOA was highest in *T. speltoides* (16.0 mmol/kg fw) and lowest in *T. tauschii* (0.21 mmol/kg fw). In a collection of 52 Chilean cultivars of *T. aestivum* and *T. durum*, Copaja et al. (1991) demonstrated that cultivars differed significantly in the production of DIMBOA, ranging from 1.4 to 10.9 mmol/kg fw Nicol et al. (1992) extended the screening work to a worldwide collection of 47 cultivars of *Triticum* (mainly *T. aestivum*), and found that the contents of DIMBOA in the shoots ranged from 0.99 to 8.07 mmol/kg fw. These results have provided valuable information on potential genetic material with high levels of DIMBOA that could be used to enhance allelopathy in wheat breeding programmes.

Further research has shown that the DIMBOA content in wheat shoot tissues is genetically inheritable. Chromosomes 4A, 4B and 5B may contain genes for DIMBOA synthesis, and chromosome 4D may contain genes inhibiting the accumulation of DIMBOA (Niemeyer and Jerez, 1997). Genetic studies of DIMBOA in maize (*Zea mays*) have also demonstrated that the accumulation of DIMBOA could be monogenic or polygenic depending on particular populations (Klun et al., 1970; Dunn et al., 1981; Simcox and Weber, 1985). Studies on the genetic control of DIMBOA could further facilitate the genetic manipulation of DIMBOA levels in wheat.

Research so far has only concentrated on the evaluation of DIMBOA content in shoots or leaves of young wheat seedlings. Little data have been accumulated for DIMBOA production in roots, and for exudation of DIMBOA by living roots

of wheat seedlings into the growth medium. Wheat accessions with relatively high levels of Hx in the roots could prove effective in the control of root pests and valuable in allelopathic suppression of weeds (Copaja et al., 1999). Furthermore, the identification of wheat roots with high contents of Hx is not adequate for the selection of varieties with allelopathic potential. Root exudate analysis is also required (Pérez and Ormeño-Núñez, 1991).

An analytical tool employing gas chromatography and tandem mass spectrometry (GC/MS/MS) has recently been developed to simultaneously identify and quantify eight known allelopathic compounds in wheat, including DIMBOA, *p*-hydroxybenzoic, vanillic, syringic, *trans*-*p*-coumaric, *cis*-*p*-coumaric, *trans*-ferulic, and *cis*-ferulic acids (Wu et al., 1999). The purposes of the present research were: (1) to use the developed analytical technique to evaluate 58 wheat accessions originating from 19 countries for the differential production of DIMBOA from shoots, roots, and in the associated agar growth medium of 17-day-old wheat seedlings; (2) to determine the relationship of DIMBOA level between the shoots and the roots of wheat seedlings; and (3) to investigate the exudation of DIMBOA from living wheat roots into the growth medium.

METHODS AND MATERIALS

Wheat Growth. Based on previous experiments, a worldwide collection of 58 wheat accessions (*T. aestivum* L.) from the Australian Winter Cereals Collection was selected and grown according to the procedure described previously (Wu et al., 2000). Briefly, twelve pre-germinated wheat seeds (surface-sterilised) from each accession were uniformly selected. They were aseptically sown on an agar surface with the embryo up, in three rows on one half of a glass beaker (500 ml) pre-filled with 30 ml of 0.3% water agar. The beaker was wrapped with a piece of parafilm and placed in a controlled growth cabinet with a daily light/dark cycle of 13 hr/11 hr and a temperature cycle at 25°C/13°C. After 7 days, a piece of pre-autoclaved white paperboard was inserted across the centre and down the middle of the beaker with the lower edge of the paperboard kept one cm above the agar surface. The beaker was again wrapped with parafilm and placed back in the growth cabinet for 10 more days.

Preparation of Shoot or Root Extracts. The procedure previously described for the preparation of shoot or root extracts was used (Wu et al., 1999). Shoots or roots of 17-day-old wheat seedlings were harvested for each accession and immediately freeze-dried. An amount of 0.100 g of shoot or root material was cut into 2-mm lengths, ground into powder, macerated with 3 ml of 0.001 M HCl, sonicated at 5°C for 15 min, and then centrifuged at 20,000 rpm at 10°C for 15 min. The supernatant was collected and extracted three times with 10 ml portions of diethyl ether. The ether was evaporated under reduced pressure at 35°C.

Collection of Root Exudates from the Agar Medium. Wheat seedlings were uprooted from their soft nutrient-free agar medium and the roots rinsed twice with 5 ml portions of distilled water to remove residual agar. The washings were pooled into the agar medium. The growth medium was collected, adjusted to pH 3.0 by dropwise addition of 0.06 M HCl, stirred thoroughly, and then sonicated at 5°C for 15 min. The agar medium was extracted three times with 60 ml portions of diethyl ether. The ether was then evaporated under reduced pressure at 35°C.

Derivatization and Quantitation. The derivatization and quantitation of wheat samples were identical to those described previously (Wu et al., 1999). Silylation of wheat shoots, roots and agar samples was accomplished by the addition of 1.0 ml of MSTFA (Alltech Australia) at 60°C for 30 mins. Silylated samples were directly analysed by GC/MS/MS. DIMBOA was identified and quantified by comparing retention times and product ion spectra with that in the user library created from the standard compound. Quantitative analysis was performed by the internal standard method (Wu et al., 1999) and reported in units of mg/kg of dry matter for wheat shoots and roots, and μg/l for water agar.

GC/MS/MS Analysis. Analyses were carried out on a Varian 3400 CX gas chromatograph coupled with a Varian Saturn 2000 ion trap mass spectrometer. Samples were introduced via a DB-5MS fused-silica capillary column of 30 m $\times$ 0.25 mm $\times$ 0.25 μm (J & W Scientific, Alltech Australia). Gas chromatographic and tandem mass spectrometric conditions for the analysis of wheat samples were identical to those reported previously (Wu et al., 1999). Mass spectral scan time from *m/z* 50 to 450 was 1.0 second (using 3 microscans). Nonresonant collision-induced dissociation (CID) was used for MS/MS. All samples were run in triplicate.

Data Analysis. All experimental data were subjected to analysis of variance by using Genstat 5 (Anonymous, 1995) and the treatment means were tested separately for least significant difference (l.s.d.) at a 5% level of probability.

RESULTS

DIMBOA Variation in Wheat Shoots. Wheat accessions produced differential amounts of DIMBOA in their shoot tissues (Table 1). There were 20 accessions with a level of DIMBOA less than 300.0 mg/kg dry matter and 8 accessions with more than 700.0 mg/kg. Thirty accessions contained DIMBOA ranging from 300.0 to 700.0 mg/kg. Accessions Canada 51 and Hartog produced no detectable DIMBOA, while the accession Khapli contained the highest amount of DIMBOA (730.4 ± 1.4 mg/kg).

DIMBOA Variation in Wheat Roots. Wheat accessions also differed significantly in DIMBOA levels in the roots of 17-day-old wheat seedlings (Table 1). There were five accessions with a level of DIMBOA below 300.0 mg/kg dry matter and 36 accessions with greater than 700.0 mg/kg. Seventeen accessions contained

TABLE 1. DIMBOA CONTENTS IN THE SHOOTS, ROOTS, AND AGAR OF 58 WHEAT ACCESSIONS[a]

Wheat accessions	Country of origin	DIMBOA (mean ± S.D.)		
		Shoots (mg/kg)[b]	Roots (mg/kg)[b]	Agar (μg/L)
Canada 51	Canada	nd*	48.3 ± 3.1	nd
Hartog	Australia	nd	491.0 ± 11.1	nd
RAC 710	Australia	39.3 ± 7.6	701.9 ± 6.1	nd
Sunstate	Australia	48.2 ± 14.0	627.3 ± 1.0	nd
Emika	Poland	75.1 ± 20.3	619.5 ± 14.0	nd
Currawong	Australia	103.7 ± 8.3	663.5 ± 5.9	nd
Triller	Australia	112.7 ± 9.8	707.4 ± 14.5	nd
Excalibur	Australia	135.6 ± 13.0	651.1 ± 1.3	30.7 ± 2.5
Robin	Australia	149.1 ± 6.0	724.2 ± 1.2	nd
Sunco	Australia	149.1 ± 26.3	700.5 ± 2.6	11.6 ± 0.5
SST 16	South Africa	151.3 ± 9.8	718.9 ± 0.4	nd
Eretria	Greece	160.3 ± 7.1	554.0 ± 25.2	13.3 ± 0.2
Janz	Australia	177.3 ± 10.6	708.4 ± 4.4	nd
Trident	Australia	180.5 ± 5.9	704.8 ± 2.5	nd
Virgilio	Italy	195.5 ± 7.7	728.2 ± 6.1	10.3 ± 2.4
Canada 4125	Canada	207.6 ± 10.6	nd	nd
Afghanistan 19	Afghanistan	214.8 ± 43.9	522.2 ± 29.3	nd
PF 8716	Brazil	251.2 ± 38.3	723.8 ± 0.8	42.0 ± 1.7
Meering	Australia	252.8 ± 22.7	155.5 ± 7.2	nd
Canada 3740	Canada	294.0 ± 8.7	704.7 ± 2.7	nd
AUS# 12788	United States	321.7 ± 2.4	699.9 ± 17.1	nd
Baroota wonder	Australia	328.3 ± 19.5	656.9 ± 8.6	nd
Cranbrook	Australia	328.6 ± 6.0	705.8 ± 13.5	15.7 ± 1.4
Batavia	Australia	367.1 ± 21.9	721.3 ± 4.9	nd
HY-65	India	389.2 ± 11.7	669.9 ± 0.7	nd
Halberd	Australia	409.6 ± 14.1	717.0 ± 9.0	nd
Afghanistan 9	Afghanistan	449.8 ± 3.8	727.8 ± 2.8	nd
Tunis 2	Tunisia	459.9 ± 15.3	728.5 ± 2.8	14.9 ± 1.0
Cadoux	Australia	485.0 ± 16.6	642.2 ± 12.9	nd
AUS# 18056	Mexico	547.3 ± 34.3	727.8 ± 1.8	nd
Jing Hong	Canada	555.2 ± 37.9	716.6 ± 5.6	nd
Dollarbird	Australia	569.5 ± 26.3	675.4 ± 6.0	nd
Matong	Australia	582.5 ± 34.8	718.5 ± 3.5	nd
WG-357	India	584.2 ± 21.3	672.9 ± 2.7	nd
Bernina	Switzerland	597.7 ± 4.6	715.9 ± 3.0	nd
Sunstar	Australia	610.9 ± 31.4	686.5 ± 25.4	nd
Opata	Mexico	620.7 ± 28.2	721.8 ± 3.2	nd
Federation	Australia	621.6 ± 14.5	713.4 ± 4.2	nd
Kite	Australia	639.1 ± 5.1	724.6 ± 2.9	nd
RAC 820	Australia	653.5 ± 20.3	718.9 ± 3.4	nd
Studena	Yugoslavia	655.2 ± 24.9	722.5 ± 1.8	nd
CD 87	Australia	656.7 ± 3.6	667.6 ± 18.6	nd

TABLE 1. CONTINUED

Wheat accessions	Country of origin	DIMBOA (mean ± S.D.)		
		Shoots (mg/kg)[b]	Roots (mg/kg)[b]	Agar (μg/L)
Lamar	United States	662.7 ± 12.0	715.9 ± 2.9	nd
Canada 56	Canada	663.4 ± 6.0	625.0 ± 28.1	nd
Wakanui	NewZealand	665.6 ± 3.7	724.5 ± 4.9	nd
Wattines	Germany	666.1 ± 4.6	639.7 ± 2.3	nd
L 1512-2721	United Arabic Republic	689.7 ± 4.5	164.6 ± 13.1	79.1 ± 5.9
AUS# 18060	Mexico	694.2 ± 13.1	720.1 ± 5.7	nd
Insignia	Australia	694.2 ± 15.3	714.1 ± 1.9	nd
SST 6	South Africa	695.8 ± 15.8	724.6 ± 1.4	8.6 ± 0.6
Sudan 8	Sudan	705.0 ± 9.5	724.4 ± 3.2	nd
AUS# 18364	South Africa	707.8 ± 9.3	730.1 ± 1.3	nd
Egret	Australia	709.0 ± 11.1	732.7 ± 0.7	nd
AUS# 12627	Peru	709.3 ± 10.6	253.4 ± 11.4	nd
Batten	NewZealand	709.8 ± 6.5	734.1 ± 3.2	nd
Altar 84	Mexico	720.3 ± 1.5	723.4 ± 2.1	nd
Tasman	Australia	728.2 ± 6.4	727.4 ± 4.9	34.2 ± 4.9
Khapli	India	730.4 ± 1.4	733.9 ± 1.4	69.8 ± 4.9
l.s.d$_{0.05}$		57.5	39.4	7.6

[a] Data presented are the means ± standard deviation (S.D.).
[b] Shoot and root results are reported as mg/kg dry matter.
*nd: not detected.

DIMBOA between 300.0 to 700.0 mg/kg. Canada 4215 produced no detectable DIMBOA in root tissues, while the Batten contained the highest amount (734.1 ± 3.2 mg/kg).

Wheat plants differentially accumulated more DIMBOA in root tissues than in shoots (Table 1). The average level of DIMBOA was 643.0 mg/kg in roots and 439.4 mg/kg in shoots. Of the 58 accessions, 50 contained higher levels of DIMBOA in roots than in shoots. Meering, AUS# 12627, L 1512-2721, Canada 56 and Canada 4125 were the exceptions. Three accessions, Altar 84, Khapli and Tasman, did not differ significantly in DIMBOA between roots and shoots.

There was no correlation in DIMBOA content between the shoots and roots when a low level of DIMBOA content was found in the shoots of wheat seedlings. However, a high level of DIMBOA is expected in root tissues when a high level of DIMBOA content is found in shoots. Among the 38 accessions with more than 300.0 mg/kg of DIMBOA in their shoots, 36 produced DIMBOA above 650.0 mg/kg (95%) in their roots. Only two accessions (L 1512-2721 and AUS# 12627) had less than 260.0 mg/kg of DIMBOA in their roots. Among the 20 accessions with less than 300.0 mg/kg of DIMBOA in shoot tissues, 14 produced more than 600 mg/kg of DIMBOA in their roots (70%), while 10 accessions (50%) produced more than 700 mg/kg of DIMBOA (Table 1).

DIMBOA Variation in Root Exudates. Wheat accessions differed significantly in the amount of DIMBOA exuded into the growth medium (Table 1). Forty-seven out of 58 accessions (81%) did not exude detectable amounts of DIMBOA through their living roots into the agar growth medium, although substantial levels of DIMBOA were found in shoot or root tissues. Only 11 accessions (19%) were capable of exuding DIMBOA into the growth medium, with amounts ranging from 8.56 μg/l of water agar for SST 6 to 79.08 μg/l of water agar for L1512-2721.

There were seven accessions with DIMBOA levels lower than 550.0 mg/kg dry mater in their root tissues (Table 1). Among them, six accessions did not exude DIMBOA into the associated growth medium, the exception being L 1512-2721. Canada 4125 contained no detectable DIMBOA in roots, nor was DIMBOA detected in the agar growth medium that had supported the seedling growth for 17 days. Among the 11 accessions capable of exuding DIMBOA from their roots, ten accessions contained DIMBOA at concentrations higher than 550.0 mg/kg dry matter in their root tissues, the exception being L 1512-2721. It is concluded that wheat seedlings do not release DIMBOA when DIMBOA levels are low in root tissues.

DISCUSSION

Significant genetic variation in the production of DIMBOA was not only found in wheat shoot tissues, but also in root tissues. Some accessions produced high levels of DIMBOA, while others had no detectable DIMBOA in shoots or roots of 17-day-old wheat seedlings (eg., shoots of Canada 51 and Hartog, and roots of Canada 4215). The extremely low level of DIMBOA in leaves of Hartog has also been reported (Nicol et al., 1992; Copaja et al., 1999). The substantial genetic variation of DIMBOA production in wheat germplasm implies that it may be possible to manipulate DIMBOA levels by means of modern biotechnology, thereby reducing the dependence on synthetic pesticides.

Exudation of allelochemicals by living plants is one of the important processes necessary for crop allelopathy to occur (Rice, 1984). In the investigation of the allelopathic potential of quackgrass (*Agropyron repens*) seedlings, Friebe et al. (1995) reported that the cyclic hydroxamic acids were important constituents in root exudates of *A. repens*. The concentration of DIMBOA was 0.4 μ mol/l in root exudates. The present study shows that DIMBOA exudation differs between wheat accessions. Only 11 out of 58 wheat accessions (19%) exuded DIMBOA into the growth medium. These results demonstrate that release of DIMBOA is accession-specific, suggesting the presence of genetic factors governing the exudation process of DIMBOA. Such an accession-dependent exudation of DIMBOA has also been reported by others. Pérez and Ormeño-Núñez (1991) claimed that DIMBOA was not detectable in the root exudates of three wheat cultivars despite its presence in

their roots. However, using hydroponic cultures and placing the plants in distilled water for two to six hours after removal from the nutrient solution, Pethós (1992) reported that wheat and maize seedlings exuded DIMBOA through their living roots.

Hydroxamic acids, including DIMBOA, isolated from crop residues are allelopathic to the germination and growth of certain weed species (Barnes and Putnam, 1987; Pérez, 1990; Blum et al., 1992). DIMBOA exuded from living roots is also one of the bioactive compounds responsible for the allelopathic activity of *A. repens* seedlings (Friebe et al., 1995). In the present study, the substantial amounts of DIMBOA in wheat shoots and roots might be involved in wheat residue allelopathy for weed suppression under conservation farming systems where wheat stubbles remain in the field. On the other hand, the exudation of DIMBOA from living roots of wheat seedlings might be genetically regulated, and affected by a number of other biotic and abiotic factors. Exudation of DIMBOA and its involvement in wheat seedling allelopathy for weed suppression needs further investigation.

The magnitude of crop allelopathic activity is not determined by one single compound, but by a mixture of many biologically-active allelochemicals (Einhellig, 1995). DIMBOA, exuded by wheat seedlings, is only one of many allelochemicals that could collectively interact with other allelochemicals to produce significant wheat allelopathy against pests, diseases and weeds. Many other allelopathic agents have been identified from wheat (Neves and Gaspar, 1990; Gaspar and Neves, 1993, 1995). These bioactive compounds could form a complex allelochemical mixture that exhibits combined effects with much greater allelopathic activity than any one individual compound (Einhellig, 1995). Further investigation of the additive or synergistic effects between DIMBOA and related compounds, in combination with other chemically-distinct compounds, is therefore needed to understand the chemical basis of crop allelopathy prior to its use in integrated pest and weed management.

Acknowledgments—This research is funded by both Charles Sturt University (CSU) and the Australian Cooperative Research Centre for Weed Management Systems. The authors are thankful for the kind provision of wheat accessions from the Australian Winter Cereals Collection, the provision of GC/MS/MS instrumentation from the Environmental and Analytical Laboratories at CSU, and the kind provision of DIMBOA standard from Dr W. S. Chilton of the Department of Botany, North Carolina State University, Raleigh, USA.

REFERENCES

ANONYMOUS. 1995. *Genstat 5 Release 3.2 Manual* (*PC/Windows NT*). Lawes Agricultural Trust (Rothamsted Experimental Station), Oxford University Press, New York, USA.

BARNES, J. P., and PUTNAM, A. R. 1987. Role of benzoxazinones in allelopathy by rye (*Secale cereale* L.). *J. Chem. Ecol.* 13:889–905.

BLUM, U., GERIG, T. M., WORSHAM, A. D., HOLAPPA, L. D., and KING, L. D. 1992. Allelopathic activity in wheat-conventional and wheat-no-till soils: Development of soil extract bioassays. *J. Chem. Ecol.* 18:2191–2221.

BOHIDAR, K., WRATTEN, S. D., and NIEMEYER, H. M. 1986. Effects of hydroxamic acids on the resistance of wheat to the aphid *Sitobion avenae. Ann. Appl. Biol.* 109:193–198.

COPAJA, S. V., NIEMEYER, H. M., and WRATTEN, S. D. 1991. Hydroxamic acid levels in Chilean and British wheat seedlings. *Ann. Appl. Biol.* 118:223–227.

COPAJA, S. V., NICOL, D., and WRATTEN, S. D. 1999. Accumulation of hydroxamic acids during wheat germination. *Phytochemistry* 50:17–24.

DUNN, G. M., LONG, B. J., and ROUTLEY, D. G. 1981. Inheritance of cyclic hydroxamates in *Zea mays L. Can. J. Plant Sci.* 61:583–593.

EINHELLIG, F. A. 1995. Allelopathy: Current status and future goals, pp.1–24, *in* Inderjit, K. M. M. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications, ACS Symposium Series No. 582, Washington, D.C.

FIGUEROA, C. C., KOENIG, C., ARAYA, C., SANTOS, M. J, and NIEMEYER, H. M. 1999. Effect of dimboa, a hydroxamic acid from cereals, on peroxisomal and mitochondrial enzymes from aphids: Evidence for the presence of peroxisomes in aphids. *J. Chem. Ecol.* 25:2465–2475.

FRIEBE, A., SCHULZ, M., KUCK, P., and SCHNABL, H. 1995. Phytotoxins from shoot extracts and root exudates of *Agropyron repens* seedlings. *Phytochemistry* 38:1157–1159.

GASPAR, E. M., and NEVES, H. C. 1993. Steroidal constituent from mature wheat straw. *Phytochemistry* 34:523–527.

GASPAR, E. M., and NEVES, H. C. 1995. Chemical constituents in allelopathic straw of wheat (*Triticum aestivum* L.). *Allelopathy J.* 2:79–87.

KLUN, J. A., GUTHRIE, W. D., HALLAUER, A. R., and RUSSELL, W. A. 1970. Genetic nature of the concentration of 2,4-dihydroxy-7-methoxy 2H-1,4-benzoxazin-3 (4H)-one and resistance to the European corn borer in a diallel set of eleven maize inbreds. *Crop Sci.* 10:87–90.

LESZCZYNSKI, B., DIXON, A. F. G., BAKOWSKI, T., and MATOK, H. 1995. Cereal allelochemicals in grain aphid control. *Allelopathy J.* 2:31–36.

NEVES, H. C., and GASPAR, E. M. 1990. Identification of active compounds in wheat straw extracts with allelopathic activity by HRGC-MS and HRGC-FTIR. *J. High Resol. Chromatogr.* 13:550–554.

NICOL, D., COPAJA, S. V., WRATTEN, S. D., and NIEMEYER, H. M. 1992. A screen of worldwide wheat cultivars for hydroxamic acid levels and aphid antixenosis. *Ann. Appl. Biol.* 121:11–18.

NIEMEYER, H. M. 1988a. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-one), defence chemicals in the gramineae. *Phytochemistry* 27:3349–3358.

NIEMEYER, H. M. 1988b. Hydroxamic acid content of *Triticum* species. *Euphytica* 37:289–293.

NIEMEYER, H. M., and JEREZ, J. M. 1997. Chromosomal location of genes for hydroxamic acid accumulation in *Triticum aestivum* L. (wheat) using wheat aneuploids and wheat substitution lines. *Heredity* 79 (Part 1):10–14.

PÉREZ, F. J. 1990. Allelopathic effect of hydroxamic acids from cereals on *Avena sativa and A. fatua. Phytochemistry* 29:773–776.

PÉREZ, F. J., and ORMEÑO-NÚÑEZ, J. 1991. Difference in hydroxamic acid content in roots and root exudates of wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.): Possible role in allelopathy. *J. Chem. Ecol.* 17:1037–1043.

PETHÓS, M. 1992. Occurrence and physiological role of benzoxazinones and their derivatives. III. Possible role of 7-methoxybenzoxazinone in the iron uptake of maize. *Acta Agron. Hung.* 41:57–64.

RICE, E. L. 1984. *Allelopathy.* 2nd ed. Academic Press, Orlando, Florida.

SIMCOX, K. D., and WEBER, D. F. 1985. Location of benzoxazinless (bx) locus in maize by monosomic and B-A translocational analyses. *Crop Sci.* 25:827–830.

WILKES, M. A., MARSHALL, D. R., and COPELAND L. 1999. Hydroxamic acids in cereal roots inhibit the growth of take-all. *Soil Biol. Biochem.* 31:1831–1836.

WU, H., HAIG, T., PRATLEY, J., LEMERLE, D., and AN, M. 1999. Simultaneous determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one by GC/MS/MS in wheat (*Triticum aestivum* L.). *J. Chromatogr. A.* 864:315–321.

WU, H., PRATLEY, J., LEMERLE, D., and HAIG, T. 2000. Laboratory screening for allelopathic potential of wheat (*Triticum aestivum*) accessions against annual ryegrass (*Lolium rigidum*). *Aust. J. Agric. Res.* 51:259–266.

Journal of Chemical Ecology, Vol. 27, No. 8, 2001

METHODOLOGICAL SETUP TO STUDY ALLELOCHEMICAL TRANSLOCATION IN RADISH SEEDLINGS

G. CHIAPUSIO and F. PELLISSIER*

University of Savoie
Department of Life Sciences
Laboratory Dynamics of Altitude Ecosystems
73 376 Le Bourget du Lac Cedex
France

(Received October 18, 2000; accepted March 29, 2001)

Abstract—[Ring-U^{14}C] *p*-hydroxybenzoic acid (POH) translocation during radish germination and early seedling growth was studied to compare two culture conditions (sterile/non-sterile) and two modes of POH extraction (ethanol/oxidizing). Quantification of POH in organs was performed by grinding them in a mortar with ethanol or by combusting them in a biological oxidizer. Comparison of these extraction methods revealed that the oxidizer provides higher POH concentrations than grinding. Uptake and translocation of POH into radish seedlings occured in the first 24 hr with the highest accumulation in cotyledons. POH in cotyledons ranged from 8 to 12.4 μmol g^{-1} FW. Two forms of POH were found in seedlings, an ethanol-soluble one and a "bound" one. After 96 hr incubation, half of the recovered POH was "bound" in roots and hypocotyls. POH recovery in seedlings was also higher under axenic rather than non-axenic conditions. POH was degraded into $^{14}CO_2$ by microorganisms under non-sterile cultures, its concentration in culture medium decreased from 10^{-3} to 10^{-4} M between the first and the fourth day of incubation. Choice between sterile and non-sterile culture conditions as a method depends on particular research objectives. Non-sterile methods can be used to reflect natural processes whereas sterile methods can be used if the objective is to determine allelochemical penetration and biological effects on target plants.

Key Words—Allelopathy, ^{14}C, allelochemical degradation, allelochemical translocation, methodology, microorganisms, *p*-hydroxybenzoic acid, radish germination.

*To whom correspondence should be addressed. e-mail: pellissier@univ-savoie.fr

INTRODUCTION

Allelopathy is the direct or indirect effect of one plant upon another through the production of chemical compounds released into the environment (Rice, 1984). Many investigations in this area are focused on the identification of released bioactive compounds by plants (Mizutani, 1999). When these compounds are clearly identified and quantified, studies pinpoint their possible phytotoxic effects upon target plants (Blum et al., 1999). Nevertheless, absorption and translocation mechanisms of such allelochemicals by target species have been poorly investigated. This work compares plant culture and chemical extraction methods in order to determine a more appropriate means for further investigating allelochemical uptake and translocation into target seedlings.

An experimental model was selected using *p*-hydroxybenzoic acid (POH) as the phytochemical of choice and radish (*Raphanus sativus* var. 18 days) as the target plant. POH is a widespread phenolic acid released into soil by root exudates, leaf leachates, and decomposed plant tissues (Dalton, 1999). It delays germination and growth of several plant species, but its effects are less severe than many more active phenolic compounds (Maffei et al., 1999). In target plants, POH mainly interferes with nucleic acid and protein metabolism (Baziramakenga et al., 1997). Radish is one of the most widely used target species in allelopathy studies, due to its rapid germination and growth, its easy dissection and sensitivity to phenolic or phytotoxic compounds (Haugland and Brandsaeter, 1996).

Bioassay is an indispensable tool in allelopathy investigations because it allows one to readily establish the specific biological activities associated with a particular allelochemical or mixture of allelochemicals. Allelochemical phytotoxicity is generally tested under non-sterile conditions. However, little information is available on possible allelochemical transformations in the culture medium by microorganisms and consequently, the real quantities absorbed by target seedlings. This is a crucial step in the assessment of phytotoxicity in plants.

A more pertinent technique is to have recourse to ^{14}C radiolabeled chemicals. However, the procedure selected to extract ^{14}C-labelled compounds is still a limited step (Van Sumere, 1989). Grinding samples in a mortar with adequate solvent or combusting samples in a biological oxidizer are two widely used methods of extraction (Chao et al., 1994; Simmen and Gisi, 1996).

Thus, objectives of the present work were to: 1) compare allelochemical absorption under sterile and non-sterile conditions; 2) quantify phytochemical penetration into target plants; 3) and compare grinding and oxidization as methods of extraction.

METHODS AND MATERIALS

General Procedure (Figure 1). We concentrated on characterizing POH kinetic translocation into radish seedlings in order to determine the influence of microorganisms on POH uptake and translocation under sterile and non-sterile cultures and, to compare two methods of POH extraction, by grinding and oxidizing.

Chemicals and Solutions. P-Hydroxybenzoic acid (POH) and [ring-U^{14}C] *p*-hydroxybenzoic acid (radiochemical purity >97%, specific activity 12 mCi mmol^{-1}) were purchased from Sigma Chemicals Ltd.

An aqueous solution of POH was prepared at 10^{-3} M with demineralized water and buffered to pH 5.5 with NaOH. [^{14}C] *p*-Hydroxybenzoic acid was dissolved in 1 ml of 99.9% ethanol (reaching a concentration of 8.7 10^{-3} mmol ml^{-1}). Ten to 15 μl of this radiolabeled POH solution were added to the non-labeled POH solution to obtain approximately 0.008 μ Ci ml^{-1} in the final solution. This mixed solution of POH and ^{14}POH was immediately used in radish culture.

Radish Culture Under Non-Sterile Condition. Twenty radish seeds (*Raphanus sativus* L., var. 18 days) were placed into a glass (7 cm diameter and 9 cm high, closed with a cap) with 5 ml of the chemical solution. In order to trap potential $^{14}CO_2$, a Petri-dish containing 5 ml of 10 N NaOH was added into the top of each

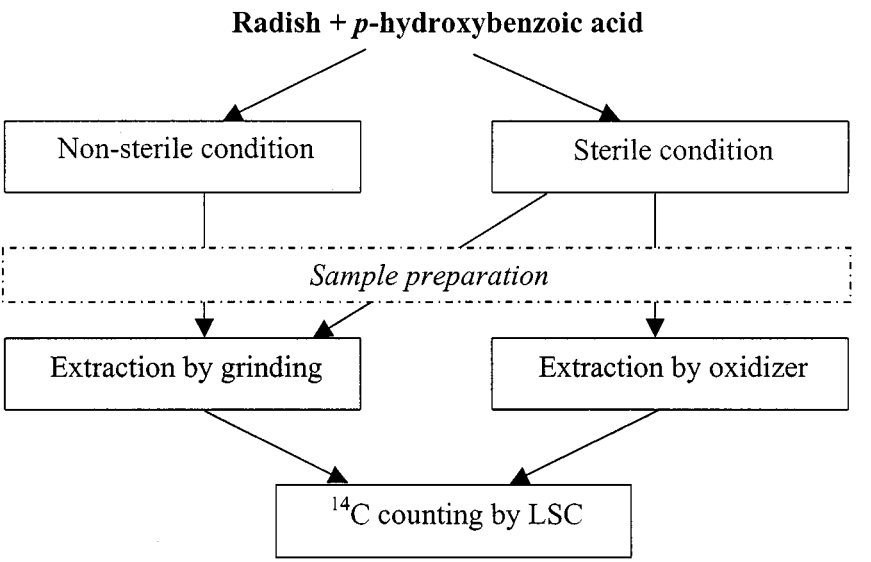


FIG. 1. General investigation procedure of POH kinetic translocation into radish seedlings using two culture conditions (sterile and non-sterile) and two types of extraction (by grinding and by oxidizer).

glass. Glasses were placed for a maximum of 4 days in a thermostatic growth chamber (20°C, photoperiod 8 h light/16 h dark).

Radish Culture Under Sterile Condition. Radish seeds were disinfected by immersion for 10 min in sterile, demineralized water and 110 volumes hydrogen peroxide (70:30 v/v), followed by rinsing (3 x's) in sterile, demineralized water. Twenty seeds were then placed into a sterile glass containing the CO_2 trap and closed with a sterile cap. Five ml of POH solution sterilized by filtration through a 0.22 μm membrane were added to each glass and 5 ml of 10 N NaOH solution were added to the CO_2 trap. Glasses were placed for a maximum of 4 days in a thermostatic growth chamber (20°C, photoperiod 8 hr light/16 hr dark). Two investigations under sterile conditions were conducted (one followed by grinding and the other by oxidizing). The average recovered ^{14}POH concentration in the culture medium was then calculated for both experiments and combined.

Sample Preparation. At the end of each incubation period (24, 48, 72 and 96 hr), radish seedlings from each glass were washed (3 x's) in ethanol to remove adsorbed compounds. Then, seedling parts were separated. Day 1 (24 hr) corresponded to germinated seeds (separation of seed and seed coat), day 2 (48 hr) to seedling radicle growth (separation of roots + hypocotyls and cotyledons), day 3 (72 hr) to seedling hypocotyl elongation and beginning of photosynthesis (separation of roots, hypocotyls and cotyledons) and day 4 (96 hr) to physiologically active seedlings (separation of roots, hypocotyls and cotyledons). Each organ type was removed from the same glass (20 seedlings), weighed and frozen together (−25°C). These constituted one sample of one organ type. There were either three or four replicates per organ type.

Extraction by Grinding. Samples were ground with a mortar and pestle. Soluble POH was extracted with 3 ml ethanol (purity 97%), a common solvent for extraction of phenolic acids (Van Sumere, 1989). Homogenates were centrifuged (3000 rpm) for 3 min at 20°C. The supernatant was set aside for further analysis after each extraction. The ground residue was extracted twice more with ethanol in the mortar and pestle. Supernatants were combined. Supernatants (5 ml) and pellets (5 ml) were mixed with 10 ml liquid scintillation cocktail (Ultima Gold, Packard). Radioactivity within samples was counted for 10 min by using a liquid scintillation counter (1500 TriCab, Packard).

Extraction by Oxidizing. This investigation was conducted with 3- and 4- day old seedlings. Frozen samples were wrapped in paper (Germaflor) for ovendrying at 80°C for 48 hr. Each sample was then combusted in a biological oxidizer (Ox-500 EG&G instruments) at 900°C for 3 min. The released $^{14}CO_2$ was directly trapped in a vial containing liquid scintillation cocktail (Oxysolve C400, Zinsser Analytic Co.) before counting as above.

Expression of Results. POH levels from radish seedlings were either expressed as concentration (μmol g^{-1} FW) or as quantity (% of applied ^{14}POH) for 20 seedlings (equivalent to one glass). Quantity is the ratio between the disintegrations per minute (DPM) of the initial^{14} POH solution which was added to each glass, and the recovery obtained from the separated organs of 20 seedlings. The total DPM counted in one glass (seedlings + culture medium + rinsing + $^{14}CO_2$) was between 85 and 100% of the initial radioactive solution added to the original culture medium. POH concentration in seedlings was calculated, based upon the equivalence of 10^{-3} POH moles represented by x DPM, detected in the final solution (see above). Concentration pattern reflects the distribution of POH in plant organs. This parameter is widely used to obtain a ratio between compound content and plant biomass, which allows comparisons between different studies. However, using only concentrations could lead to erroneous conclusions because seedling biomass *per se* is necessary to understand plant distribution of POH in target organs (Koricheva, 1999). Table 1 shows the biomass of each radish organ. It reveals the importance of cotyledon biomass which was about three times more than roots or hypocotyls ($P < 0.05$). Thus, POH content was used in order to detect preferential organ accumulation.

Statistical Analyses. As data were not normally distributed and variances were heterogeneous, the Mann-Whitney U non-parametric test ($P < 0.05$) was used for analysis. Three categories of tests were performed to compare: 1) POH content (concentration and quantity) in different organs within the same incubation day; 2) differences in POH concentration from one incubation day to the next, for the same organ; and 3) differences in POH concentration between methods (sterile *vs*. non-sterile and grinding *vs*. oxidizing).

TABLE 1. BIOMASS (MG FRESH WEIGHT ± SD) OF DISSECTED RADISH SEEDLINGS GROWN WITH 10^{-3}M MIXED SOLUTION (POH + ^{14}POH) UNDER STERILE AND NON-STERILE CONDITIONS

Incubation period (in days)	Medium	Roots + hypocotyls	Roots	Hypocotyls	Cotyledons
D2	Sterile	109 ± 9			284 ± 13*
	Non-Sterile	101 ± 32			277 ± 40*
D3	Sterile		104 ± 20	61 ± 7	264 ± 33*
	Non-Sterile		76 ± 21	77 ± 9	329 ± 32*
D4	Sterile		156 ± 18	107 ± 24	326 ± 11*
	Non-Sterile		168 ± 26	147 ± 9	458 ± 19*

Data represent means of at least 3 replicates per incubation time and per culture condition.
*Indicates significantly higher biomass compared to other organs ($P < 0.05$).

RESULTS

Recovery of Unincorporated POH and Released $^{14}CO_2$. Under non-sterile conditions, POH concentrations in the culture medium drastically decreased from 1.7 (D1) to 0.2 (D4) μmol ml^{-1} (Figure 2). The initial POH concentration, 10^{-3}M, fell to 10^{-4}M. Meanwhile, $^{14}CO_2$ release increased from 0.01 ± 0.005 μmol ml^{-1} at D1 to 0.03 ± 0.02 μmol ml^{-1} at D3 followed by a significant increase up to 0.05 ± 0.02 μmol ml^{-1} at D4 (data not shown). Under sterile conditions, no $^{14}CO_2$ was released for any incubation period and POH concentration decreased from 1.6 to 0.7 μmol ml^{-1} over 4 days, a 50% decrease.

POH Translocation under Non-Sterile Conditions and Extraction by Grinding. POH uptake began with seed germination (D1 : 1.8 μmol g^{-1} FW in germinated seeds, data not shown) and was translocated into all radish organs. POH concentration in each organ was compared between incubation days. Cotyledons contained the highest POH levels (10.3 μmol g^{-1} FW), but only on the third day of incubation (Table 2). POH on D4 was only half of the D3 POH concentration in roots, hypocotyls and cotyledons. The total concentration in seedlings also declined by 50% between D3 and D4.

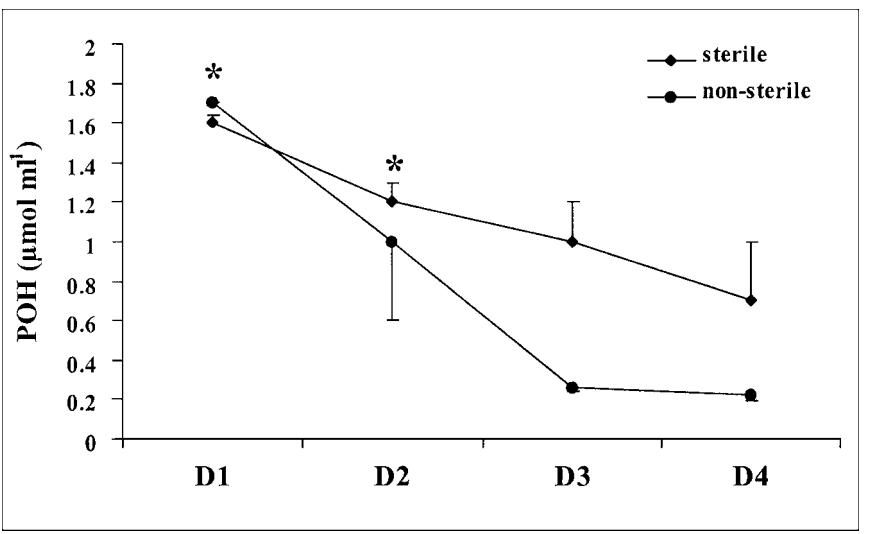


FIG. 2. POH concentration (expressed as μmol ml$^{-1} \pm$ SD) recovered in the radish culture medium under sterile and non-sterile conditions during the four incubation periods (D1 to D4, refers to day 1 to 4). Data represent means of at least 3 replicates per incubation time and per culture condition. * Refers to difference between sterile and non-sterile conditions being significant ($P < 0.05$).

TABLE 2. POH TRANSLOCATION IN RADISH SEEDLINGS UNDER NON-STERILE CONDITION OF INCUBATION. POH CONCENTRATION (EXPRESSED AS μMOL G^{-1} FRESH WEIGHT $\pm$ SD) AND QUANTITY (EXPRESSED AS % OF APPLIED ^{14}POH FOR 20 SEEDLINGS $\pm$ SD) WERE MEASURED AFTER GRINDING EXTRACTION METHOD

	POH concentration (μmol g^{-1}FW)			POH applied (%)		
	Day 2	Day 3	Day 4	Day 2	Day 3	Day 4
Plant Organs						
Roots + Hypocotyls	3.3 ± 0.7			5 ± 1		
Roots		5.9 ± 1.4	2.8 ± 1.4		8 ± 1	5 ± 2
Hypocotyls		5.8 ± 0.9	2.7 ± 1.3		5 ± 0.4	4 ± 1
Cotyledons	4.7 ± 1.8	10.3 ± 1.8*	5.5 ± 1.9	18 ± 8*	35 ± 3*	23 ± 8*
Total	8.1 ± 2.5	22 ± 4	10.9 ± 4.5	22 ± 9	47 ± 4	32 ± 11

Data represent means of at least 3 replicates per incubation time. *Indicates inside a same incubation time, POH content of the organ is different from the others ($P < 0.05$).

POH Translocation under Sterile Conditions and Extraction by Grinding. POH was absorbed by germinating seeds at D1 and accumulated to a concentration of 1.9 ± 0.04 μmol g^{-1} FW (data not shown). Comparisons between POH recovery in organs for each day revealed that POH was concentrated in cotyledons at D2 and D3 (8.2 and 11.2 μmol g^{-1} FW, respectively (Table 3)). POH occurred both in roots (7.7 μmol g^{-1} FW) and cotyledons (8.1 μmol g^{-1} FW) by D4. Total POH concentration in seedlings increased by 7 from D1 to D2 and by 2 from D2 to D3, before declining at D4. Consequently, the highest POH concentration in radish seedlings was found on D3, with about 24 μmol g^{-1} FW.

POH Translocation under Sterile Conditions and Extraction by Oxidizing. Contrary to the results obtained by grinding, no significant differences were found between the POH concentration at D3 and D4 in all organs (Table 4). This was confirmed in whole seedlings where statistical differences of POH recovery were not found between D3 and D4.

Comparison of POH Translocation between Sterile and Non-Sterile Conditions. POH recovery was different between radish organs, but only on the fourth day of incubation (Tables 2 and 3). Hypocotyls contained twice as much POH under sterile conditions than they did under non-sterile conditions, and roots had three times more. Higher accumulation of POH under sterile conditions was also obtained when POH accumulation was compared in whole seedlings.

Comparison of POH Translocation between Grinding and Oxidizing Extractions. POH recovery in radish seedlings was higher following oxidizing extraction than the grinding extraction (Tables 3 and 4). For example, POH content on D3 was 1.5 times higher in hypocotyls and 2 times higher in roots. On D4, only amounts

TABLE 3. POH TRANSLOCATION IN RADISH SEEDLINGS UNDER STERILE CONDITION OF INCUBATION. POH CONCENTRATION (EXPRESSED AS μMOL G^{-1} FRESH WEIGHT $\pm$ SD) AND QUANTITY (EXPRESSED AS % OF APPLIED ^{14}POH FOR 20 SEEDLINGS $\pm$ SD) WERE MEASURED AFTER GRINDING EXTRACTION METHOD

	POH concentration (μmol g^{-1}FW)			POH applied (%)		
	Day 2	Day 3	Day 4	Day 2	Day 3	Day 4
Plant Organs						
Roots + Hypocotyls	5 ± 0.8			7 ± 2		
Roots		7.2 ± 0.3	7.7 ± 1.6		8 ± 2	18 ± 2
Hypocotyls		5.2 ± 0.2	$4.7 \pm 0.4^*$		6 ± 1	9 ± 1
Cotyledons	$8 \pm 1.4^*$	$11.2 \pm 1^*$	8.1 ± 1.9	$31 \pm 6^*$	$52 \pm 7^*$	$46 \pm 2^*$
Total	12.9 ± 2	23.9 ± 1.4	19.4 ± 2.1	37 ± 8	65 ± 9	72 ± 3

Data represent means of at least 3 replicates per incubation time. * Indicates inside a same incubation time, POH content of the organ is different from the others ($P < 0.05$).

in roots and cotyledons were statistically greater after oxidizing extraction. This better recovery was also observed on D3 (1.5 times more) and on D4 (2.5 times more) in whole seedlings.

POH Storage in Radish Organs. In terms of POH content in radish organs, these results suggest cotyledons are POH sinks on D2 to D4 (Tables 2–4). Depending on method and incubation time, the percentage of ^{14}POH recovered in cotyledons from 20 seedlings varied from 18 to 52%. Generally, POH recoveries in roots and hypocotyls were similar (between 5 to 18%).

TABLE 4. POH TRANSLOCATION IN RADISH SEEDLINGS UNDER STERILE CONDITION OF INCUBATION. POH CONCENTRATION (EXPRESSED AS μMOL G^{-1} FRESH WEIGHT $\pm$SD) AND QUANTITY (EXPRESSED AS % OF APPLIED ^{14}POH FOR 20 SEEDLINGS $\pm$ SD) WERE MEASURED AFTER OXIDIZER EXTRACTION METHOD

	POH concentration (μmol g^{-1} FW)			POH applied (%)		
	Day 2	Day 3	Day 4	Day 2	Day 3	Day 4
Plant Organs						
Roots + Hypocotyls						
Roots		15.7 ± 4.1	14.8 ± 3.1		5 ± 1	11 ± 5
Hypocotyls		$7.1 \pm 0.9^*$	$5.5 \pm 0.9^*$		5 ± 1	6 ± 1
Cotyledons		12.4 ± 2.4	12.4 ± 1		$30 \pm 2^*$	$38 \pm 3^*$
Total		35.1 ± 5.9	33.2 ± 2.4		40 ± 4	53 ± 7

Data represent means of at least 3 replicates per incubation time. * Indicates inside a same incubation time, POH content of the organ is different from the others ($P < 0.05$).

DISCUSSION

This work characterized POH translocation in radish seedlings. Results of all experiments revealed that POH uptake begins with germination and is translocated into all organs of radish seedlings. The highest POH concentrations were found in cotyledons (8 to 12.4 μmol g^{-1} FW) and/or roots (7.7 to 15.7 μmol g^{-1} FW).

Initially, POH uptake depended upon the presence (non-sterile culture) or absence (sterile culture) of microorganisms. Indeed, $^{14}CO_2$ release occurred from D1 to D4 under non-sterile culture conditions, but not in sterile cultures. This suggests that $^{14}CO_2$ was released as microorganisms degraded POH. POH is known to be transformed by bacteria and/or fungi into protocatechuic acid and then to aliphatic compounds such as acetyl-CoA and succinate (Bartz and Weltring, 1985). It seems that POH mineralization begins during the first hours of incubation because $^{14}CO_2$ was detectable at D1. This process is also well described in soil where bioavailable phenolic compounds are rapidly degraded by microbes. Martin and Haider (1980), showed that 95% of ^{14}C carboxyl carbon of POH added into non-sterile soil was lost as $^{14}CO_2$ within one week, and the main degradation product was protocatechuic acid. Testing ^{14}C Ring-U protocatechuic acid degradation in soil, they also found that it was readily degraded into $^{14}CO_2$ (35% in one week). POH mineralization depended on experimental conditions (soil specificity) and microbial species. For example, POH mineralization by *Arthrobacter sp.* ranged from 32% to 80% in one day depending on the type of soil (Johnson et al., 1998).

Because this experiment showed that POH was degraded under non-sterile conditions, the lowest POH concentrations were recovered in the culture medium and in radish organs compared to sterile culture. Non-Sterile bioassays are mostly used in allelopathy studies. Therefore, the presence of microorganisms should be determined to interpret the tested allelochemical effect.

The highest POH concentration in seedlings was obtained under sterile conditions using the oxidizing extraction method. Of course, when samples are oxidized, every ^{14}C is recovered. This is quite impossible with the grinding extraction method. From D3 to D4, stability of POH concentration in organs was noticed. Concentrations were equal in roots and cotyledons but, the biomass of cotyledons was about 3 times more. Cotyledons appeared to be the main POH sink.

Being water soluble and lipophilic, POH is able to pass through cell membranes (partition coefficient in octan-1-ol/water : $\log P = 1.1$). In radish seedlings, POH uptake can occur by roots and then is able to penetrate through the root symplast of endodermic cells, apoplast and finally reach the xylem (Delrot and Bonnemain, 1991). POH storage in cotyledons could occur both in vacuoles

(as aglycosidic form) and probably in walls or membranes in "bound" form. This leads to various potential cellular targets of POH in allelopatically stressed plants. Thus, various physiological disorders can occur simultaneously in such plants.

This study further revealed that POH was recovered in radish seedlings in two different chemical forms: an ethanol "soluble" form and a "bound" form that is unextractable by ethanol. Differences between grinding and oxidizing results could be explained in terms of its ethanol solubility. POH concentration differences between these two extraction methods revealed the amount of "bound" POH. For example, half of POH recovered in roots on D3 was bound (i.e., 8.5 μmol g^{-1}). On D4 it was also half of the POH recovered in roots and cotyledons, 9.8 μmol g^{-1} FW and 6.9 μmol g^{-1} FW, respectively. No statistical differences were found for hypocotyls suggesting that hypocotyls only act as a translocating organ between roots and cotyledons. Photosynthesis is supposedly fully active in seedlings by D4, implying that physiological processes such as enzymatic reactions also are active. The conversion of POH into the "bound" form could be more efficient than in younger seedlings. Moreover, secondary compounds are known to be covalently linked to plant cell walls (Hutzler et al., 1998), in waxes (Schmutz et al., 1994), or on external surfaces of plant organs (Cuadra and Harborne, 1996).

This investigation confirmed that POH was taken up by roots and translocated into different organs of radish seedlings. POH quantification was more efficient by oxidizing plant organs than grinding them in ethanol. The oxidizing extraction technique gave more precise results to quantify allelochemicals in plants. Differences between concentrations obtained by these two methods of extraction gave preliminary indication of two forms, soluble and bound, of POH in cells. Both techniques are then complementary to characterize and quantify forms of POH within the plant. It is also important to consider both concentration and quantity to better understand POH translocation in cells, organs and whole plant. Cotyledons seem to act as POH sink.

The role of microorganisms and seedlings in POH degradation is still not clear at this stage. Further, this study was performed counting radiolabeled carbons and stating that all of those belong to POH. Nevertheless, it is likely that metabolic products of POH were counted as ^{14}POH because any detected ^{14}C was considered in these analyses. Thus, further investigation with a radiolabel TLC-scanner is necessary for the differentiation of all ^{14}C molecules (Dayan et al., 1997).

Acknowledgments—The authors would like to thank Dr. Christiane Gallet (University of Savoie) for her constructive remarks and Dr. Christian Gauvrit (INRA, Dijon) and his team in welcoming us in their laboratory to perform oxidizing extraction. They also wish to thank the two anonymous referees for their valuable review of this work.

REFERENCES

BARZ, W., and WELTRING, K. M. 1985. Biodegradation of aromatic extractives of wood, pp. 607–662, *in* T. Higuchi (ed.). Biosynthesis and Biodegradation of Wood Components. Academic Press, San Diego, California.

BAZIRAMAKENGA, R., LEROUX, G. D., SIMRD, R. R., and NADEAU, P. 1997. Allelopathic effects of phenolic acids on nucleic acid and protein levels in soybean seedlings. *Can. J. Bot*. 75:445–450.

BLUM, U., AUSTIN, M. F., and SHAFER, S. R. 1999. The fates and effects of phenolic acids in a plant-microbial-soil model system, pp. 159–166, *in* F. A. Macias, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds.). Recent Advances in Allelopathy. Volume I: A Science for the Future. Servicio de Publicaciones-Universidad de Cadiz, Cadiz, Spain.

CHAO, J. F., HSIAO, A. I., QUICK, W. A., and HUME, J. A. 1994. Effect of decapitation on absorption, translocation, and phytotoxicity of imazamethabenz in wild oat (*Avena sativa* L.). *J. Plant Growth Regul*. 13:153–158.

CUADRA, P., and HARBORNE, J. B. 1996. Changes in epicuticular flavonoids and photosynthetic pigments as a plant response to UV-B radiation. *Z. Naturforsch. A* 51:671–680.

DALTON, B. R. 1999. The occurrence and behavior of plant phenolic acids in soil environments and their potential involvement in allelochemical interference interactions: Methological limitations in establishing conclusive proof of allelopathy, pp. 57–74, *in* Inderjit, K. M. M. Dakshini and L. C. Foy (eds.). Principles and Practices in Plant Ecology: Allelochemical Interactions. CRC Press, Boca Raton, Florida.

DELROT, S., and BONNEMAIN, J. L. 1991. Le transport à longue distance des herbicides dans la plante, pp. 51–77, *in* R. Scalla (ed.). Les herbicides: Mode d'action et principes d'utilisation. INRA, Paris, France.

DAYAN, F. E., DUKE, S. O., WEETE, J. D., and HANCOCK, H. G. 1997. Selectivity and mode of action of carfentrazone-ethyl, a novel phenyl triazolinone herbicide. *Pestic. Sci*. 51:65–73.

HAUGLAND, E., and BRANSAETER, L. O. 1996. Experiments on bioassay sensitivity in the study of allelopathy. *J. Chem. Ecol*. 22:1845–1859.

HUTZLER, P., FISCHBACH, R., HELLER, W., JUNGBLUT, T. P., REUBER, S., SCHMITZ, R., VEIT, M., WEISSENBÖCK, G., and SCHNITZLER, J. P. 1998. Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *J. Exp. Bot*. 49:953–965.

JOHNSON, T. A., SIMS, G. K., ELLSWORTH, T. R., and BALLANCE, A. R. 1998. Effects of moisture and sorption on bioavailability of *p*-hyhroxybenzoic acid to *Arthrobacter* sp. in soil. *Microbiol. Res*. 153:349–353.

KORICHEVA, J. 1999. Interpreting phenotypic variation in plant allelochemistry: Problems with the use of concentration. *Oecologia* 119:467–173.

MAFFEI, M., BERTEA, C. M., GARNERI, F., and SCANNERINI, S. 1999. Effect of benzoic acid hydroxy- and methoxy-ring substituents during cucumber (C*ucumis sativus* L.) germination. I. Isocitrate lyase and catalase activity. *Plant Sci*. 141:139–147.

MARTIN, J. P., and HAIDER, K. 1980. Microbial degradation and stabilization of ^{14}C-labeled lignins, phenols, and phenolic polymers in relation to soil humus formation, pp. 77–100, *in* T. K. Kirk, T. Higuchi, and H. Chang (eds.). Lignin biodegradation: Microbiology, chemistry and potential applications. CRC Press, Boca Raton, Florida.

MIZUTANI, J. 1999. Plant ecochemicals which may play important roles in complex interactions between higher plants, pp. 149–158, *in* F. A. Macias, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds.). Recent Advances in Allelopathy. Volume I: A Science for the Future. Servicio de Publicaciones-Universidad de Cadiz, Cadiz, Spain.

RICE, E. L. 1984. Allelopathy, 2nd ed. Academic press, New York.

SCHMUTZ, A., BUCCALA, A., JENNY, T., and RYSER, U. 1994. The phenol in the wax and in the suberin of green cotton fibres and their function. *Acta Hort*. 381:269–275.

SIMMEN, U. R. S., and GISI, U. 1996. Uptake and distribution in germinating wheat of [14C]SAN 789F and [14C] cyproconazole applied as seed treatment. *Crop Prot*. 15:275–281.

VAN SUMERE, C. F. 1989. Phenols and phenolic acids, pp. 29–73, *in* P. M. Dey and J. B. Harborne (eds.). Methods in Plant Biochemistry, Vol. 1: Plant Phenolics. Academic Press, New York.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

ELEVATIONAL TRENDS IN DEFENSE CHEMISTRY, VEGETATION, AND REPRODUCTION IN *Sanguinaria canadensis*

ALISSA K. SALMORE* and MARK D. HUNTER

Institute of Ecology
University of Georgia
Athens, Georgia 30602

(Received June 22, 2000; accepted May 10, 2001)

Abstract—Evaluation of biotic interactions along geographic gradients reveals that pressure on plant populations by herbivores and pathogens increases as latitude decreases, and is accompanied by a parallel increase in the number and toxicity of alkaloid-bearing plants. We compared rhizome alkaloid content with plant reproductive and vegetative characters in *Sanguinaria canadensis* (Papaveraceae) along an elevational gradient over two growing seasons to ascertain 1) if alkaloid production in bloodroot varies among populations and systematically with elevation, and 2) if there exists a correlation between isoquinoline alkaloid, vegetative and reproductive production. In general, alkaloid content in bloodroot rhizomes declines with elevation, increases with rhizome water content, varies by site, and fluctuates seasonally with plant growth and reproduction. Alkaloid content was positively correlated with vegetative and reproductive effort with few exceptions. Analysis of total protopine and benzophenanthridine alkaloid concentrations revealed generally similar patterns as those of individual alkaloid concentrations, although significant differences did appear between individual alkaloid concentrations.

Key Words—*Sanguinaria canadensis*, bloodroot, isoquinoline alkaloids, elevation, plant defense, elaiosome.

INTRODUCTION

Abiotic and biotic environmental factors, as well as plant genotype, phenology and ontogeny can all influence the allocation of resources within plants (Coleman and Jones, 1991). Geographic gradients are useful in studying biotic interactions as

* To whom correspondence should be addressed. Current address: Great Lakes WATER Institute, 600 E. Greenfield Ave., Milwaukee, WI 53204.

they constitute natural experiments by providing variations in abiotic factors under which the biotic interactions can be evaluated. This has proven particularly effective for investigating facets of plant-herbivore interactions. There exists compelling evidence that pressures on plant populations by herbivores and pathogens increase with decreasing latitude (reviews by Levin, 1976; Vermeij and Veil, 1978; Jeanne, 1979; Gaines and Lubchenco, 1982); in a parallel fashion, there is an increase in the percent of alkaloid-bearing plants within and among plant families and an increase in toxicity of those alkaloids with decreasing latitude (Levin, 1976; Levin and York, 1978). These relationships are reflected in elevational gradients as well. Herbivory often declines with increasing elevation (Breulheide and Scheidel, 1999; Fuentes-Contreras et al., 1999 but see Reynolds and Crossley, 1997; Suzuki, 1998; Hengxiao et al., 1999), with evidence for decreasing alkaloid chemical defenses with increasing elevation (Chandra and Purohit, 1980; Carey and Wink, 1994).

Such patterns support hypotheses regarding selection on and costliness of plant defense. Considerable effort has been dedicated to determining the direct costs of chemical defense in terms of metabolism, growth, and reproduction, with the goal of illustrating a decrease in fitness with increasing defense in the absence of herbivores, but studies have shown a range of conflicting results (reviews by Gershenzon, 1994; Bergelson and Purrington, 1996). Mauricio and Rausher (1997) recently demonstrated that *Arabidopsis thaliana* plants released from insect and pathogen pressure experience a decrease in selection pressure on glucosinolate concentration and trichome density. It follows, then, that with decreasing herbivore and pathogen pressure toward higher latitudes or elevations, chemical defenses may similarly decline or show higher variation due to the release from selection pressure. Moreover, a relative increase in vegetative or reproductive effort in higher latitudes or elevations should be apparent if defenses are costly.

We present here a study of variation in isoquinoline alkaloid chemistry, vegetative growth, and reproduction among populations of *Sanguinaria canadensis* along an elevational gradient. The goals of our research are 1) to describe variation in isoquinoline alkaloid concentrations among populations and systematically with elevation in *S. canadensis* (bloodroot), and 2) to determine if there is a negative correlation between alkaloid production and efforts toward vegetative growth and reproduction.

Natural History. S. canadensis (bloodroot) is a perennial herb that occurs in a widespread but patchy distribution in eastern North America from Florida to Nova Scotia, and produces several highly bioactive isoquinoline alkaloids in the rhizomes, stems, and leaves, noticeable as a bright orange-red latex. These broad-spectrum defense chemicals deter or kill bacteria, fungi, insects, nematodes, and protozoa (Miller and Feeny, 1983; review in Downum, 1992; Schmeller et al., 1997). Plants reproduce both by seeds and by vegetative propagation of the rhizome. Pods are produced from May through early July and may contain up to 50 seeds, each displaying an oil-rich seed appendage (elaiosome). The elaiosomes

contain fatty acids, amino acids, and diglycerides that attract and stimulate ants to act as dispersal agents (Marshall et al., 1979; Skidmore and Heithaus,1988; Gunther and Lanza, 1989). Germination rates in *S. canadensis* are greater after elaiosome removal by ants (Lobstein and Rockwood, 1993) and ant dispersal may influence genetic relatedness of local bloodroot populations (Pudlo et al., 1980).

METHODS AND MATERIALS

Field Sites. We monitored populations of bloodroot along an elevational transect between Athens, Georgia, and Franklin, North Carolina, during the 1998 and 1999 growing seasons. All populations were located within mesic coves in deciduous hardwood forest. Cover for these sites ranged from 68 to 92% after canopy leaf emergence, estimated using a densiometer estimation. Low-elevation populations ranged from 195 to 215 m in Clarke, Oglethorpe, and Oconee Counties,Georgia; mid-elevation populations between 340 and 550 m were located in Habersham and Rabun Counties, Georgia; high-elevation sites were found between 760-1280 m in Macon County, North Carolina within the Coweeta Hydrologic Laboratory LTER boundaries. Samples included 4 to 12 individuals per population (i.e., per site). Up to 14 sites were included per elevation. Spring 1999 samples ($n = 206$) included a second sampling of plants from November 1998 ($n = 120$), as well as newly sampled plants.

Buds and leaves emerged from the soil between February and April each year. After emergence, plants were tagged around the base of the leaf stem. In both years of study, field data were collected on rhizome width and length, flower and leaf phenology, leaf number and size at time of seed-set, and number of flowers and seeds produced. Sites were visited starting in February when plants emerged in the spring. Each site was visited weekly during flowering, twice monthly during early seed-set, and bi-weekly as seeds ripened. As the pods opened on the plant, seeds were collected, and diaspore and elaiosome weights were recorded. Elaiosome tissue was dissected from the seed and weighed. In November 1998, tissue samples were taken during plant dormancy for chemical analysis ("Fall 1998" samples). In 1999, March-May tissue samples were taken at the time of seed collection ("Spring 1999" samples). To take tissue samples, we first uncovered the rhizome and measured length and width; the roots were left undisturbed. We excised 0.5-1g of tissue from the middle of the rhizome equidistant from the base of the growing bud and the end of the living rhizome tissue. We then weighed the tissue sample using a portable digital balance, and placed it in a foil- covered glass vial with 10 ml of 100% HPLC-grade methanol. Vials were stored at -20° C until processed for chemical analysis.

Chemical Analysis. Tissue samples for HPLC analysis were homogenized up to one minute in the original vial using an Omni homogenizer fitted with a

saw-tooth rotor. The resulting slurry was filtered using a 13 mm nylon syringe-tip filter, transferred to a clean vial, and stored at -20°C until HPLC analysis. The homogonizer was rinsed with 1 ml of methanol, adding to the total volume of the samples. No further pre-purification of the samples was performed. Due to the photosensitivity of sanguinarine, extraction procedures were conducted under low-light conditions, and vials were either amber glass or wrapped in aluminum foil. Filtered samples (40 μl each) were analyzed at 284 nm using an HPLC Separations Module 2690 and Waters 996 Photodiode Array Detector (Waters Assoc, Milford, MA). Alkaloids were separated using a Phenomenex Silica C18 column (5 μm, 1.5×46) with a mobile phase of 0.1 N tartaric acid (0.125% SDS) in 1:1 v:v in acetonitrile; the flow rate was 0.5 ml/min (Hashimioto et al.,1986; Mahady et al., 1993). External standards were obtained for sanguinarine ($R_T = 11.87$), berberine ($R_T = 14.94$), and chelerythrine ($R_T = 16.13$) from Sigma Chemical Co. (St. Louis, MO). Remaining peaks were tentatively identified as protopine ($R_T = 8.59$), allocryptopine ($R_T = 9.42$), chelirubine ($R_T = 11.13$), sanguirubine ($R_T =$ approx. 14.79), chelilutine ($R_T = 19.53$), and sanguilutine ($R_T = 21.49$) by comparing elution times and relative peak areas from previous papers (Hashimoto et al., 1986; Thorne et al., 1986; Mahady et al., 1993). Because of extensive work on the pharmacological properties of bloodroot alkaloids, these identifications should be robust but we stress that we did not identify them ourselves. Consequently, we consider these identifications as tentative and their concentrations are given as sanguinarine equivalents. In some HPLC profiles, the peaks for sanguirubine coeluted with berberine and resolved with inconsistent clarity; we therefore excluded them from subsequent analyses.

Statistical AnalysIs. Data were analyzed using generalized linear model in SAS (PROC GENMOD; Anonymous, 1996) by eliminating variables and interaction terms sequentially from the model to provide the most simple and parsimonious models available (Agresti, 1996). Alkaloid concentrations were analyzed individually and as alkaloid group totals. The protopine group totals include protopine and allocryptopine; the benzophenanthridine group totals include chelirubine, sanguinarine, chelerythrine, chelilutine, and sanguilutine.

RESULTS

Variation in Alkaloid Characters. Alkaloid concentrations either declined or were unrelated to elevation. Total benzophenanthridine group concentrations decreased with increasing elevation for both Fall 1998 and Spring 1999, whereas total protopine group concentrations were unrelated to elevation for either date (Table 1; Figure 1). Individual concentrations of sanguinarine and chelerythrine were unrelated to elevation in Fall 1998, but the remaining individual alkaloid concentrations declined with increasing elevation. In Spring 1999, sanguinarine

TABLE 1. BETWEEN-YEAR VARIATION IN ALKALOID CONCENTRATIONS AND CORRELATION WITH ELEVATION AND SITE FOR 1998 AND 1999 IN *Sanguinaria canadensis*

	1998	1999	$\chi^2(p)$
Protopine			
Year effect[a]	1.48 ± 0.17	1.17 ± 0.06	4.44(.0351)
Elevation effect[b]	19.51(.0001) −	ns	
Site effect[b]	68.2(.0001)	52.69(.0004)	
Allocryptopine			
Year effect[a]	1.26 ± 0.14	1.07 ± 0.06	ns
Elevation effect[b]	12.24(.0005) −	ns	
Site effect[b]	49.02(.0005)	49.85(.0010)	
Total protopines			
Year effect[a]	2.27 ± 0.29	2.26 ± 0.11	ns
Elevation effect[b]	ns	ns	
Site effect[b]	47.43(.044)	51.38(.0006)	
Chelirubine			
Year effect[a]	1.78 ± 0.18	0.87 ± 0.04	35.24(.0001)
Elevation effect[b]	5.83(.0157) −	ns	
Site effect[b]	46.39(.0011)	ns	
Sanguinarine			
Year effect[a]	4.34 ± 0.27	4.1 ± 0.14	ns
Elevation effect[b]	ns	19.97(.0001)	
Site effect[b]	57.83(.0001)	48.77(.0013)	
Chelerythrine			
Year effect[a]	2.94 ± 0.19	2.29 ± 0.08	13.83(.0002)
Elevation effect[b]	ns	ns	
Site effect[b]	37.8(.0136)	37.14(.0314)	
Chelilutine			
Year effect[a]	1.99 ± 0.24	1.16 ± 0.06	21.41(.0001)
Elevation effect[b]	22.00(.0001) −	ns	
Site effect[b]	47.12(.0009)	39.58(.0171)	
Sanguilutine			
Year effect[a]	2.09 ± 0.25	1.25 ± 0.07	16.31(.0001)
Elevation effect[b]	26.08(.0001) −	ns	
Site effect[b]	44.23(.0022)	41.42(.005)	
Total benzophenanthridines			
Year effect[a]	13.65 ± 1.57	8.83 ± 0.30	13.65(.0001)
Elevation effect[b]	17.25(.0001) −	6.75(.0094)	
Site effect[b]	72.09(<.0001)	36.04(<.0408)	

Note: Alkaloid concentrations were measured over 3 consecutive days in November 1998 and as seeds set throughout Spring 1999. −: negative correlation; +: positive correlation. For 1998 site effect, $df = 25$, and for 1999 site effect, $df = 23$. For all other independent variables, $df = 1$.

[a] Values are mean % dry weight ± S.E. $\chi^2(p)$.

[b] Values are $\chi^2(p)$.

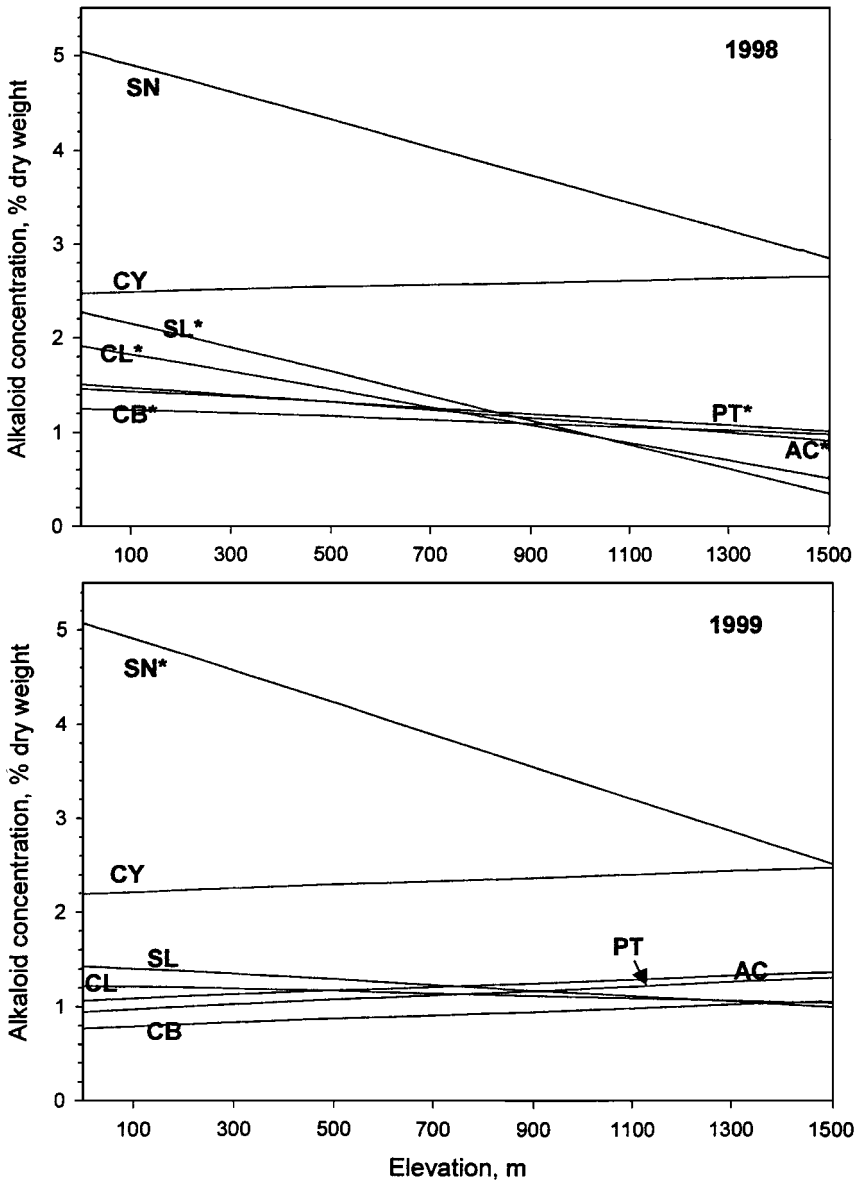


FIG. 1. Comparison of trends in alkaloid concentrations of *Sanguinaria canadensis* over an elevational gradient between November 1998 and Spring 1999. PT: protopine, AC: allocryptopine, CB: chelirubine, SN: sanguinarine, CY: chelerythrine, CL: chelilutine, SL: sanguilutine. ∗ indicates significant relationships.

was the only individual alkaloid that significantly declined with elevation; the concentrations of all other alkaloids were unrelated to elevation (Table 1; Figure 1). Total benzophenanthridine group and total protopine group concentrations differed among sites in both 1998 and 1999. Individual alkaloid concentrations, with the exception of Spring 1999 chelirubine concentrations, showed variation by site for both dates (Table 1). Alkaloid content of rhizomes declined from dormancy (Fall 1998) to active growth (Spring 1999), with the exception of total protopine and sanguinarine concentrations (Table 1).

Variation in Vegetative and Reproductive Characters. Elevational trends observed in vegetative and reproductive characters varied with character and sample date. Leaf size increased with elevation in 1998, but decreased with elevation in 1999. Rhizome length and width were unrelated to elevation for either date (Table 2). Mean rhizome width was greater in 1999. Notably, water content of rhizomes declined with elevation for both dates. In general, the majority of vegetative characters did not vary according to site, although in some instances, weak correlations were evident for rhizome width, rhizome length, and leaf size (Table 2).

In 1998, the number of flowers per plant and elaiosome weight were unrelated to elevation, whereas they both declined with increasing elevation in 1999 (Table 2). The number of seeds per plant increased with elevation in both years. Reproductive characters varied more strongly by site in 1999 than in 1998, although seed weight variation was unrelated to among-site differences (Table 2). Elaiosome weight was higher and average flowers per plant lower in 1999. Reproductive characters were unrelated to percent water in the rhizome in both 1998 and 1999.

Alkaloids vs Vegetative and Reproductive Characters. Alkaloid content generally increased with, or was unrelated to, vegetative and reproductive effort, with a few exceptions. Total Fall 1998 benzophenanthridine and protopine group concentrations increased with rhizome size, and total protopine group concentrations decreased with leaf size in 1998 (Table 3). Total protopine concentrations sampled in Spring 1999 were unrelated to vegetative characters, whereas total benzophenanthridine concentrations increased with rhizome size and decreased with leaf size. Total benzophenanthridine and protopine group concentrations increased with rhizome water content in both fall 1998 and Spring 1999. With the exception of chelirubine, individual alkaloid concentrations reflected the same correlation for both sample dates (Table 3).

Total alkaloid concentrations in Fall 1998 were higher in plants that had produced more flowers and heavier seeds during the 1998 growing season. Individually, all alkaloids increased with seed weight, allocryptopine increased with number of flowers per plant, and chelerythrine increased with number of flowers and number of seeds per plant in 1998 (Table 4). In contrast, protopine, allocryptopine, and sanguilutine concentrations decreased with an increase in the number of seeds per plant in 1998. No relationships were found between either total benzophenanthridine or total protopine group concentrations and Spring 1999

TABLE 2. BETWEEN-YEAR VARIATION IN REPRODUCTIVE AND VEGETATIVE CHARACTERS AND CORRELATION WITH ELEVATION AND SITE FOR 1998 AND 1999 IN *Sanguinaria canadensis*

	1998	1999	$\chi^2(p)$
Elaiosome weight			
Year effect, mean g ± S.E., $\chi^2(p)$	0.0018 ± .0001	.0036 ± .0001	78.75(.0001)
Elevation effect[a]	ns	26.90(.0001) −	
Site effect[a]	56.46(.0001)	112.8(.0001)	
Seed weight, g			
Year effect, g ± S.E., $\chi^2(p)$	0.012 ± .0004	.012 ± .0001	ns
Elevation effect[a]	ns	21.99(.0001) +	
Site effect[a]	152.75(.0001)	ns	
No. of seeds/plant			
Year effect, No. ± S.E., $\chi^2(p)$	11.8 ± .92	13.8 ± .75	ns
Elevation effect[a]	9.40(.0022) +	24.18(.0001) +	
Site effect[a]	32.13(.0212)	43.25(.0065)	
No. of flowers/plant			
Year effect, No. ± S.E., $\chi^2(p)$	2.08 ± .25	1.35 ± .04	18.54(.0001)
Elevation effect[a]	ns	5.75(.0165) −	
Site effect[a]	ns	39.26(.0256)	
Leaf width, cm			
Year effect, cm ± S.E., $\chi^2(p)$	12.56 ± .45	11.77 ± .26	ns
Elevation effect[a]	7.79(.0053) +	35.10(.0001) −	
Site effect[a]	ns	ns	
Rhizome width, cm			
Year effect, cm ± S.E., $\chi^2(p)$	1.25 ± .03	1.17 ± .02	5.69(.0171)
Elevation effect[a]	ns	ns	
Site effect[a]	34.32(.0335)	ns	
Rhizome length, cm			
Year effect, cm ± S.E., $\chi^2(p)$	7.6 ± .74	6.55 ± .25	ns
Elevation effect[a]	ns	ns	
Site effect[a]	ns	5.09(.0241)	
% Water of total rhizome weight			
Year effect, % total weight ± S.E., $\chi^2(p)$	79.5 ± .8	78.5 ± .43	ns
Elevation effect[a]	10.39(.0013) −	37.14(.0001) −	
Site effect[a]	40.11(.0072)	ns	

Note: Characters were measured at seed set in the 1998 and 1999 growing seasons; measures of rhizome size and sampling of tissue to determine % water in the rhizomes were completed over 3 consecutive days in November 1998 and as seeds set throughout Spring 1999. −: negative correlation; +: positive correlation. For 1998 site effect, $df = 25$, and for 1999 site effect, $df = 23$. For all other independent variables, $df = 1$.

[a] Values are $\chi^2(p)$.

reproductive characters. Individually, sanguinarine concentration increased with elaiosome weight in 1999 although no other relationships between individual alkaloid concentration and reproductive characters were apparent for that sample date (Table 4).

TABLE 3. CORRELATION OF ALKALOID CONCENTRATIONS WITH VEGETATIVE CHARACTERS IN *Sanguinaria canadensis*

	PT	AC	Total PR	CB	SN	CY	CL	SL	Total BZ
No. of leaves/plant									
1998	ns	ns	ns	ns	ns	ns	ns	ns	ns
1999	ns	ns	ns	**6.58(.0103)**	ns	ns	ns	ns	ns
Average leaf size									
1998	**10.47(.0012)**	ns	**7.8(.0052)**	ns	ns	ns	ns	ns	**8.51(.0035)**
1999	ns	ns	ns	ns	ns	ns	ns	ns	**9.17(.0025)**
Rhizome width									
1998	ns	ns	ns	ns	ns	ns	ns	ns	ns
1999	9.40(.0022)	.98(.0017)	ns	24.30(.0001)	ns	9.66(.0019)	29.23(.0001)	154.8(.0001)	6.24(.0125)
Rhizome length									
1998	6.55(.0105)	10.01(.0016)	ns	ns	ns	ns	ns	ns	7.89(.005)
1999	**4.67(.0306)**	ns	ns	ns	ns	ns	ns	ns	ns
% Water weight of rhizome									
1998	ns	ns	40.93(.0001)	29.96(.0001)	178.6(.0001)	16.78(.0001)	23.45(.0001)	16.86(.0001)	95.6(.0001)
1999	7.11(.0077)	7.14(.0075)	20.11(.0021)	9.49(.0021)	44.24(.0001)	27.74(.0001)	27.74(.0001)	4.49(.0341)	4.49(.0001)

Note: PT: protopine, AC: allocryptopine, Total PR: Total protopine group, CB: chelirubine, SN: sanguinarine, CY: chelerythrine, CL: chelilutine, SL: sanguilutine, Total BZ: Total Benzophenanthridine group. Negative correlations are indicated in bold text. For all independent variables, $df = 1$.

TABLE 4. CORRELATION OF ALKALOID CONCENTRATIONS WITH REPRODUCTIVE CHARACTERS IN *Sanguinaria canadensis*

	PT	AC	Total PR	CB	SN	CY	CL	SL	Total BZ
Elaiosome weight									
1998	ns	ns	ns	ns	ns	ns	ns	ns	ns
1999	ns	ns	ns	ns	4.3(.0379)	ns	ns	ns	ns
Seed weight									
1998	22.96(.0001)	19.43(.0001)	6.57(.0104)	19.43(.0001)	6.97(.0083)	23.67(.0001)	31.99(.0001)	10.58(.0011)	16.21(.0001)
1999	ns	ns	ns	ns	ns	ns	ns	ns	ns
No. of flowers/plant									
1998	ns	3.96(.0466)	4.55(.0329)	ns	ns	5.22(.0223)	ns	4.29(.0382)	12.64(.0004)
1999	ns	ns	ns	ns	ns	ns	ns	ns	ns
No. of seeds/plant									
1998	**11.38(.0007)**	**23.22(.0001)**	ns	ns	ns	4.02(.0449)	ns	**5.66(.0173)**	ns
1999	ns	ns	ns	ns	ns	ns	ns	ns	ns
No. of flower × No. of seeds									
1998	ns	ns	ns	ns	ns	4.8(.0283)	ns	ns	ns
1999	ns	ns	ns	ns	ns	ns	ns	ns	ns
No. of seeds × seed weight									
1998	ns	18.69(.0001)	ns	ns	ns	ns	ns	ns	ns
1999	ns	ns	ns	ns	ns	ns	ns	ns	ns

Note: PT: protopine, AC: allocryptopine, Total PR: Total protopine group, CB: chelirubine, SN: sanguinarine, CY: chelerythrine, CL: chelilutine, SL: sanguilutine, Total BZ: Total Benzophenanthridine group. Negative correlations are indicated in bold text. For all independent variables, $df = 1$.

Overall Predictive Model. In a model assessing the relative contributions of elevation, site, reproductive, and vegetative characters to rhizome alkaloid content of bloodroot, rhizome water content and site explained most of the variance in both total benzophenanthridine and total protopine group concentrations for both 1998 and 1999 (Table 5). The strongest predictor of individual alkaloid concentrations in Fall 1998 was seed weight in most cases. Elevation and percent water in the rhizome were related to individual concentrations in Spring 1999 (Table 5).

DISCUSSION

Elevational Variation. Our results provide further evidence for an elevational cline in alkaloid production. Because we did not quantify herbivory or pathogen load, it cannot be determined if lower rhizome alkaloid content observed at higher elevations is a result of release from selective pressure, but our results are consistent with this hypothesis. Significant site effects suggest there are local influences on alkaloid production apart from larger climatic forces that accompany elevational gradients, or genetic variation in the alkaloid production of local populations.

The strong increase in alkaloid concentration with rhizome water content may result from nitrogen- or micronutrient-limitations. Water availability can constrain nutrient absorption because diffusion rate is the limiting step in the uptake of scarce nutrients (Landers et al., 1997) and soil nutrients influence alkaloid production (Waterman and Mole, 1989; Ohnmeiss and Baldwin, 1994; Baldwin et al., 1998; Salmore and Hunter, 2001). The observed increase in seed number and leaf size with elevation suggests that any nutrient limitation to alkaloid biosynthetic pathways may be a consequence of a within-plant allocation shift, rather than an overall nutrient limitation to the whole plant. Furthermore, the ability to generate secondary compounds under nutrient enrichment may be constrained genetically (reviews in Waller and Nowacki, 1978 and Roberts and Wink, 1998).

The decrease in rhizome alkaloid content between fall and spring sample dates supports the findings of Bennett et al. (1990), that showed the lowest concentration alkaloids in the rhizome at late seed-set. During active growth, alkaloid production may appear to decline due to an increase in biomass, even though total production may be equivalent or greater in the spring. Little change in rhizome size was observed between years to account for this. Translocation of alkaloids from the rhizome to fine roots or above-ground parts could cause this pattern, but currently no evidence exists for long-distance transport of alkaloids in *S. canadensis* (Kutchan et al., 1985). Alternatively, alkaloid production in fine roots or above-ground parts could increase at the expense of alkaloids produced in the rhizome.

Competition for substrate during the production of alkaloids derived from similar metabolic pathways may explain some of the variation in individual alkaloid concentrations. The isoquinoline alkaloids found in bloodroot are biosynthetically

TABLE 5. BEST PREDICTORS OF ALKALOID CONCENTRATIONS IN *Sanguinaria canadensis* AMONG ELEVATION, SITE, REPRODUCTIVE, AND VEGETATIVE CHARACTERS

	Fall 1998				Spring 1999			
	Variable	*df*	χ^2	*p*	Variable	*df*	χ^2	*p*
Protopine	+Seed weight	1	22.73	.0001	−Elevation	1	7.00	.0081
	+%H_2O	1	4.24	.0393	+%H_2O	1	12.62	.0004
Allocryptopine	+Seed weight	1	12.22	.0005	−Elevation	1	9.4	.0021
	+Rhizome Length	1	6.97	.0083	+%H_2O	1	14.98	.0001
Total protopines	Site	25	65.12	<.0001	Site	23	78.53	<.0001
	+%H_2O	1	14.24	.0002	+%H_2O	1	3.89	.0486
	+Rhizome Length	1	4.95	.0206	−Rhizome Length	1	20.11	<.0001
	−Leaf size	1	9.76	.0018	+Elaiosome weight	1	4.03	.0448
Sanguinarine	+Seed weight	1	3.90	.0481	−Elevation	1	5.56	.0184
	+%H_2O	1	4.31	.0378	+%H_2O	1	24.81	.0001
Chelerythrine	+Seed weight	1	34.44	.0001	−Elevation	1	10.12	.0015
	−Elevation	1	5.4	.0201	+%H_2O	1	28.39	.0001
Chelirubine	+Seed weight	1	19.43	.0001	−Elevation	1	8.29	.004
					+%H_2O	1	9.48	.0021
Chelilutine	+Seed weight	1	24.03	.0001	+%H_2O	1	19.85	.0001
	−Elevation	1	5.09	.0240				
Sanguilutine	+Seed weight	1	28.70	.0001	+%H_2O	1	12.36	.0004
	−Elevation	1	17.16	.0001				
Total benzophenanthridines	Site	25	63.49	<.0001	Site	23	37.06	.0320
	+%H_2O	1	81.37	<.0001	+%H_2O	1	34.4	<.0001
	+Rhizome length	1	4.17	.0412				

Note: %H_2O: Percent water content of rhizome; +: positive correlation between characters; −: negative correlation.

closely related; protopine is a key substrate for the more highly oxidized benzophenanthridine alkaloids, and these derived molecules may compete for substrate as well (Zenk, 1994; Roberts, 1998).

Correlations Between Defense, Growth, and Reproduction. Contrary to our prediction, sanguinarine was positively correlated with seed and elaiosome size, all alkaloids increased with seed weight, and higher alkaloid concentrations occured in larger rhizomes. If herbivory drives the level of defense investment (Feeney, 1976; Rhoades and Cates, 1976), then better defended plants escape herbivory and are able to produce higher quality seeds, or if resource availability determines the amount of nutrients available (Bryant et al., 1983; Coley et al.,1985), then bloodroot plants that are able to invest in higher quality seeds also are able to invest more in sanguinarine production. Quantification of herbivory and pathogen loads would provide insight into the mechanism driving patterns of alkaloid production. An increase in 1998 concentrations of protopine, allocryptopine, and sanguilutine with a decline in the number of seeds produced in the previous growing season may provide evidence for a trade-off, but this pattern is evident only in the 1998 sample date and for three of the seven alkaloids. A more likely cause for this correlation is the production of sanguinarine, which requires available protopine pools, and competes for a biosynthetic dihydro-intermediate with more oxidized alkaloids (Roberts, 1998) like sanguilutine.

Conclusion. Alkaloid concentrations in *Sanguinaria canadensis* provide further evidence for an elevational cline in the production of defensive compounds, although the results of this study demonstrate that rhizome alkaloid content varies with seasonal plant activity. The manifestation of trade-offs involving alkaloid production, in turn, is affected by these fluctuations and may lead to different conclusions based on the date that samples were taken. Total alkaloid group concentrations provide insight into main biosynthetic activities, while patterns seen in individual alkaloids illustrate complex within-organism metabolic allocation. Alkaloid content data resulting from growing clones or transplants of *S. canadensis* over several years under an experimental manipulation of herbivores and pathogens would better delineate trade-offs between defense, growth, and reproduction.

Acknowledgments—The authors thank M. Thomas, R. Klaper, and A. Reynolds for assistance in the field, and T. Maddox, T. Ackerman, and M. Madritch for technical assistance. We are grateful to A. Shenk, S. Sanders, and J. Schmidt for permission to visit their property. Invaluable access to analytical equipment was graciously offered by Prof. W. Randle. The paper was much improved by helpful comments from R. Klaper, M. Thomas, and two anonymous reviewers. Finally, we thank the UGA Museum of Natural History Josh Laerm Award for financial assistance.

REFERENCES

ANONYMOUS. 1996. Statistical Analysis Software. Version 6.12. SAS Institute, Carey, NC.
AGRESTI, A. 1996. An Introduction to Categorical Data Analysis. Wiley, New York, NY.

BALDWIN, I. T., GORHAM, D., SCHMELZ, E. A., LEWANDOWSKI, C. A., and LYNDS, G.Y. 1998. Allocation of nitrogen to an inducible defense and seed production in *Nicotiana attenuata*. *Oecologia* 115:541–552.

BENNETT, B. C., BELL, C.R., and BOULWARE, R. T. 1990. Geographic variation in alkaloid content of *Sanguinaria canadensis* (Papaveraceae). *Rhodora* 92:57–69.

BERGELSON, J., and PURRINGTON, C. B. 1996. Surveying patterns in the cost of resistance in plants. *Am. Nat.* 148:536–558.

BREULHEIDE, H., and SCHEIDEL, U. 1999. Slug herbivory as a limiting factor for the geographical range of *Arnica montana*. *J. Ecol.* 87:839–848.

BRYANT, J. P., CHAPIN III, F. S., and KLEIN, D. R. 1983. Carbon-nutrient balance of plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CAREY, D. B., and WINK, M. 1994. Elevational variation of quinolizidine alkaloid contents in a lupine (*Lupinus argenteus*) of the Rocky Mountains. *J. Chem. Ecol.* 20:849–857.

CHANDRA, P., and PUROHIT, A. N. 1980. Berberine contents and alkaloid profile of *Berberis* species from different altitudes. *Biochem. Syst. Ecol.* 8:379–380.

COLEMAN, J. S., and JONES, C. G. 1991. A phyocentric perspective of phytochemical induction by herbivores, pp. 3–46, *in* D. W. Tallamy and M. J. Raupp (eds.). Phytochemical Induction by Herbivores. Wiley, New York.

COLEY, P. D., BRYANT, J. D., and CHAPIN III, F. S. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

DOWNUM, K. R. 1992. Tansley Review No. 43: Light-activated plant defense. *New Phytol.* 122:401–420.

FEENY, P. 1976. Plant apparency and chemical defense, pp. 1–14, *in* J. Wallace and R. Mansell (eds.). Biochemical Interactions Between Plants and Insects: Recent Advances Phytochemistry, Vol. 10. Plenum, New York.

FUENTES-CONTRERAS, E., GIANOLI, E., CABALLERO, P. P., and NIEMEYER, H. M. 1999. Influence of altitude and host-plant species on gall distribution in *Colliguaja* spp. (Euphorbiaceae) in central Chile. *Rev. Chil. Hist. Nat.* 72:305–313.

GAINES, S. D., and LUBCHENCO, J. 1982. A unified approach to marine plant-herbivore interactions. II. Biogeography. *Annu. Rev. Ecol. Syst.* 13:111–138.

GUNTHER, R. W., and LANZA, J. 1989. Variation in attractiveness of *Trillium* diaspores to a seed-dispersing ant. *Am. Midl. Nat.* 122:321–328.

GERSHENZON, J. 1994. Metabolic costs of terpenoid accumulation in higher plants. *J. Chem. Ecol.* 20:1281–1327.

HASHIMOTO, Y., OKADA, M. I., SHOME, U., and KATO, A. 1986. The analysis of cell contents of some papaveraceous plants by newly devised automated histochemical chromatography.*Anal. Lett.* 19:2253–2260.

HENGXIAO, G, MCMILLIN, J. D., WAGNER, M. R., ZHOU, J., ZHOU, Z., and XU, X. 1999. Altitudinal variation in foliar chemistry and anatomy of yunnan pine, *Pinus yunnanensis*, and pine sawfly (Hym., Diprionidae) performance. *J. Appl. Entol.* 123:465–471.

JEANNE, R. C. 1979. Latitudinal trends in ant predation. *Ecology* 60:1211–1224.

KUTCHAN, T. M., AYABE, S., and COSCIA, C. J. 1985. Cytodifferentiation and papaver alkaloid accumulation, pp. 280–294, *in* J. D. Phillipson, M. F. Roberts, and M. H. Zenk (eds.). The Chemistry and Biology of Isoquinoline Alkaloids. Springer-Verlag, Berlin.

LANDERS, H., CHAPIN III, F.S., and PONS, T. L. 1997. Plant Physiological Ecology. Springer-Verlag, New York.

LEVIN, D. 1976. Alkaloid-bearing plants: An ecogeographic perspective. *Am. Nat.* 110:261–284.

LEVIN, D., and YORK, B. M. 1978. The toxicity of plant alkaloids: An ecogeographic perspective. *Biochem. Syst. Ecol.* 6:61–76.

LOBSTEIN, M. B., and ROCKWOOD, L. L. 1993. Influence of elaiosome removal on germination in five ant-dispersed plant species. *Virg. J. Sci.* 44:59–73.

MAHADY, G. B., SCHILLING, A. B., and BEECHERR, C. W. W. 1993. XXII *Sanguinaria canadensis* L. (Sanguinarius): In vitro culture and the production of benzophenanthridine alkaloids, pp. 313–327, *in* Y. P. S. Bajaj (ed.). Biotechnology in Agriculture and Forestry. Vol. 24: Medicinal and Aromatic Plants V. Springer-Verlag, Berlin.

MARSHALL, D. L., BEATTIE, A. J., and BOLLENBACHER, W. E. 1979. Evidence for diglycerides as attractants in an ant-seed interaction. *J. Chem. Ecol*. 5:355–359.

MAURICIO, R., and RAUSHER, M. D. 1997. Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution* 5:1435–1444.

MILLER, J. S., and FEENY, P. 1983. Effects of benzylisoquinoline alkaloids on the larvae of polyphagous Lepidoptera. *Oecologia* 58:332–339.

OHNMEISS, T. E., and BALDWIN, I. T. 1994. The allometry of nitrogen allocation to growth and an inducible defense under nitrogen-limited growth. *Ecology* 75:995–1002.

PUDLO, R. J., BEATTIE, A. J., and CULVER, D. C. 1980. Population consequences of changes in an ant-seed mutualism in *Sanguinaria canadensis. Oecologia* 146:32–37.

REYNOLDS, B. C., and CROSSLEY, D. A. 1997. Spatial variation in herbivory by forest canopy arthropods along an elevation gradient. *Environ. Entomol.* 26:1232–1239.

RHOADES, D. F., and CATES, R. G. 1976. Toward a general theory of plant antiherbivore chemistry, pp. 168–213, *in* J. Wallace and R. Mansell (eds.). Biochemical Interactions Between Plants and Insects: Recent Advances in Phytochemistry, Vol. 10, Plenum, New York.

ROBERTS, M. F. 1998. Enzymology of alkaloid biosynthesis, pp. 109–146, *in* M. F. Roberts and M. Wink (eds.). Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum, New York.

ROBERTS, M. F., and WINK, M. 1998. Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum, New York.

SALMORE, A. K., and HUNTER, M. D. 2001. Environmental and genotypic influences on isoquinoline alkaloid content in *Sanguinaria canadensis. J. Chem. Ecol*. 27(9):xxx–xxx.

SCHMELLER, T., LATZ-BRUNING, B., and WINK, M. 1997. Biochemical activities of berberine, palmatine, and sanguinarine mediating chemical defense against micoroganisms and herbivores. *Phytochemistry* 44:257–266.

SKIDMORE, B. A., and E. R. HEITHAUS. 1988. Lipid cues for seed-carrying by ants in *Hepatica americana. J. Chem. Ecol*. 4:2185–2190.

SUZUKI, S. 1998. Leaf phenology, seasonal changes in leaf quality and herbivory pattern of *Sanguisorba tenuifolia* at different altitudes. *Oecologia* 117:169–176.

THORNE, E. M., BOULWARE, R. T., HARKRADER, R. J., and SOUTHARD, G. L. 1986. HPLC analysis of sanguinarine in oral health care products. *J. Soc. Cosmet. Chem*. 37:279–286.

VERMEIJ, G. J., and VEIL, J. A. 1978. Latitudinal pattern in bivalve shell gaping. *Malacologia* 17:57–61.

WALLER, G. R., and NOWACKI, E. K. 1978. Alkaloid Biology and Metabolism in Plants. Plenum, New York.

WATERMAN, P. G., and MOLE, S. 1989. Soil nutrients and plant secondary compounds, pp. 241–254, *in* Mineral nutrients in tropical forest and savanna ecosystems symposium. Blackwell Scientific, Cambridge, MA. Special publication of the British Ecological Society, No. 9.

ZENK, M. H. 1994. The formation of benzophenanthridine alkaloids. *Pure Appl. Chem.* 66:2023–2028.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

ENVIRONMENTAL AND GENOTYPIC INFLUENCES ON ISOQUINOLINE ALKALOID CONTENT IN *Sanguinaria canadensis*

ALISSA K. SALMORE and MARK D. HUNTER*

Institute of Ecology
University of Georgia
Athens, Georgia 30602

(Received June 22, 2000; accepted May 12, 2001)

Abstract—In a common garden, we investigated genetic and environmental influences on alkaloid production using *Sanguinaria canadensis* as a model. Nutrient and shade regimes were applied to replicated clones over one growing season, and induction of alkaloid production in bloodroot was tested on a whole-plant basis using jasmonic acid as an elicitor. Alkaloid concentrations increased with decreasing light intensity and fertilizer levels. Induction was not achieved by foliar application of jasmonic acid. Genetic influences represented by clone effects may be indicated by variation in alkaloid concentration by clone, but this experimental design did not allow us to distinguish genetic from pre-experiment environmental influences on the rhizomes.

Key Words—*Sanguinaria canadensis*, bloodroot, alkaloids, sanguinarine, phototoxicity, shade, jasmonic acid.

INTRODUCTION

Since the response of plants to herbivory is limited by the availability of resources, the allocation of limiting nutrients defines the quality and quantity of plant defenses (Bryant et al., 1983; Coley et al., 1985). Evidence exists that some alkaloid synthesis is relatively constant (Ralphs et al., 1998) or can be functionally variable (review by Eilert, 1998), where chemical or physical changes following damage can occur within a matter of hours, or after a long period as a response in the next growing season (Coleman and Jones, 1991; Baldwin, 1998). Such inducible defenses may be adaptive because individuals that express a costly defense only when needed can otherwise increase allocation to growth and reproduction

*To whom correspondence should be addressed.

0098-0331/01/0900-1729$19.50/0

(Feeney, 1976; Fagerstrom et al., 1987; Agrawal,1998; Baldwin, 1998), or because herbivores experience reduced performance in dealing with variability in defense systems and therefore avoid such plants (Karban et al., 1997).

Induction has been demonstrated experimentally for specific alkaloids in cell culture (Gundlach et al., 1992; Cline et al., 1993; Facchini, et al., 1996) and in whole plant systems (VanDam, 1993; Aerts et al., 1994; Baldwin, 1996; Baldwin et al., 1998). *Sanguinaria canadensis* cell cultures respond to fungal cell wall, hormone, and micronutrient elicitors with increased production of the isoquinoline alkaloid sanguinarine (Cline et al., 1993; Facchini et al., 1996). In cell cultures of *S. canadensis*, jasmonic acid increased alkaloid-synthesis enzyme activity to double that of control levels, whereas methyl jasmonate increased activity three- to four-fold higher (Ignatov et al., 1996).

Although environment may modify the production of defenses, the regulation of quantity and quality of production falls under genetic control (Waller and Nowacki, 1978; Saito and Murakoshi, 1998) with evidence for polygenic inheritance and chemical heterosis in hybrid studies (review by Levin, 1976). Furthermore, since phenotype reflects the interaction of a developmental program and genotype with a particular environment (Sultan, 1995), genetically controlled defense characters may vary with environment (Maddox and Cappuccino, 1986).

The purpose of this study was to assess the relative influence of genes and environment on the chemical defenses of *Sanguinaria canadensis* (bloodroot). We ask: what is the contribution of gene and environment to the production of these alkaloids? Is alkaloid production inducible on a whole-plant basis in *S. canadensis* and does induction vary by genotype? For these experiments, we propagated clones of individual plants under varying environmental conditions and subsequently collected information on alkaloid content and plant phenology.

Natural History. S. canadensis (Bloodroot) is a perennial herb that occurs in mesic deciduous hardwood forest and has a widespread but patchy distribution in eastern North America from Florida to Nova Scotia. Plants reproduce by seeds and via vegetative propagation of a fleshy rhizome. Bloodroot plants produce and store several highly bioactive benzophenanthridine alkaloids in their rhizomes, which act as broad-spectrum defense chemicals that deter or kill bacteria, fungi, insects, nematodes, and protozoa (Miller and Feeny, 1983; review by Downum, 1992; Schmeller et al., 1997). Pods are produced from May through early July and contain up to 50 seeds that have oil-rich seed appendages (elaiosomes) that are attractive to several ant species (Beattie and Culver, 1981).

METHODS AND MATERIALS

Experimental Design. In February 1998, we obtained rhizomes from Sunlight Gardens in Andersonville, Tennessee, and planted them in fertilizer-free potting

mix. Initial relatedness for the rhizomes is unknown, and given a common source, this could mean that the initial sample expressed low genetic variation. We assembled the potting mix with 25% composted pine bark mulch, 45% peat, 15% vermiculite, and 15% perlite (Conrad Fafard, Inc., Agawan, MA). Each rhizome was assigned to be fertilized at either no, medium (200 ppm), or high (400 ppm) levels using standard Hoaglan's NPK 20-10-20 fertilizer (Peters, St. Louis, MO). Fertilizer was applied bi-weekly for two months (through the growing season). When plants became dormant, they were maintained with water only. All plants were propagated in a greenhouse for the first year (Figure 1, A and B).

Ten rhizomes from each fertilizer group were assigned to the induction experiment. The remaining rhizomes from each fertilizer group were designated for the shade experiment (Figure 1C). In February 1999, when plants were still dormant, we divided each rhizome into a set of three clones (Figure 1, D and E). These pieces were measured for length and width, and tissue samples were collected from the middle of the rhizome as each rhizome was divided for HPLC analysis; up to 1 g wet weight of rhizome tissue was removed with a razor blade and placed directly into a glass scintillation vial with 10 ml of 100% HPLC-grade methanol. Samples were stored at -20°C until HPLC analysis. Clones were replanted in fertilizer-free potting mix in separate 6″ plastic pots and maintained with water for the duration of the experiments. Weekly observations on leaf, flower, and fruit number, and leaf appearance were recorded. Bloodroot facultatively self-pollinates in the absence of pollinators (Lyon, 1992); therefore, no hand-pollinations were performed. Most plants initiated seed pods, but they all eventually aborted in both the induction and shade experiments. In October 1999, the experimental plants were harvested. Rhizome length and width were measured, and a final rhizome tissue sample was taken as before and stored in 100% methanol at -20°C until analysis by HPLC.

Shade Experiment. Outdoor shade houses for the treatments were constructed by using black nylon 50% and 80% shade cloth (Synthetic Industries; Gainesville, GA) and 1″ id. PVC pipe anchored to 2.4 m by 4.6 m wooden ground frames. The no-shade treatment was placed in a ground frame without a shade cloth-PVC pipe structure. Weed cloth was placed on the ground to prevent additional shading by volunteer plants and to discourage infestation by fire ants.

One rhizome from each of the clone sets was randomly assigned to 80%, 50%, and 0% shade treatments. All clones were randomly placed within a single block in each treatment. Plants were watered 2 to 3 times during the week to maintain soil moisture. Aphids colonized some of the plants for 3 weeks mid-season, and presence/absence data were recorded.

Induction Experiment. One clone from each of the 10 sets was randomly assigned to a jasmonic acid, water, and control treatment. All clones were randomly placed within a single block on a greenhouse bench. Jasmonic acid was obtained from Sigma Chemical, Inc. (St. Louis, MO), dissolved in 1 ml 100% ethanol, and diluted to a 100 mM stock solution with distilled, deionized water and stored at

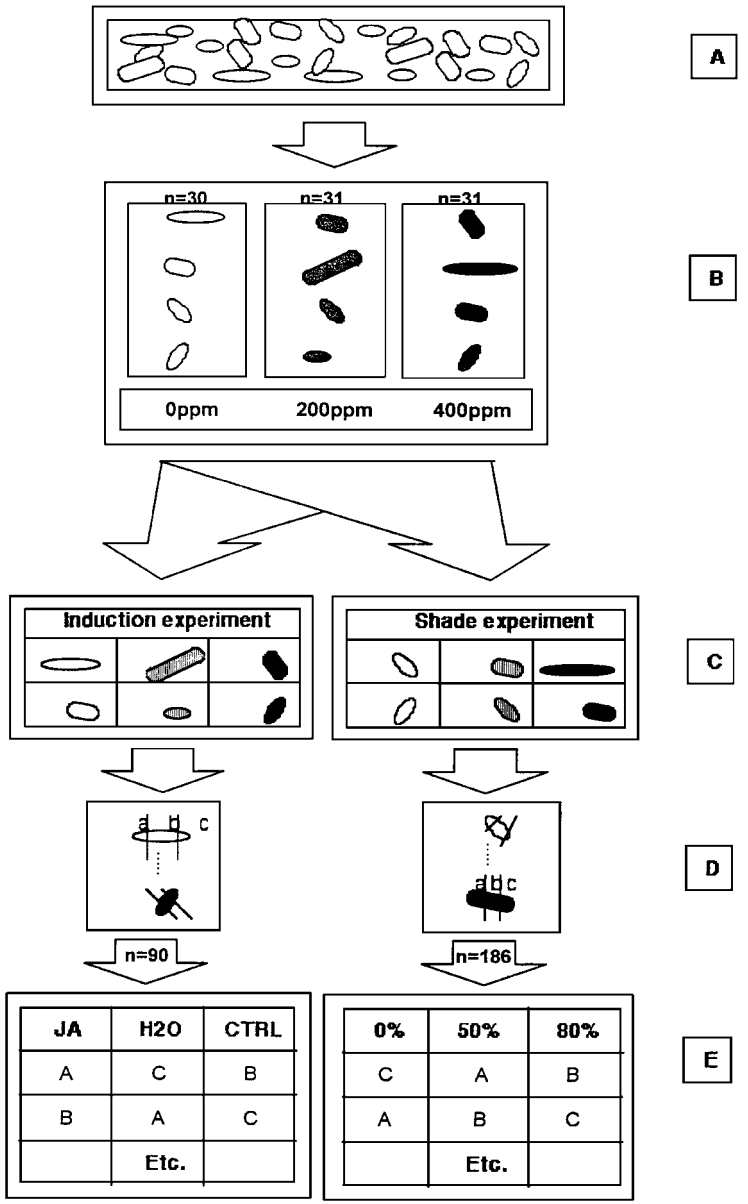


FIG. 1. Experimental design. Initial pool of rhizomes (A) assigned to fertilizer treatments (B) and propagated in the greenhouse for 1 year. Rhizomes assigned to either induction or shade experiment (C), cut into clone sets (D), and randomly assigned to treatment blocks (E).

−20°C. Jasmonic acid-treated (JA) clones received via spray bottle 5 ml of 1 mM JA in distilled, deionized water over the entire above-ground plant (Thaler et al., 1996); water-treatment clones received an application of 5 ml distilled, deionized water; control plants had no foliar application. Foliar treatments were applied by spray bottle once a week and began after the primary leaf of the individual clone was fully expanded after emerging from the soil. Treatments continued until dormancy (approximately 15 weeks). Plants were removed from the block during treatment to prevent cross-contamination by aerial spray. All plants were watered in the morning before the treatment and one additional day during the week to maintain soil moisture.

Chemical Analysis. Rhizome tissue samples were reduced to small pieces in the original vial using a scalpel. Any liquid or tissue remaining on the scalpel was rinsed back into the vial with 1 ml 100% methanol. The resulting slurry was filtered with a 13 mm nylon syringe-tip filter, transferred to a clean vial, and stored at −20°C. No further pre-purification of the samples was performed. Due to the photosensitivity of sanguinarine, extraction procedures were conducted under low-light conditions and all vials used were either amber glass or wrapped in aluminum foil. Samples (40 ul each) were analyzed with an HPLC Waters Assoc. (Milford, MA) Separations Module 2690 and Waters 996 Photodiode Array Detector, reading at 284 nm. Alkaloids were separated by using a Phenomenex Silica C18 column (5 um, 1.5 × 46 mm) with a mobile phase of 0.1N tartaric acid (0.125% SDS) in 1:1 v:v in acetonitrile; the flow rate was 0.5 ml/min (Hashimoto et al.,1986; Mahady et al., 1993). Standards were obtained for sanguinarine, berberine, and chelerythrine from Sigma Chemical Co. (St. Louis, MO). Remaining peaks were tentatively identified as protopine, allocryptopine, chelirubine, sanguirubine, chelilutine, sanguilutine and by comparing the elution time and relative peak areas from previous papers (Hashimoto et al., 1986; Thorne et al., 1986; Mahady et al., 1993). Because of extensive work on the pharmacological properties of bloodroot alkaloids, these identifications should be robust, but we stress that we did not identify them ourselves. Consequently, we consider these identifications as tentative and their concentrations are given as sanguinarine equivalents.

Statistical Analysis. Data were analyzed by using generalized linear model procedures in SAS (PROC GENMOD; Anonymous, 1996). Variables and interaction terms were sequentially eliminated from the model to provide the most simple and parsimonious model available (Agresti, 1996). Alkaloid concentrations were analyzed individually and as alkaloid group totals. The protopine group included protopine and allocryptopine; the benzophenanthridine group included chelirubine, sanguinarine, chelerythrine, sanguilutine, and chelilutine. Clone and fertilizer effects were analyzed in separate models. Although all clones in 1999 experiments were represented in each treatment, the individuals from which these clones were derived received differing fertilizer levels during 1998.

RESULTS

Alkaloids present in *S. canadensis* rhizome tissue from our experimental plants include (in order of elution) protopine ($R_T = 8.48$), allocryptopine ($R_T = 9.29$), chelirubine ($R_T = 10.94$), sanguinarine ($R_T = 11.71$), sanguirubine ($R_T =$ approx. 14.67), berberine ($R_T = 14.71$), chelerythrine ($R_T = 15.84$), chelilutine ($R_T = 19.20$), and sanguilutine ($R_T = 21.09$). Sanguinarine and chelerythrine constitute the most abundant alkaloids. Alkaloid concentrations overall were consistently lower after the shade and induction experiments compared to the pre-experiment levels (Table 1). In some HPLC profiles, the peaks for sanguirubine and berberine were small, eluted very close together, and resolved with inconsistent clarity; we therefore excluded them from subsequent analyses.

Shade Experiment. Because not all clones were in all fertilizer treatments, clone and fertilizer effects could not be directly compared and were analyzed in separate statistical models. Most individual alkaloid concentrations and total benzophenanthridine group alkaloid concentrations were influenced by clone (Table 2), with up to 10-fold variation in the production of alkaloids among clones (Figure 2). Chelirubine and total protopine group concentrations were unrelated to clone.

In either clone or fertilizer models, total benzophenanthridine alkaloid concentrations increased with increasing shade, which followed the individual concentrations of sanguinarine and chelerythrine (Figure 3, Table 2). Total protopine group concentration was higher in the 50% and 80% shade treatment, although alkaloid concentrations among these two treatments were not statistically different.

TABLE 1. COMPARATIVE MEANS (±SE) OF POSTFERTILIZER ALKALOID CONCENTRATIONS GIVEN AS PERCENT DRY WEIGHT FOR FEBRUARY 1999 AND OCTOBER 1999

	Shade experiment		Induction experiment	
Alkaloid	February 1999	October 1999	February 1999	October 1999
Protopine	0.71 ± 0.26	0.37 ± 0.16	0.74 ± 0.38	0.32 ± 0.03
Allocryptopine	0.70 ± 0.27	0.37 ± 0.17	0.77 ± 0.03	0.34 ± 0.03
Total PR alkaloids	1.41 ± 0.53	0.74 ± 0.33	1.51 ± 0.41	0.66 ± 0.06
Sanguinarine	3.52 ± 1.9	1.0 ± 0.53	4.45 ± 0.14	1.38 ± 0.11
Chelerythrine	2.02 ± 1.05	0.70 ± 0.36	2.57 ± 0.07	0.99 ± 0.05
Chelirubine	0.82 ± 0.35	0.41 ± 0.18	0.87 ± 0.03	0.37 ± 0.03
Chelilutine	1.57 ± 0.67	0.59 ± 0.24	1.83 ± 0.07	0.78 ± 0.05
Sanguilutine	0.96 ± 0.39	0.53 ± 0.21	1.03 ± 0.04	0.49 ± 0.03
Total BZ alkaloids	8.89 ± 4.36	2.59 ± 1.79	10.75 ± 0.63	4.01 ± 0.54

Note: Protopine, allocryptopine, chelirubine, chelilutine, and sanguilutine concentrations are calculated as sanguinarine equivalents. Total PR: total protopine alkaloids; Total BZ: total benzophenanthridine alkaloids.

TABLE 2. FACTORS THAT SIGNIFICANTLY INFLUENCE ALKALOID CONCENTRATION IN *Sanguinaria canadensis*: SHADE EXPERIMENT

	df	PT	AC	Total PR	SN	CY	CB	CL	SL	Total BZ
Model including clone effect on alkaloid concentration										
Clone	60	63.49 (.0287)	70.31 (.012)	ns	114.15 (.0001)	117.65 (.0001)	ns	97.42 (.0016)	87.87 (.0110)	125.25 (<.0001)
Shade	2	ns	ns	10.24 (.0060)	9.13 (.0104)	11.3 (.0035)	ns	ns	ns	10.24 (.0060)
% Water in rhizome	1	ns	ns	ns	ns	ns	4.35 (.0369)	ns	ns	ns
Rhizome width	1	5.8 (.0160)	ns	6.48 (.0109)	ns	ns	ns	5.2 (.0224)	ns	ns
Rhizome length	1	ns	ns	ns	ns	ns	5.36 (.0206)	ns	ns	ns
Model including fertilizer effect on alkaloid concentration										
Fertilizer	2	ns	ns	ns	ns	ns	11.77 (.0028)	8.66 (.0131)	13.38 (.0012)	7.21 (.0272)
Shade	2	11.01 (.0044)	8.64 (.0132)	10.24 (.0060)	8.37 (.0152)	7.6 (.0224)	ns	2.51 (.0285)	ns	8.92 (.0116)
% Water in rhizome	1	ns	ns	ns	6.23 (.0125)	ns	7.5 (.0062)	11.01 (.0009)	6.14 (.0132)	6.14 (.0132)
Rhizome width	1	4.03 (.0445)	ns	6.48 (.0109)	ns	ns	ns	ns	ns	ns
Rhizome length	1	ns	5.11 (.0238)	ns	ns	ns	ns	ns	ns	ns
Fertilizer × shade		ns	ns	ns	ns	ns	ns	8.31 (.0401)	ns	ns

Note: For all analyses, $df = 2$ for fertilizer and shade; $df = 1$ for % water in rhizome, rhizome width, rhizome length, and interaction terms. PT: protopine, AC: allocryptopine, Total PR: Total protopine alkaloids, CB: chelirubine, SN: sanguinarine, CY: chelerythrine, CL: chelilutine, SL: sanguilutine, Total BZ: total benzophenanthridine alkaloids. Statistics given as $X^2(p)$.

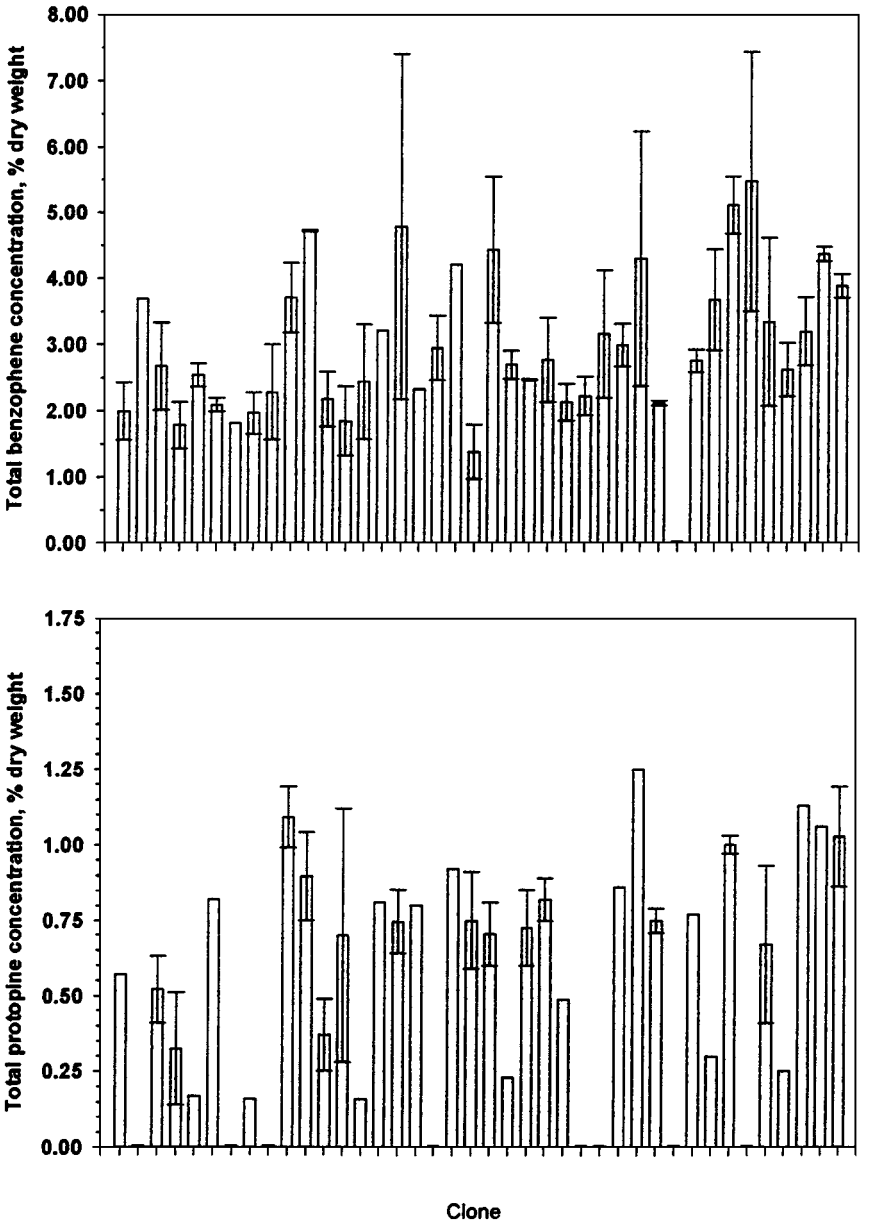


FIG. 2. Variation in total benzophene and protopine concentration among clones in *Sanguinaria canadensis*, across all fertilizer and shade treatments. Error bars are standard errors. For single clone samples ($N = 1$), data are without error bars.

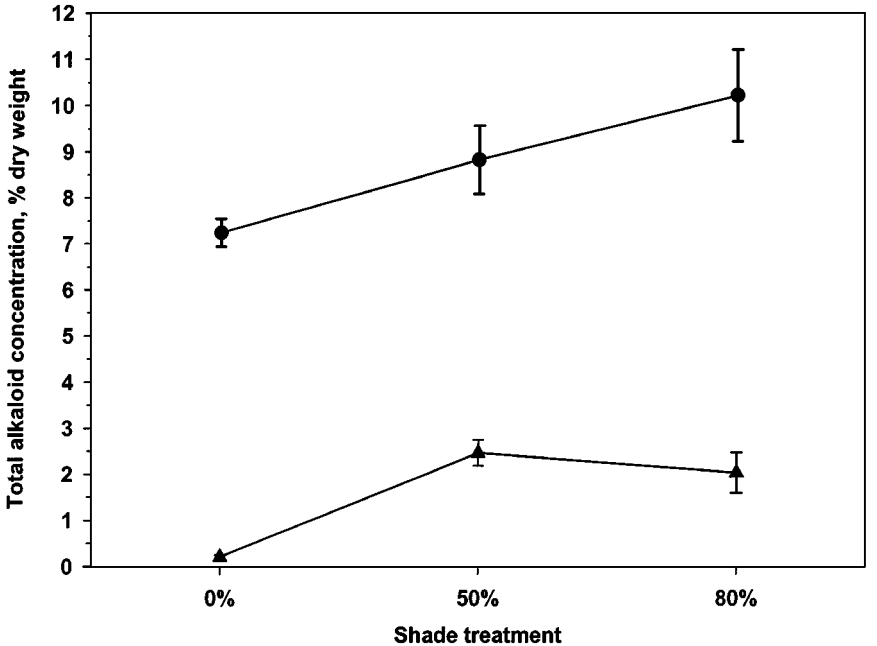


FIG. 3. The effect of shade on alkaloid concentrations in *Sanguinaria canadensis*. Total benzophenanthridine group concentration = •; total protopine group concentration = ▲. Points are the means (±SE) of 5, 61, and 58 replicates per treatment for 0%, 50%, and 80% shade, respectively. High mortality occurred in the 0% shade treatment.

This had the net effect of a weak increase in total protopine alkaloids with increasing shade (Table 2). Protopine, allocryptopine, and chelilutine concentrations increased with shade when modeled with fertilizer as a main effect, but this relationship was not significant in the clone model (Table 2).

Total benzophenanthridine group concentration was influenced by previous year fertilizer and increased with rhizome water content; neither influenced total protopine group concentrations. Zero and medium fertilizer levels produced higher mean alkaloid concentrations than plants that were provided with a high level of fertilizer (Figure 4). Fertilizer levels explained most of the variance in chelirubine, chelilutine, and sanguilutine concentrations along with rhizome water content. Sanguinarine, chelirubine, chelilutine, and sanguilutine concentrations increased with increasing rhizome water content in the model including fertilizer; however when clone was included as a variable in the model, only chelirubine concentration significantly increased with rhizome water content (Table 2).

Total protopine group alkaloid concentration, as well as individual protopine and chelilutine concentrations decreased with increasing rhizome width, and

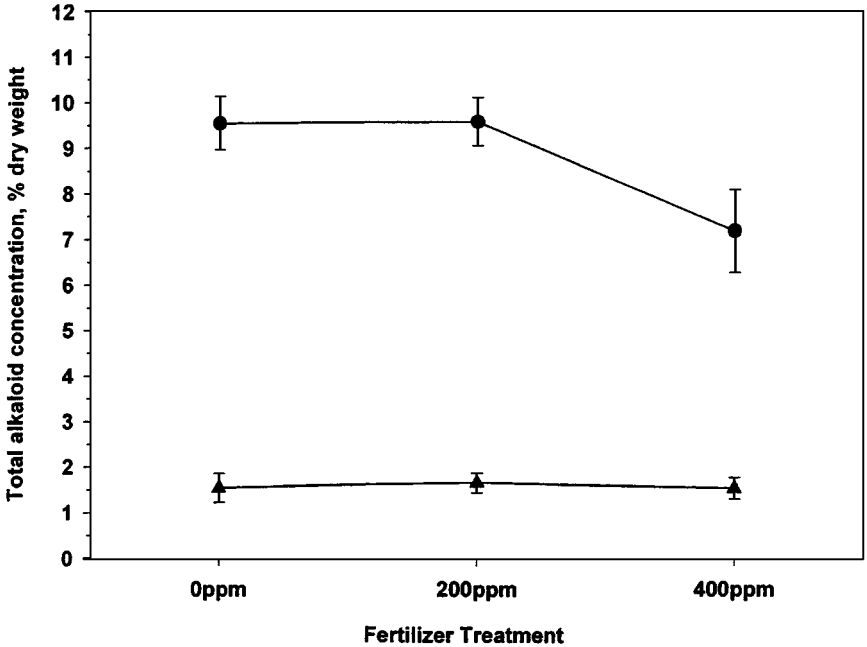


FIG. 4. Alkaloid concentration response to different fertilizer levels in *Sanguinaria canadensis*: Shade experiment. Total benzophenanthridine group concentration = •; total protopine group concentration = ▲. Points are the means (±SE) of 45, 43, 34 replicates for no, low, and high fertilizer treatments, respectively.

allocryptopine and chelirubine concentrations decreased with increasing rhizome length. Total benzophenanthridine concentration was unrelated to rhizome size. The relationship between chelirubine and chelilutine and rhizome size was not significant in the clone model (Table 2).

Induction Experiment. As in the shade experiment, not all clones were included in all fertilizer pre-treatments, so clone and fertilizer effects were analyzed in separate statistical models. All individual and total alkaloid group concentrations varied significantly by clone in this experiment (Table 3) with alkaloid concentrations varying up to 10-fold among clones (Figure 5).

Jasmonic acid applications did not increase the concentration of any of the alkaloids, individually or as group totals, over the concentrations of the control treatments (Figure 6, Table 3).

Individual and total benzophenanthridine alkaloid concentrations decreased with increasing fertilizer levels (Figure 7, Table 3), although fertilizer did not influence total protopine group concentration over all. The interaction between fertilizer and rhizome width significantly influenced protopine, allocryptopine, chelirubine,

TABLE 3. FACTORS THAT SIGNIFICANTLY INFLUENCE ALKALOID CONCENTRATION IN *Sanguinaria canadensis*: JASMONIC ACID EXPERIMENT

	df	PT	AC	Total PR	SN	CY	CB	CL	SL	Total BZ
Model including clone effect on alkaloid concentration										
Clone	29	41.12 (.0054)	52.61 (.0003)	57.71 (.0012)	66.76 (.0001)	66.82 (.0001)	58.9 (.0008)	41.06 (.0680)	43.12 (.0444)	72.93 (<.0001)
Jasmonic acid	2	ns	ns	ns	ns	ns	ns	ns	ns	ns
% Water in rhizome	1	ns	ns	ns	ns	ns	ns	5.52 (.0188)	8.9 (.0028)	ns
Rhizome width	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Rhizome length	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Clone × rhizome length	1	40.26 (.0069)	52.73 (.0002)	ns	ns	ns	58.74 (.0009)	ns	ns	ns
Clone × % water	1	ns	ns	ns	ns	ns	ns	43.4 (.0417)	44.37 (.0339)	ns
Model including fertilizer effect on alkaloid concentration										
Fertilizer	2	11.90 (.0025)	8.06 (.0177)	0.68 (.7113)	11.85 (.0027)	11.90 (.0025)	12.69 (.0018)	12.46 (.0020)	10.58 (.0050)	12.39 (.0020)
Jasmonic acid (JA)	2	ns	ns	5.20 (.0742)	ns	ns	ns	ns	ns	ns
% Water in rhizome	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Rhizome width	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Rhizome length	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fertilizer × rhizome width	1	7.63 (.0220)	8.53 (.0140)	ns	ns	ns	12.82 (.0016)	ns	11.01 (.0040)	ns
JA × fertilizer	4	ns	ns	12.73 (.0127)	ns	ns	ns	ns	ns	ns

Note: PT: protopine, AC: allocryptopine, Total PR: Total protopine alkaloids, CB: chelirubine, SN: sanguinarine, CY: chelerythrine, CL: chelilutine, SL: sanguilutine, Total BZ: total benzophenanthridine alkaloids. Statistics given as $X^2(p)$.

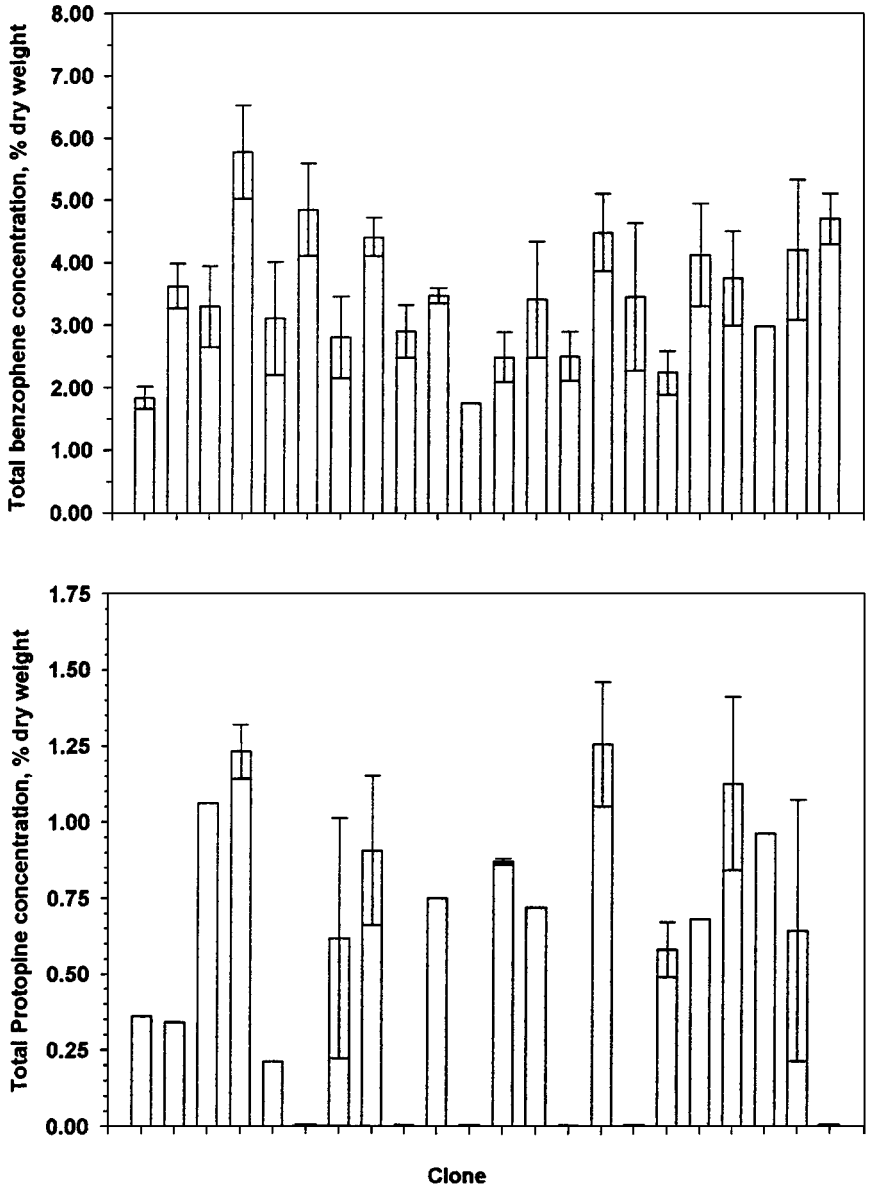


FIG. 5. Variation in total benzophene and protopine concentration among clones in *Sanguinaria canadensis*, across all fertilizer and induction treatments. Error bars are standard errors. For single clone samples ($N = 1$), data are without error bars.

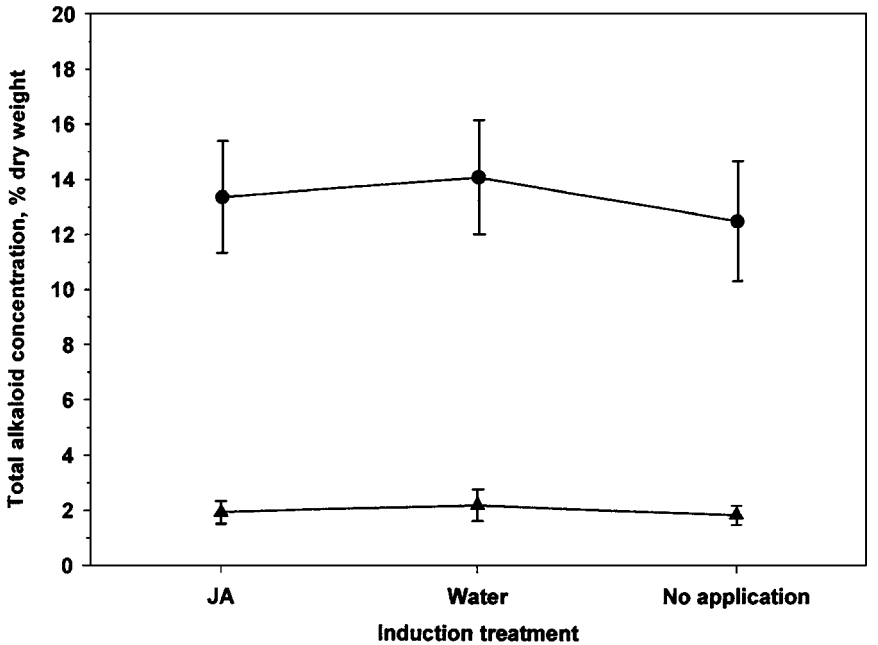


FIG. 6. The effect of foliar application of jasmonic acid on alkaloid concentrations in *Sanguinaria canadensis*. Total benzophenanthridine group concentration = •; total protopine group concentration = ▲. Points are the means (± SE) of 30 replicates per treatment.

and sanguilutine (Table 3); these alkaloids increased weakly with increasing rhizome width under medium fertilizer levels, but decreased weakly with increasing rhizome width under high fertilizer levels. No relationship between alkaloid concentration and rhizome width was apparent in the non-fertilized treatment.

The interaction between clone and rhizome length significantly influenced protopine, allocryptopine, and chelirubine levels, although total protopine and benzophenanthridine group concentrations were not influenced by rhizome size in this experiment (Table 3). The change in individual protopine, allocryptopine, and chelirubine concentrations with rhizome length varied among clones in no predictable manner.

Total benzophenanthridine concentrations increased weakly with rhizome water content, but no significant relationship between total protopine levels and rhizome water content was found. Chelilutine concentration increased and sanguilutine concentration decreased with increasing rhizome water content in the clone model (Table 3). An interaction between clone and rhizome water content explained a minor part of the variance in chelilutine and sanguilutine concentrations (Table 3). The change in chelilutine and sanguilutine concentration with increasing rhizome water content varied unpredictably among clones.

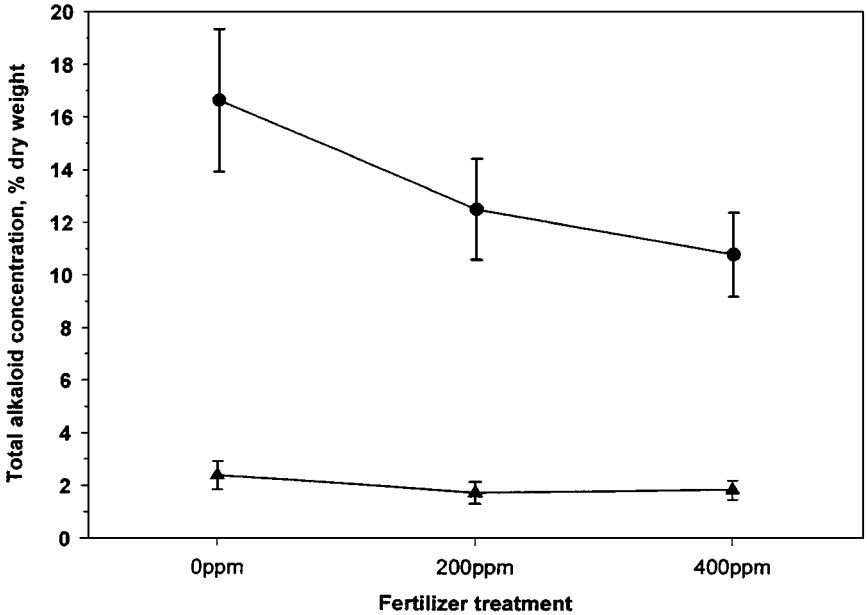


FIG. 7. Alkaloid concentration in response to fertilizer levels in *Sanguinaria canadensis*: Jasmonic acid experiment. Total benzophenanthridine group concentration = •; total protopine group concentration = ▲. Points are the means (±SE) of 30 replicates per treatment. For single clone samples (n = 1), data are without error bars.

DISCUSSION

Shade Experiment. Total benzophenanthridine alkaloid concentrations, mainly sanguinarine and chelerytherine, decreased with increasing sunlight while the protopine alkaloids only weakly reflected this trend. Benzophenanthridine alkaloids are highly photoreactive toxins and may cause increasing autotoxicity with increasing light levels (Arnason et al., 1992). One target of photosensitized alkaloids is cell membranes (Downum, 1992). When benzophenanthridine alkaloids are sensitized by light, an oxygen singlet is released by each alkaloid molecule, which can oxidize unsaturated fatty acids, cholesterol, and membrane-associated proteins (review by Downum, 1992). Degradation of vacuole membranes that sequester alkaloids could lead to an autotoxic event. If photosensitive alkaloids cause intercellular damage at high light levels, then plants under higher light conditions may maintain lower concentrations of alkaloids in their tissues to avoid autotoxicity. However, since high mortality occurred in the 0% shade treatment, the plants in that treatment may have produced lower alkaloid concentrations due to poor vigor from abiotic stress rather than due to a down-regulation response to high light. The

contribution of autotoxicity to the decline in vigor could not be determined from the data collected.

The shade experiment also showed that different alkaloids varied in their response to ambient light levels in *S. canadensis*. In contrast to sanguinarine and chelerythrine, concentrations of chelirubine, chelilutine, sanguilutine, and total protopine alkaloid concentrations varied weakly or were unaffected by light level. The parallel patterns seen between more similar alkaloids (e.g., sanguinarine and chelerythrine; chelirubine, chelilutine, and sanguilutine; protopine and allocryptopine) reflect the common metabolic pathways that produce these compounds. Protopine serves as a precursor for several more highly oxidized alkaloids, such as sanguinarine and chelirubine (Roberts, 1998), and all the isoquinoline alkaloids found in bloodroot are biosynthetically closely related (Roberts, 1998). The alkaloids in bloodroot may be produced in parallel, compete for substrate or common enzymes, or provide substrate for the formation of other alkaloids.

Induction Experiment. In our experiments, bloodroot alkaloids were not induced by jasmonic acid. Although jasmonate-induced alkaloid production has been demonstrated in bloodroot cell culture (Ignatov, 1996), no whole-plant benzophenanthridine alkaloid induction has been recorded for this or any other plant species. It may, therefore, be misleading to extrapolate results from cell culture experiments to whole-plant induction, or from one biosynthetic pathway to others. It may be possible that the lack of induction by jasmonic acid in our experiments may have been due to inappropriate application techniques and not lack of inducibility. The amount of jasmonic acid sprayed per application in this experiment was equivalent to the amount used as a high but non-toxic level known to result in significant induction responses in tomato plants (Thaler et al., 1996, 1999). However, foliar application of jasmonic acid to the waxy leaves of bloodroot may not have effectively introduced the jasmonic acid to plant tissues. A more appropriate application method could be to add the solution directly into the roots, as was successful with nicotine induction in *Nicotiana attenuata* (Baldwin, 1998).

Additionally, the time lag between jasmonic acid application and rhizome tissue collection may have been sufficiently long that any temporary response to the jasmonic acid applications was missed. *Nicotiana* spp. respond in a few hours to a few days to methyl jasmonate exposure (Baldwin et al., 1998), and tomatoes show elevated levels of the defensive enzyme polyphenol oxidase between eight days to five weeks following application of jasmonic acid (Thaler, 1999).

Patterns Across Experiments. Alkaloid concentrations varied among bloodroot clones across all treatments in both experiments (e.g., Figures 2 and 5). While this variation likely results from genetic effects, we cannot discount the unknown effects of the abiotic conditions under which seedlings developed. An experimental design that includes all clones in all treatments and establishes relatedness among different clones would offer greater insight into the genetic component of alkaloid

production in bloodroot. We were able to demonstrate, however, that alkaloid concentrations exhibit some plasticity in response to abiotic factors in *S. canadensis*.

Over the course of the experimental period, concentrations of alkaloids declined across all treatments for both shade and induction experiments (Table 1). Since plants were maintained without fertilizer for an entire growing season, nutrient scarcity most probably limited their metabolism and, subsequently, alkaloid production.

In both experiments, plants that received high fertilizer levels in the previous year had lower alkaloid concentrations than those that received low or moderate fertilizer levels. Supplemental nutrients in the fertilizer treatments may have caused a shift in allocation from storage and defense to growth (Tuomi et al., 1984; Herms and Mattson, 1992; Marino et al., 1997), resulting in a dilution of alkaloid content (Figure 7) due to increased biomass. Some evidence to support this theory may be found in the decrease in a subset of individual alkaloid concentraitons with an increase in rhizome size in both the induction and shade experiments (Tables 2 and 3). Much of the alkaloid-bearing latex stored in the rhizome of bloodroot is found in a thick layer of vacuolar cells just below the epidermis (Wink and Roberts, 1998). There are vacuolar cells present throughout the interior of the rhizome, but the density is much lower. If bloodroot alkaloids are synthesized and/or stored mostly in the vacuolar cell layer, then an increase in rhizome diameter would appear to cause a decline in alkaloids stored there due to a shift in the diameter to volume ratio. As rhizome length and width were not correlated, the decrease in some of the alkaloid concentrations with increasing rhizome length may be related to the number of leaves present on longer rhizomes. Alternatively, high (400 ppm) fertilizer levels may have injured fine roots due to nutrient burn, leaving those plants less vigorous.

CONCLUSION

Light intensity and mineral nutrition influence alkaloid concentration in *Sanguinaria canadensis*. Whole-plant response of bloodroot to a jasmonic acid elicitor was not observed. Phototoxicity may explain increased alkaloid production and plant vigor under higher shade. Since concentrations of individual alkaloids varied differentially in experimental treatments, there may exist direct or sequential competition for substrate or enzymes during alkaloid biosynthesis. Or, if no association exists, alkaloids may be functionally uncoupled to some extent. Genetic influences represented by clone effects may be indicated by variation in alkaloid concentration by clone, but this experimental design did not allow us to distinguish genetic from pre-experiment environmental influences on the rhizomes.

Acknowledgments—The authors thank M. Thomas, R. Klaper, J. Johnston, A. Tull, M. Zimmerman, T. Maddox, T. Ackerman, A. Reynolds, and M. Madritch for technical assistance. Invaluable access to analytical equipment was graciously offered by Prof. W. Randle. The paper was much improved by helpful comments from R. Klaper, M. Thomas, and two anonymous reviewers. Finally, we thank the UGA Museum of Natural History Josh Laerm Award for financial assistance.

REFERENCES

ANONYMOUS. 1996. Statistical Analysis Software. Version 6.12. SAS Institute, Carey, NC.

AERTS, R. J., GISI, D., DECAROLIS, E., DELUCA, V., and BAUMANN, T. W. 1994. Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus* and *Cinchona* seedlings. *Plant J.* 5:635–642.

AGRESTI, A. 1996. An Introduction to Categorical Data Analysis. Wiley, New York.

AGRAWAL, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 279:1201–1202.

ARNASON, J. T., GUÈRIN, B., KRAML, M. M., MEHTA, B., REDMOND, R. W., and SCIANO, J. C. 1992. Phototoxic and photochemical properties of sanguinarine. *Photochem. Photobiol.* 55:35–38.

BALDWIN, I. T. 1996. Methyl lasmonate-induced nicotine production in *Nicotiana attenuata*: Inducing defenses in the field without wounding. *Ent. Exp. et Appl.* 80:213–220.

BALDWIN, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. USA* 95:8113–8118.

BALDWIN, I. T., GORHAM, D., SCHMELZ, E. A., LEWANDOWSKI, C. A., and LYNDS, G. Y. 1998. Allocation of nitrogen to an inducible defense and seed production in *Nicotiana attenuata. Oecologia* 115:541–552.

BEATTIE, A. J., and CULVER, D. C. 1981. The guild of myrmecochores in the herbaceous flora of West Virginia forests. *Ecology* 62:107–115.

BRYANT, J. P., CHAPIN III, F. S., and KLEIN, D. R. 1983. Carbon-nutrient balance of plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CLINE, S. D., MCHALE, R. J., and COSCIA, C. J. 1993. Differential enhancement of benzophenanthridine alkaloid content in cell-suspension cultures of *Sanguinaria canadensis* under conditions of combined hormonal deprivation and fungal elicitation. *J. Nat. Prod.* 56:1219–1228.

COLEMAN, J. S., and JONES, C. G. 1991. A phytocentric perspective of phytochemical induction by herbiovres, pp. 3–46, *in* D. W. Tallamy and M. J. Raupp (eds.). Phytochemical Induction by Herbivores. Wiley, New York.

COLEY, P. D., BRYANT, J. D., and CHAPIN III, F. S. 1985. Resource availability and plant antiherbivore defense. *Science* 230:895–899.

DOWNUM, K. R. 1992. Tansley Review No. 43: Light-activated plant defense. *New Phytol.* 122:401–420.

EILERT, U. 1998. Induction of alkaloid biosynthesis and accumulation in plants and *in vitro* cultures in response to elicitation, pp. 219–238, *in* M. F. Roberts and M. Wink (eds.). Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum, New York.

FACCHINI, P. J., JOHNSON, A. G., POUPART, J. J., and DELUCA, V. 1996. Uncoupled defense gene expression and antimicrobial alkaloid accumulation in elicited opium poppy cell cultures. *Plant Physiol.* 111:687–697.

FAGERSTROM, T., LARSSON, S., and TENOW, O. 1987. On optimal defense in plants. *Funct. Ecol.* 1:73–81.

FEENY, P. 1976. Plant apparency and chemical defense, pp. 1–14, *in* J. Wallace and R. Mansell (eds.). Biochemical Interactions Between Plants and Insects: Recent Advances in Phytochemistry, Vol. 10, Plenum, New York.

GUNDLACH, J., MULLER, M. J., KUCHAN, T. M., and ZENK, M. H. 1992. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci.* 89:2389–2393.

HASHIMOTO, Y., OKADA, M. I., SHOME, U., and KATO, A. 1986. The analysis of cell contents of some papaveraceous plants by newly devised automated histochemical chromatography. *Anal. Lett.* 19:2253–2260.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: To grow or defend. *Q. Rev. Biol.* 67:283–334.

IGNATOV, A., CLARK, W. G., CLINE, S. D., PSENAK, M., KRUEGER, R. J., and COSCIA, J. 1996. Elicitation of dihydrobenzophenanthridine oxidase in *Sanguinaria canadensis* cell cultures. *Phytochemistry* 43:1141–1144.

KARBAN R., AGRAWAL, A. A., and MANGEL, M. 1997. The benefits of induced defenses against herbivores. *Ecology* 78:1351–1355.

LEVIN, D. 1976. Alkaloid-bearing plants: An ecogeographic perspective. *Am. Nat.* 110:261–284.

LYON, D. L. 1992. Bee pollination of facultatively xenogamous *Sanguinaria canadensis* L. *Bull. Torrey. Bot. Soc.* 119:368–375.

MAHADY, G. B., SCHILLING, A. B., and BEECHER, C. W. W. 1993. *Sanguinaria canadensis* L. (Sanguinarius): *In vitro* culture and the production of benzophenanthridine alkaloids, pp. 313–327, *in* Y. P. S. Bajaj (ed.). Biotechnology in Agriculture and Forestry. Vol. 24: Medicinal and Aromatic Plants V. Springer-Verlag, Berlin.

MADDOX, G. D., and CAPPUCCINO, N. 1986. Genetic determination of plant susceptibility to a herbivorous insect depends on environmental context. *Evolution* 40:863–866.

MARINO, P. C., EISENBERG, R. M., and CORNELL, H. V. 1997. Influence of sunlight and soil nutrients on clonal growth and sexual reproduction of the understory perennial herb *Sanguinaria canadensis* L. *Bull. Torrey Bot. Soc.* 124:219–227.

MILLER, J. S., and FEENY, P. 1983. Effects of benzylisoquinoline alkaloids on the larvae of polyphagous Lepidoptera. *Oecologia* 58:332–339.

RALPHS, M. H., MANNERS, G. D., and GARDNER, D. R. 1998. Influence of light and photosynthesis on alkaloid concentration in larkspur. *J. Chem. Ecol.* 24:167–182.

ROBERTS, M. F. 1998. Enzymology of alkaloid biosynthesis, pp. 109–146, *in* M. F. Roberts and M. Wink (eds.). Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum, New York.

SAITO, K., and MURAKOSHI, I. 1998. Genes in alkaloid metabolism, pp. 147–158, *in* M. F. Roberts and M. Wink (eds.). Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum, New York.

SCHMELLER, T., LATZ-BRUNING, B., and WINK, M. 1997. Biochemical activites of berberine, palmatine, and sanguinarine mediating chemical defense against microorganisms and herbivores. *Phytochemistry* 44:257–266.

SULTAN, S. E. 1995. Phenotypic plasticity and plant adaptation. *Acta Bot. Neerl.* 44:363–383.

THALER, J. S. 1999. Induced resistance in agricultural crops: Effects of Jasmonic acid on herbivory and yield in tomato plants. *Environ. Entomol.* 28:30–37.

THALER, J. S., FIDANTSEF, A. L., DUFFEY, S. S., and BOSTOCK, R. M. 1999. Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *J. Chem. Ecol.* 25:1597–1609.

THALER, J. S., STOUT, M. J., KARBAN, R., and DUFFEY, S. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentrum*) in the laboratory and field. *J. Chem. Ecol.* 22:1767–1781.

THORNE, E. M., BOULWARE, R. T., HARKRADER, R. J., and SOUTHARD, G. L. 1986. HPLC analysis of sanguinarine in oral health care products. *J. Soc. Cosmet. Chem.* 37:279–286.

TUOMI, J., NIEMELA, P., and HAUKIOJA, E. 1984. Nutrient stress: An explanation for plant anti-herbivore responses to defoliation. *Oecologia* 61:208–210.

VAN DAM, N. M., VANDERMEIJDEN, E., and VERPOORTE, R. 1993. Induced responses in 3 alkaloid-containing plant-secies. *Oecologia* 95:425–430.

WALLER, G. R., and NOWACKI, E. K. 1978. Alkaloid Biology and Metabolism in Plants. Plenum, New York.

WINK, M., and ROBERTS, M. F. 1998. Compartmentation of Alkaloid Synthesis, Transport, and Storage, pp. 239–262, *in* Alkaloids: Biochemistry, Ecology, and Medicinal Applications. Plenum, New York.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

INTERACTIONS OF APHID HERBIVORY AND NITROGEN AVAILABILITY ON THE TOTAL FOLIAR GLYCOALKALOID CONTENT OF POTATO PLANTS

D. A. FRAGOYIANNIS,[1,*] R. G. MCKINLAY,[2] and J. P. F. D'MELLO[2]

[1]*Institute of Ecology and Resource Management,*
The University of Edinburgh,
School of Agriculture Building,
West Mains Road EH9 3JG,
Edinburgh, UK

[2]*Scottish Agricultural College,*
West Mains Road EH9 3JG,
Edinburgh, UK

(Received October 18, 2000; accepted April 25, 2001)

Abstract—In plant growth room (PGR) and open-air pot (OAP) experiments, potato cvs King Edward and Maris Piper were grown under two nitrogen levels or two different nitrogen release patterns. Plants were subjected to infestation by peach potato aphids *Myzus persicae* (Homoptera: Aphididae). Total glycoalkaloid (GA) levels were measured in the foliage of both infested and non-infested plants, before, during and after aphid infestation. In the PGR experiment, aphid infestation reduced the amounts of total GAs in both cultivars. This reduction is attributed to the sugar deficiency induced in the plants owing to the dense aphid colonization. Results from the OAP experiment showed a temporal increase of GAs produced by potato cv. King Edward plants subjected to aphid infestation. Elevated amounts of nitrogen in the nutrient solutions (PGR experiment) reduced total GAs, while no differences were observed between manure and fertilizer treated plants (OAP experiment). It is concluded that the source of available nitrogen does not affect foliar GA synthesis in potatoes, and as a consequence, does not affect its endogenous chemical defense against insect herbivory. The case for insect-induced chemical defense mechanisms as triggered by low rates of aphid infestation is discussed.

Key Words—Aphid, Aphididae, *Myzus persicae*, potato, *Solanum tuberosum*, glycoalkaloids, solanine, chaconine, insect herbivory, nitrogen, chemical defense.

*Current address for correspondence: Nikosthenous 12, TK 11635, Athens Greece

0098-0331/01/0900-1749$19.50/0

INTRODUCTION

Part of the defensive mechanisms of plants against pathogens or herbivorous insects is related to the synthesis of secondary plant metabolites or allelochemicals. In many cases, the increased allelochemical content of damaged plant tissue is considered to be the reaction of plants to stress induced by fungi or phytophagous insects. Phytochemical responses to insect herbivory have been reported for alkaloids in wild tobacco (Baldwin, 1989; Baldwin et al., 1990), furanocoumarins in wild parsnip (Zangerl, 1990; Zangerl and Berenbaum, 1995) glucosinolates in swede, kale and oilseed rape (Birch et al., 1992) and most recently hydroxamic acids in wheat (Gianoli and Niemeyer, 1997a,b).

The main products of secondary metabolism in potato *Solanum tuberosum* (L.) are glycoalkaloids (GAs). GAs are steroid glycosides formed when the alkaloid solanidine is attached to carbohydrate moieties. Depending on cultivar, 95% or more of the total glycoalkaloid (GA) fraction in tubers and vegetative parts, consists of the α-forms of solanine and chaconine (Jadhav et al., 1981).

The inhibitory effects of GAs on both fungal and insect pests of potato suggest their significance as natural pesticides (Jadhav et al., 1981). There are a limited number of studies investigating the effects of insect herbivory on the GA content of *Solanaceae*. In particular, Hlywka et al. (1994) suggested that insect herbivory, either increases (Colorado potato beetle, *Leptinotarsa decemlineata*) or has no effect (potato leafhopper, *Empoasca fabae*) on the total GA content of potato tubers, depending on the type of damage. By contrast Zullo et al. (1984) reported that infestation of *S. americanum* plants by the aphid *Aphis fabae* ssp. *solanella* reduced total GAs in stems, leaves, and fruits.

The objective of this work was to examine the effects of aphid infestation on the GA content in the foliage of potato plants during their early stages of growth. As nitrogen availability has been shown to affect GA concentration in potato foliage (Fragoyiannis, 1999), the foliar GA content of potato plants subjected to extensive damage by the aphid *Myzus persicae* was examined. Plants were fertilized with two different levels and sources of nitrogen.

METHODS AND MATERIALS

In plant growth room (PGR) and open-air pot (OAP) experiments, the potato *Solanum tuberosum* L. cultivars King Edward (KE) and Maris Piper (MP) were infested by the aphid *Myzus persicae*. In the PGR experiment, a semi-hydroponic set-up was established involving two different nitrogen regimes - low and high. In the OAP experiment, the same amount of nitrogen was available to potato plants in the form of fertilizer and manure. In both experiments, GAs produced by aphid-infested plants were compared to those produced by aphid-free plants.

Plant Growth Room Experiment. Small tuber pieces of approximately similar size developing one sprout per piece (surplus sprouts were excised) were cut from mother tubers of clonal origin. Each growing sprout was planted in one plastic pot (diameter: 15 cm; depth: 14 cm) using perlite (1:1 mix of horticultural to seed grade) as growing substrate. The pots stood on plastic rectangular trays ($22 \times 16.5 \times 4.5$ cm), were placed in a plant growth room (20°C, 50% R.H., and long day 16:8 conditions) and watered with nutrient solutions. Two nutrient solutions, N_{Low}, N_{High}, representing nitrogen concentrations of 100 and 300 mg total N/l, respectively applied. The total amounts of N added per pot were 200 and 600 mg for N_{Low} and N_{High}, respectively. Phosphorus and potassium applications remained constant at 40 mg/l and 300 mg/l, respectively, for both N treatments. Equal amounts of distilled water were added to all replicates to compensate water evaporation. In all solutions, the NO_3^- to NH_4^+ ratio was the same at 4:1. This proportion ensured the tendency for slight acidification of the growing media. The insignificant buffering capacity of perlite (Hall and Smith, 1994) was counterbalanced by adding ground magnesian limestone (1.5 g/l perlite). Trace element availability was supported by adding fritted trace elements (WM 253A, 0.5 g/l perlite). Pots were randomly relocated every second day. Growing one or, at most, two sprouts per pot allowed ease of watering and random plant relocation without damaging plants in the restricted space of the plant growth room. Two different fertilization treatments (N_{Low} and N_{High}) were combined with aphid-infested and non-infested plants harvested 15, 45, or 60 days after planting.

Open-Air Pot Experiment. Potato tubers (*S. tuberosum* L. cvs. King Edward and Maris Piper) were planted in pots (diameter: 23 cm; depth: 23 cm) under open field conditions. The pots contained topsoil, classified as poorly drained clay soil of Stirling Series (SG), randomly sampled from two neighboring farms one of which practiced organic agriculture, the other conventional agriculture. Semi-composted sheep manure was obtained from the Scottish Agricultural College farm at Bush Estate, Lothian Region. Soil samples and sheep manure were analyzed for pH, extractable ammonium N, nitrate N and total N after sieving and mixing. Soil analyses revealed that the soil derived from the conventional farm had higher N content owing to previously applied ammonium nitrate. The N content of the soil derived from the organic farm was amended by adding a small amount (approx. 13 g/pot) of sheep manure, thus ensuring that the total available N in both soil samples was the same at 415 mg total N/pot. The two different fertilization treatments (fertilizer and manure) were combined with aphid-infested and non-infested plants harvested 20, 40 or 60 days after planting.

Aphid Infestation. Thirty standardized adults (approximately five/leaf) derived from a clone lineage culture of the aphid *M. persicae* were transferred to the treatments subjected to infestation in the PGR and OAP experiments, once the second true leaf had fully expanded. Due to differences in N fertilization, N_{High} plants reached this stage faster compared to N_{Low}, hence they were infested first

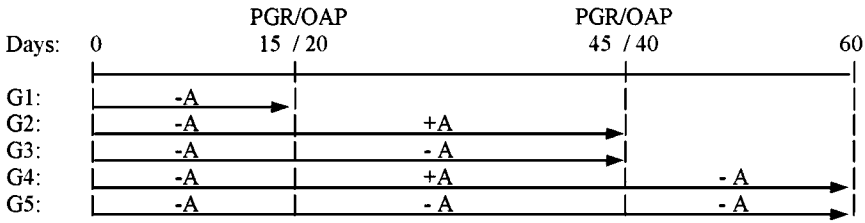


FIG. 1. Schematic representation of the *Myzus persicae* infestation groups (G1, G2, G3, G4, and G5) applied to the potato plants in plant growth room (PGR) and open-air pot (OAP) experiments, plotted against times of harvesting; +A: aphid-infested period, −A: aphid-free period.

with the aphids. In the OAP experiment, part of the aphid infestation (five aphids on the first true leaf) were protected from aphid predators using a nylon mesh pocket. The rest were set free to colonize the plant. Removal of aphids from the aphid-infested G2 and G4 plants was achieved by spraying the plants with a 0.5% soft soap solution. A schematic representation of the aphid infestation treatments applied to the potato plants and times of harvesting is given in Figure 1.

Sample Preparation and Analysis. Potato leaves, petioles and growing tops were excised, weighed, packed in muslin sachets and immersed in liquid nitrogen. The tissues remained in the freezer (−40°C) prior to lyophilisation for approximately 72 hr/sample. Each sample was ground using a water cooled mill and stored in the freezer. The extraction and analytical techniques used for the quantification of α-solanine and α-chaconine were based on those of Hellenäs (1986). Total GAs were the sum of α-solanine and α-chaconine in each sample. High performance liquid chromatography (HPLC) analysis equipment consisted of a high pressure isocratic pump (Spectra Physics Analytical P1000), programmable wavelength detector (Spectra 200), sample injector (Gilson 232 BIO) combined with a dilutor (Gilson 401) fitted with a 20 μl loop, and a datajet integrator (Spectra Physics SP4270). A stainless steel column (Phase Separations model Spherisorb), packed with ODS2-hypersil (Shandon, 3 μm) was used for analysis. The mobile phase was acetonitrile/water: 34:66 (v/v) and 0.5 ml/l ethanolamine adjusted to pH 4.55–4.56 with orthophosphoric acid (12% solution). The chromatograph was set to deliver solvent at 1 ml/min at 0.002 absorbance units full scale (AUFS) output. Peaks were detected by UV absorbance at 202 nm. All chemicals used were 'pure UV grade' (Fisons Ltd. UK). Both experiments were replicated three factor with N applied at 2 levels (N_{Low}/N_{High} in the PGR and manure/fertilizer in the OAP) to 5 levels of aphid/harvest plants (G1, G2, G3, G4 and G5) to potato cultivars King Edward and Maris Piper. Treatment combinations were repeated four times. In total, 80 pots were placed in the plant growth room in two cages (4.5 m × 1 m × 1 m) made of nylon mesh isolating the infested from the

non-infested plants and another 80 pots in open-air conditions. Pots were randomly relocated every second day. PGR experimental data were analysed using the Analysis of Variance procedure for factorial design of MINITAB® (vs. 11.1). OAP experimental data were analyzed using the General Linear Model procedures of MINITAB® (vs. 11.1), in order to deal with a few missing values derived from hail-damaged plots. Significant differences between treatment means were identified using t-tests.

RESULTS

In the PGR experiment, elevated amounts of N in the nutrient solution reduced GA levels in both cultivars (Table 1). By contrast, the main effect of N source in the OAP experiment was not significant. As a result, the type of N (e.g., manure or fertilizer), did not affect foliar GA synthesis (Table 2). In both

TABLE 1. EFFECTS OF NITROGEN FERTILIZATION, CULTIVAR, AND APHID INFESTATION ON POTATO LEAF GLYCOALKALOID (GA) CONTENT IN THE PLANT GROWTH ROOM EXPERIMENT

Glycoalkaloid content (mg/100 g fresh weight)					
Nitrogen fertilization		Cultivar		Aphid infestation	
N_{Low}	140.9	KE	139.7	G1 − A	75.9
N_{High}	58.0	MP	59.3	G2 + A	49.9
				G3 − A	128.6
SED	4.83	SED	4.83	G4 + A	57.3
				G5 − A	185.8
				SED	5.4

	Analysis of variance		
Source	*df*	Mean square	*F* ratio
Nitrogen fertilization (N)	1	137540	295.41***
Cultivar (cv.)	1	129442	278.02***
Aphid infestation (A)	4	52341	112.42***
N × cv.	1	23227	49.89***
N × A	4	13693	29.41***
cv. × A	4	5478	11.77***
N × cv. × A	4	3612	7.76***
Error	60	466	

N_{Low}: 100 mg N/liter; N_{High}: 300 mg N/liter; KE: potato cv. King Edward; MP: potato cv. Maris Piper; SED: standard error of difference between means for 60 degrees of freedom.
***$P < 0.001$.

TABLE 2. EFFECTS OF NITROGEN FERTILIZATION, CULTIVAR, AND APHID INFESTATION ON POTATO LEAF GLYCOALKALOID (GA) CONTENT IN THE OPEN-AIR POT EXPERIMENT

Glycoalkaloid content (mg/100 g fresh weight)					
Nitrogen source		Cultivar		Aphid infestation	
Manure	53.8	KE	91.0	G1 − A	55.5
Fertilizer	56.6	MP	19.4	G2 + A	56.3
				G3 − A	43.6
SED	2.95	SED	2.95	G4 + A	60.4
				G5 − A	60.1
				SED	4.67

	General linear model			
Source	*df*	Adj. mean square	*F* ratio	*P*
Nitrogen source (N)	1	134	0.77	NS
Cultivar (cv.)	1	89278.2	512	<0.001
Aphid infestation (A)	4	683.7	3.92	<0.01
N × cv.	1	518.1	2.97	NS
N × A	4	1148.1	6.58	<0.001
cv. × A	4	728.7	4.18	<0.01
N × cv. × A	4	365.2	2.09	NS
Error	53	174.4		

KE: potato cv. King Edward; MP: potato cv. Maris Piper; SED: standard error of difference between means for 53 degrees of freedom; NS: not significant.

experiments, cv. King Edward produced elevated amounts of foliar GAs compared to those produced by cv. Maris Piper. In the PGR experiment, aphid-infested plants had reduced amounts of total foliar GAs compared to non-infested (G2 vs. G3) (Table 1). This lasted even after the extermination and removal of aphids from the plants (G4 vs. G5). Although aphid infestation induced an increase in GAs in the OAP experiment (G2 vs G3), this increase was temporary because differences were not observed between G4 and G5 plants (Table 2). An analysis of the interactions between the main effects is presented separately for each experiment.

Plant Growth Room Experiment

Analysis of variance indicated that all three 2-way interactions (N fertilization × cultivar, N fertilization × aphid infestation and cultivar × aphid infestation) and one 3-way interaction (N fertilization × cultivar × aphid infestation) were significant (Table 1).

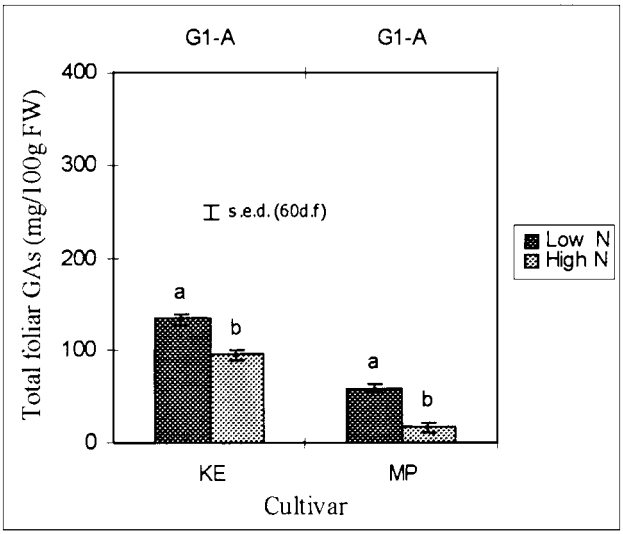


FIG. 2. Total foliar fresh weight glycoalkaloid (GA) content (mean ± SE, four replicates of potato cvs. King Edward (KE) and Maris Piper (MP) grown in low (100 mg N/liter) and high (300 mg N/liter) nitrogen fertilization levels harvested 15 days after planting (G1, plant growth room experiment). Means in columns of the same potato cv. followed by different letters are significantly different (t-test following balanced ANOVA).

Plants from both cultivars grown in low N nutrient solution harvested 15 days after planting (G1) had higher levels of GAs than those grown in high N solution (Figure 2).

Similar differences were observed between high and low N treatments in non-infested plants (G3 and G5) harvested at the next two time points (30 and 45 days after infestation, Figures 3a, 3b). Aphid infestation reduced GA levels in either low or high N treated plants of both cultivars that were harvested 30 days after infestation (G2 vs G3, Figure 3a). An exception was observed on potato cv. Maris Piper; a significant difference was not observed between aphid-infested and non-infested plants grown in high N solution. The effects of aphid infestation lasted even after aphid extermination. The differences between aphid-infested and non-infested plants were significant for both N levels and both cultivars (G4 vs G5, Figure 3b).

Open-Air Pot Experiment

Significant effects were observed in the interactions N source × aphid infestation, and potato cultivar × aphid infestation. The N source × cultivar and the N source × cultivar × aphid infestation interactins were not significant (Table 2).

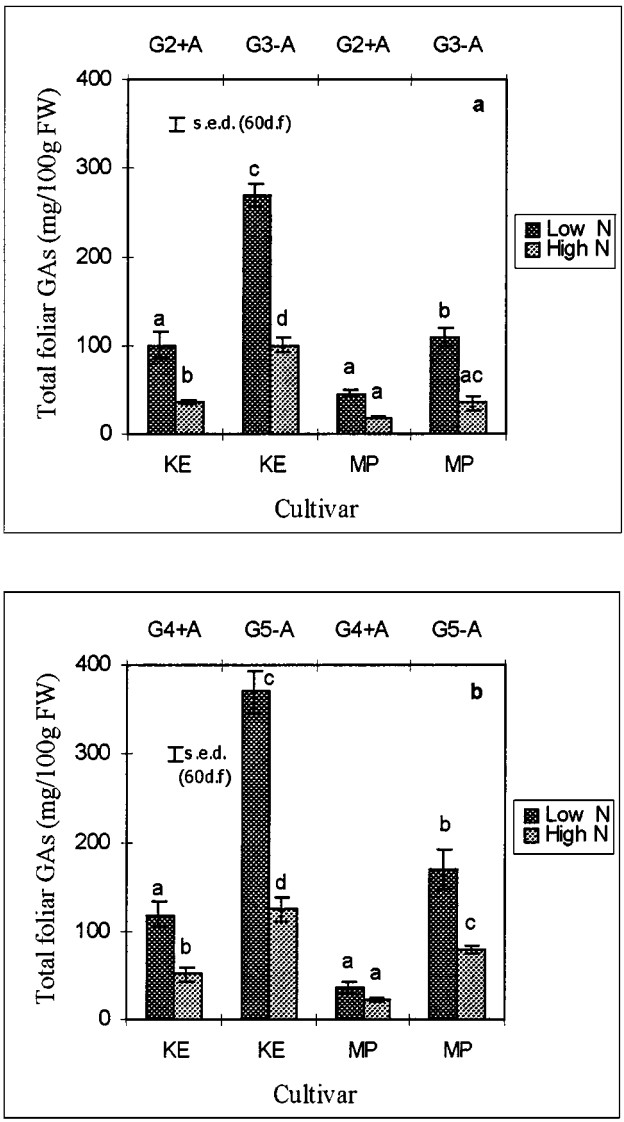


FIG. 3. Total foliar fresh weight glycoalkaloid (GA) content (mean ± SE, four replicates) of aphid-infested (G2 + A and G4 + A) and non-infested (G3 − A and G5 − A) potato cvs. King Edward (KE) and Maris Piper (MP) grown in low (100 mg N/liter) and high (300 mg N/liter) nitrogen fertilization levels and harvested 30 (Figure 3a) and 45 (Figure 3b) days after aphid infestation (plant growth room experiment). Means in columns of the same potato cv. followed by different letters are significantly different (t-test following balanced ANOVA).

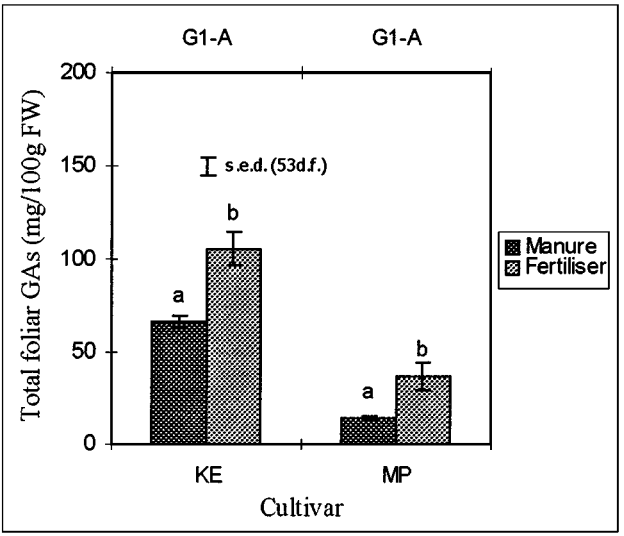


FIG. 4. Total foliar fresh weight glycoalkaloid (GA) content (mean ± SE, four replicates) of potato cvs. King Edward (KE) and Maris Piper (MP) grown with manure and fertilizer harvested 20 days after planting (G1, open-air pot experiment). Means is columns of the same potato cv. followed by different letters are significantly different (t-test following linear model).

Manure-treated potato cv. King Edward plants, harvested 20 days after planting (G1) produced less GAs compared to those treated with fertilizer (Figure 4). A difference also was observed between manure- and fertilizer-treated potato cv. Maris Piper plants.

Manure-treated plants of both cultivars harvested 20 days after infestation (G2 and G3), produced similar GA levels to fertilizer-treated plants (Figure 5a). In addition, effects of N source on the foliar GA production were not observed between manure- and fertilizer-treated plants of any cultivar harvested at the next time point (G4 and G5 Figure 5b).

The effect of cultivar × aphid infestation interaction indicates that apart from cutivar, aphid infestation affected foliar GA production. In particular, an increase of foliar GAs was observed in both manure- and fertilizer-treated aphid-infested cv. King Edward plants, compared to non-infested, harvested 20 days after infestation (G2 and G3 Figure 5a). These differences ceased in cv. King Edward plants harvested at the next time point (G4 and G5 Figure 5b). Aphid infestation did not have any effect on cv. Maris Piper. Differences between aphid-infested and non-infested cv. Maris Piper plants harvested at 20 (G2 and G3) and 40 (G4 and G5) days after infestation were not significant.

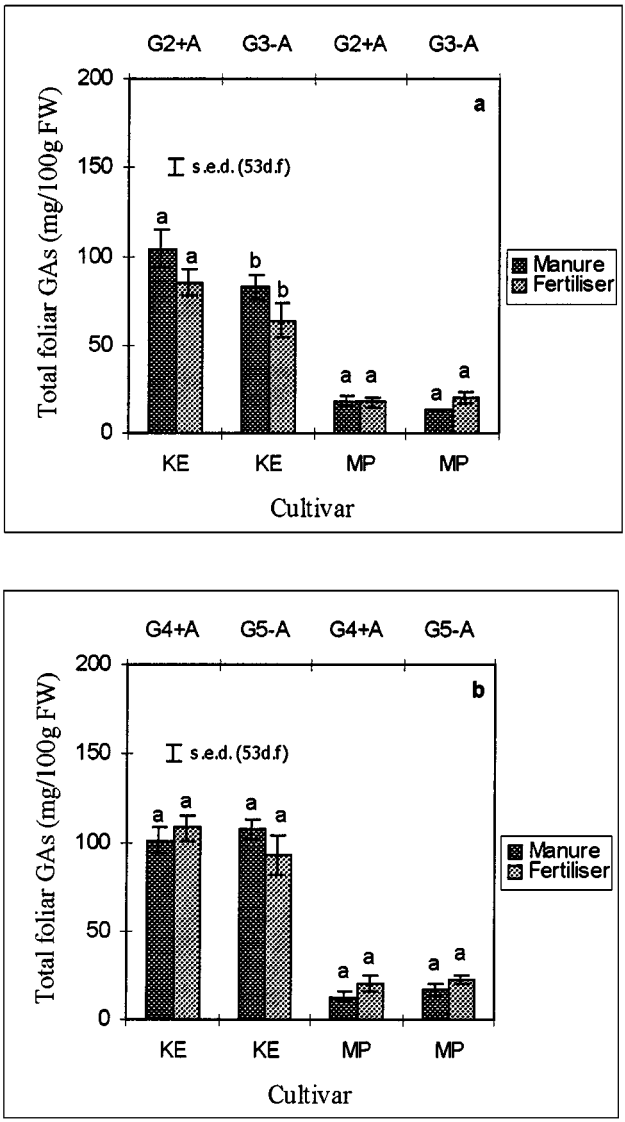


FIG. 5. Total foliar fresh weight glycoalkaloid (GA) content (mean ± SE, four replicates) of aphid-infested (G2 + A and G4 + A) and non-infested (G3 − A and G5 − A) potato cvs. King Edward (KE) and Maris Piper (MP) grown with manure and fertilizer and harvested 20 (Figure 5a) and 40 (Figure 5b) days after aphid infestation (open-air pot experiment). Means in columns of the same potato cv. followed by different letters are significantly different (t-test following linear model).

DISCUSSION

Mechanical damage has the potential to cause wound defensive reactions by plants including the accumulation of secondary defensive substances. It has been reported (Olsson, 1986) that GA synthesis in potato tubers is increased as a result of enhanced physiological activity of cells neighboring sites of mechanical damage. During aphid stylet penetration, usually considered to be intercellular, some cell walls are grazed, and/or ruptured (Spiller et al., 1985; Kimmins and Tjallingii, 1985), and cells are destroyed (Pollard, 1973). Such reactions have been documented in field and growth room studies, where defoliation damage by Colorado potato beetles *Leptinotarsa decemlineata* resulted in increased tuber GA concentrations (Hlywka et al., 1994). Damage by potato leafhoppers *Empoasca fabae* did not have any apparent effect on the tuber GA content (Hlywka et al., 1994). The difference was attributed to the type of herbivory stress induced by each of these species: Colorado potato beetles are very destructive chewing insect pests, whereas potato leafhoppers are phloem feeders. Since aphids are phloem feeders, it is possible that the mechanical damage associated with stylet penetration of potato plants by *M. persicae* could have altered GA concentration in the foliage.

The results obtained from the PGR experiment showed that GA levels of aphid-infested plants were reduced in both potato cultivars and both N levels applied. This was not only an immediate and direct effect of the dense colonization of the plants, but also an after-effect that followed elimination of aphids. It has been suggested that synthesis of solanidine - the aglycon moiety of the GA molecule - occurs in plastids (Ramaswamy et al., 1976). One response of plants to phloem-feeding insects is the degeneration and disappearance of chloroplasts in the area neighboring stylet punctures (Miles, 1989; Pollard, 1973). Aphid herbivory can induce photosynthetic stress at the level of the chloroplast and limit the photosynthetic rate, while other cellular components like mitochondria and plasma or vacuolar membranes are damaged. This damage leads to cell death and, therefore, chloroplast death (Miles, 1989). The reduction observed in the foliar GA levels could be a result of a reduction in solanidine biosynthesis, following a decrease in the number of undamaged plastids occurring in potato leaf cells.

Structural differences between solanine and chaconine and between their α-, β-, and γ- forms are related to carbohydrate moiety composition (Nair et al., 1981). Synthesis of the γ-, β-, and α- forms of solanine and chaconine occurs when the aglycon steroid solanidine molecule is glucosylated in a stepwise synthesis with one, two or three sugars (Osman and Zacharius, 1979). The presence of soluble sugars that exist in the GA molecule in sufficient quantities during the final steps of GA formation may play a crucial role in the amount of GAs that will finally be synthesized and expressed. Aphids interfere with photosynthetic efficiency in the infested plant and provide a rich medium for fungal growth through their excretion products (Schepers, 1989). Due to the normally low nitrogen/carbon ratio

of phloem sap, aphids also have to adapt to a nutrient source with a suboptimal supply of nitrogen during much of the growing season. To extract enough nitrogen and acquire sufficient protein, aphids eliminate large quantities of phloem sap and excrete carbohydrate-rich honeydew (Llewellyn et al., 1974). Hence, it is possible that lack of glucose, rhamnose and galactose - which make up the sugar part of the GA molecule - resulting from both photosynthesis rate reduction and carbohydrate absorption by the aphids, led to a reduction in the amount of GAs foliage.

The results from the PGR experiment suggest that heavy aphid infestation negatively affects the defensive system of potato plants, and that this is related to secondary metabolism. This effect was obvious for both low and high levels of available nitrogen. However, it appears that reduction of GA synthesis was greater when nitrogen was applied at high or excessive quantities. Nitrogen availability appears to play a crucial role related to the general health status of the potato crop. In the OAP experiment, manure-treated potato cv. King Edward plants, harvested early in G1 treatment, had decreased amounts of GAs compared to fertilizer-treated plants (Figure 4). This was observed as well for the cultivar Maris Piper. The amount of green matter harvested in G1 treatment was almost the same for both manure and fertilizer treatments for each cultivar (data not shown). This suggests that the increase of GA production in G1 treatment observed in fertilizer grown plants could not be related to differences in the amount of green matter produced by manure and fertilizer treatments. A possible explanation may be attributed to the different rates of N release and plant uptake between plants treated with manure and with fertilizer, at the early stages of plant growth. No differences were observed in foliar GA production between manure-treated and fertilizer-treated, aphid-infested and non-infested plants during and after the period of aphid infestation. Hence, the source of available N does not play any role in GA synthesis in the subsequent stages of plant growth.

The PGR experimental results are in agreement with those obtained by Zullo et al. (1984) who reported decreased GA levels in stem, leaf and fruits of *Solanum americanum* plants infested by the aphid *Aphis fabae* ssp. *solanella*. The OAP experiment showed that the two cultivars reacted in a different way during aphid infestation. An increase in the amounts of GAs was observed in the aphid-infested, manure- or fertilizer-treated King Edward plants; such an increase was not observed in Maris Piper. In addition, no after-effects of aphid infestation were observed in either of the cultivars examined in the OAP experiment. The discrepancy between the PGR and OAP experiments may be attributed to the restricted aphid infestation that took place in the field; the presence of aphid predators and parasitoids combined with the average low summer temperatures did not allow the expected dense aphid colonization to occur. No decrease, in foliar GA production was observed as a result of low aphid infestation in either potato cultivar.

When damage is caused by arthropod herbivore feeding, leaves distant from the site of injury often accumulate compounds toxic or distasteful to the arthropod

(Tallamy and Raupp, 1991). It is possible that such systemic 'induced resistance' to aphids may have occurred for potato cv. King Edward in the OAP experiment. A signal internal to the plant may have induced the increase of the foliar GAs in both manure- and fertilizer-treated plants of potato cv. King Edward. In addition, removal of the aphids from the plants brought GAs back to the levels prior to aphid infestation. However, it seems that for some reason such a signal was not detected in the cultivar Maris Piper. A similar signal possibly was generated during the early stages of aphid infestation in the PGR experiment. The subsequent dense aphid colonization of plants presumably concealed expression and resulted in a decrease of the foliar GAs owing to a reduced solanidine and/or carbohydrate synthesis.

The results suggest that low densities of aphid infestation stimulate a temporary increase of potato foliar GAs, and the existence of an induced defensive allelochemical response in potato. Further studies are required to examine the mechanisms and characteristics of this response. An implication of these results is that in organic farming where nitrogen availability is restricted, plants may be effectively protected from pests owing to the presence of the natural defense system. In conventional farming systems where nitrogen availability is high, plants may be more susceptible to pest infestation because of a reduction of natural defensive toxins.

Acknowledgments—This research was funded by a grant from the Commission of the European Communities, Directorate General for Agriculture (Contract No. AIR-3-BM93-2418). We wish to thank Dr G. Percival of the Scottish Agricultural College Auchincruive, for his technical advice on GA analyses and Mr E.A. Hunter of Biomathematics and Statistics, Scotland, The University of Edinburgh, James Clerk Maxwell Building, Edinburgh, for statistical advice.

REFERENCES

BALDWIN, I. T. 1989. The mechanism of damage-induced alkaloids in wild tobacco. *J. Chem. Ecol.* 15:1661–1680.

BALDWIN, I. T., SIMS C. L., and KEAN S. E. 1990. The reproductive consequences associated with inducible alkaloidal responses in wild tobacco. *Ecology* 71:252–262.

BIRCH, A. N. E., GRIFFITHS, D. W., HOPKINS, R. J., SMITH, W. H. M., and MCKINLAY, R. G. 1992. Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly *(Delia floralis)* larvae. *J. Sci. Food Agric.* 60:1–9.

FRAGOYIANNIS, D. A. 1999. Interaction effects between the peach potato aphid *Myzus persicae* and secondary plant metabolites occurring in potato *Solanum tuberosum* L. Ph.D. Thesis. The University of Edinburgh.

GIANOLI, E., and NIEMEYER, H. M. 1997a. Characteristics of hydroxamic acid induction in wheat triggered by aphid infestation. *J. Chem. Ecol*. 23:2695–2705.

GIANOLI, E., and NIEMEYER, H. M. 1997b. Lack of costs of herbivory-induced defenses in a wild wheat: Integration of physiological and ecological approaches. *Oikos* 80:269– 275.

HALL, D. A., and SMITH, C. A. 1994. An evaluation of perlite-based substrates for ornamental pot plant production. *Acta Horticulturae* 361:486–490.

HELLENÄS, K. E. 1986. A simplified procedure for quantification of potato glycoalkaloids in tuber extracts by HPLC; comparison with ELISA and a colorimetric method. *J. Sci. Food Agric*. 37:776–782.

HLYWKA, J. J., STEPHENSON, G. R., SEARS, M. K., and YADA, R. Y. 1994. Effects of insect damage on glycoalkaloid content in potatoes (*Solanum tuberosum*). *J. Agric. Food Chem*. 42:2545–2550.

JADHAV, S. J., SHARMA, R. P., and SALUNKHE, D. K. 1981. Naturally occuring toxic alkaloids in foods. *CRC Crit. Rev. Toxicol.* 9:21–67.

KIMMINS, F. M., and TJALLINGII, W. F. 1985. Ultrastructure of sieve element penetration by aphid stylets during electrical recording. *Entomol. Exp. Appl*. 39:135–141.

LLEWELLYN, M., RASHID, R., and LECKSTEIN, P. 1974. The ecological energetics of the willow aphid, *Tuberolachnus salignus* (Gmelin): Honeydew production. *J. An. Ecol*. 43:19–29.

MILES, P. W. 1989. Specific responses and damage caused by Aphidoidea, pp. 23–47, *in* A. K. Minks and P. Harrewijn (eds.). Aphids Their Biology, Natural Enemies and Control. 2C. Elsevier Amsterdam.

NAIR, P. M., BEHERE, A. G., and RAMASWAMY, N. K. 1981. Glycoalkaloids of *Solanum tuberosum* Linn. *J. Sci. Ind. Res*. 40:529–535.

OLSSON, K. 1986. The influence of genotype on the effects of impact damage on the accumulation of glycoalkaloids in potato tubers. *Potato Res.* 29:1–12.

OSMAN, S. F., and ZACHARIUS, R. M. 1979. Biosynthesis of potato glycoalkaloids. *Am. Potato J*. 56:475.

POLLARD, D. G. 1973. Plant penetration by feeding aphids. *Bull. Ent. Res*. 62:631–714.

RAMASWAMY, N. K., BEHERE, A. G., and NAIR, P. M. 1976. A novel pathway for the synthesis of solanidine in the isolated chloroplast from greening potatoes. Eur. *J. Biochem*. 67:275–282.

SCHEPERS, A. 1989. Control of aphids; chemical control, pp. 89–122, *in* A. K. Minks and P. Harrewijn (eds.). Aphids Their Biology, Natural Enemies and Control. 2C. Elsevier Amsterdam.

SPILLER, N. J., KIMMINS, F. M., and LLEWELLYN, M. 1985. Fine structure of aphid stylet pathways and its use in host plant resistance studies. *Entomol. Exp. Appl*. 38:293–295.

TALLAMY, D. W., and MCLAUD, E. S. 1991. Squash beetles, Cucumber beetles, and inducible cucumber responses, p. 431, *in* D. W. Tallamy and M. J. Raupp (eds.). Phytochemical Induction by Herbivores. Wiley, New York.

ZANGERL, A. R. 1990. Furanocoumarin induction in wild parsnip: Evidence for an induced defense against herbivores. *Ecology* 71:192–1932.

ZANGERL, A. R., and BERENBAUM, M. R. 1995. Spatial temporal and environmental limits on xanthotoxin induction in wild parsnip foliage. *Chemoecology* 5(6):37–42.

ZULLO, M. A. T., TEIXEIRA, J. P. F., SPOLADORE, D. S., LOURENCAO, A. L., and ARANHA, C. 1984. Content of glycoalkaloids in *Solanum americanum* infested by *Aphis fabae solanella. Bragantia* 43:255–259.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

EFFECTS OF PHYTIC ACID AND XANTHOTOXIN ON GROWTH AND DETOXIFICATION IN CATERPILLARS

E. S. GREEN, A. R. ZANGERL, and M. R. BERENBAUM*

Department of Entomology
University of Illinois at Urbana-Champaign
320 Morrill Hall
505 S. Goodwin Avenue
Urbana, Illinois 61801

(Received October 27, 2000; accepted April 24, 2001)

Abstract—Phytic acid is abundant in the fruits and seeds of many plants and is found in foliage to a lesser extent. Among its several properties, phytic acid is a potent chelator of essential minerals and proteins; thus, the possibility exists that heme-based enzymes such as cytochrome P450 monooxygenases in herbivores are detrimentally affected by phytic acid via chelation of dietary iron. Mortality, growth performance, and P450-mediated metabolism of xanthotoxin, a plant allelochemical, were examined in the presence of phytic acid in three lepidopteran species: a polyphagous seed-feeding species (*Heliothis virescens*), a polyphagous foliage-feeding species (*Trichoplusia ni*), and a species oligophagous on immature reproductive structures of two genera of Apiaceae (*Depressaria pastinacella*). While first instar *H. virescens* experienced no increase in mortality after 120 hours on a diet containing 1% phytic acid compared to a control diet, both *T. ni* and *D. pastinacella* experienced virtually complete mortality over the same time period. Ultimate instars of all three species experienced reductions in relative growth rates (RGR) and relative consumption rates (RCR) in the presence of phytic acid, although the only species to experience reduced digestive efficiency (ECI) was *H. virescens.* Cytochrome P450-mediated metabolism of xanthotoxin was reduced 60% in the presence of phytic acid in *D. pastinacella*, although metabolism remained unaffected in the two noctuids. These studies suggest a defensive function of phytic acid in addition to its primary functions of phosphorus storage, energy storage, and cell wall precursor source.

Key Words—phytic acid, herbivory, cytochrome P450, mineral bioavailability, plant resistance, primary metabolite, secondary metabolite.

*To whom correspondence should be addressed. E-mail: maybe@life.uiuc.edu

0098-0331/01/0900-1763$19.50/0

INTRODUCTION

Phytic acid (myo-inositol-1,2,3,4,5,6-hexakis-dihydrogenphosphate) is a common constituent of oilseeds, legumes, and cereals. In germinating seedlings, phytate, the salt form of phytic acid, serves as the major storage form of phosphorus, often accounting for more than 85% of the total phosphorus in legumes and grains upon seed ripening (Reddy et al., 1989). Phytic acid is a potent chelator of essential minerals, including iron, calcium, magnesium, and zinc, as well as a chelator of positively charged proteins. Once bound to phytic acid, minerals and proteins form insoluble complexes that are unavailable for absorption under normal physiological conditions. Thus, the bioavailability of minerals and protein in an animal's diet may be reduced by the concomitant consumption of phytic acid.

Although the antinutritive effects of phytic acid on vertebrates are well-documented (Zhou and Erdman, 1995; Pillauf and Rimbach, 1997), the effects of this ubiquitous plant constituent on insects are less well-known. Studies of seeds in storage indicate that phytic acid may be an insect antifeedant; increased phytic acid content, for example, is associated with reduced risk of infestation by *Bruchus pisorum* in peas (Marzo et al., 1997). Apparent increases in phytic acid content of infested legume and cereal seeds in storage may be due to selective removal of seed parts free of phytic acid by insects (mung bean, chickpea, pigeon pea, Modgil and Mehta, 1997; wheat and sorghum, Jood et al., 1995).

Effects of phytic acid on insect infestation of plants in field situations are less well-documented. In addition to acting as an antifeedant, phytic acid may act to reduce digestibility of foliage and fruits by forming complexes with dietary proteins. As a mineral chelator, phytic acid is known to reduce bioavailability of such minerals as calcium, magnesium, zinc, and iron (Reddy et al., 1989). Of particular importance to insect herbivores, and potentially vulnerable to the action of phytic acid, are the cytochrome P450 iron-based heme proteins. Cytochrome P450 monooxygenases (P450) constitute an iron-based enzyme system that both detoxifies hostplant allelochemicals and other xenobiotics and synthesizes endogenous hormones (Berenbaum, 1991). Because phytic acid is an iron chelator, the possibility exists that phytic acid may interfere with cytochrome P450 function. It is conceivable that iron deficiency could result in a reduction of cytochrome P450 activity and an overall decrease in allelochemical metabolism.

To determine whether phytic acid may serve as a defense against consumers of foliage and fruits, survivorship and growth parameters were measured for three lepidopteran larvae that vary in the frequency with which they encounter phytic acid. The tobacco budworm, *Heliothis virescens* (Noctuidae), is a fruit feeder that attacks tobacco, tomatoes, and other solanaceous plants as well as cotton and some legumes (Metcalf and Metcalf, 1993). As a consumer of legume fruits, then, *H. virescens* likely frequently encounters high levels of phytic acid in its diet. Phytic acid is present in several of its hosts, including legumes, at levels ranging

from 0.28–1.26% in chickpeas to 1.00–2.22% in soybeans (Reddy et al., 1989). The cabbage looper, *Trichoplusia ni* (Noctuidae), is a generalist folivore on herbaceous plants of over 80 known species (Tietz, 1972; Eichlin and Cunningham, 1978). Although phytic acid can be found in foliage of a range of plants, it generally occurs at levels considerably lower than those found in seeds (e.g., 0.002% in fluted pumpkin, Ladeji et al., 1995; 0.009% in *Launaea taxaracifolia* to 0.655% in *Euphorbia hirta*, Wallace et al., 1998). The parsnip webworm, *Depressaria pastinacella* (Oecophoridae), feeds on buds, flowers, and immature fruits of two genera of Apiaceae, *Pastinaca* and *Heracleum*. Although mature fruits of *P. sativa* vary in phytic acid content, often exceeding 0.5% fresh weight, levels of phytic acid are low to undetectable in buds, flowers, and immature fruits, the principal food of this specialist (A. Zangerl and M. Berenbaum, unpublished).

Despite differences in diet breadth, all three of these species rely on cytochrome P450 monooxygenases for detoxification of furanocoumarins, secondary metabolites found in several (*H. virescens*, *T. ni*) or all (*D. pastinacella*) of their hostplants (Berenbaum, 1995). Thus, we measured rates of metabolism of xanthotoxin, a furanocoumarin, in the presence and absence of phytic acid in these species in order to determine whether it can act as an inhibitor of detoxification enzymes.

MATERIALS AND METHODS

All three lepidopterans can be reared on the same artificial diet (Nitao and Berenbaum, 1988), which can easily be modified to reflect various degrees of nutritional quality. Three different experiments were conducted with artificial diets in order to assess impacts of phytic acid on mortality rates, growth performance, and xenobiotic metabolism.

First Instar Survivorship. To determine if phytic acid affects mortality rates during early development, a first instar survivorship experiment was performed. For all three species, neonates were allowed to feed on an artificial diet containing 1% fresh weight phytic acid (Sigma, St. Louis, MO) or on an artificial diet with no additives as a control. Sample size for each diet type was 20 for *D. pastinacella* and *H. virescens*, and 25 for *T. ni*. Mortality was assessed every 24 hours up to 120 hr. All larvae developed at approximately the same rate. A two-dimensioned contingency table with Yates continuity correction was analyzed by chi square to determine if mortality differed between control and phytic acid treatments for all three species.

Gravimetric Estimates of Performance. To assess the effects of phytic acid on growth and feeding efficiency, gravimetric estimates of performance were determined for all three caterpillar species. Ultimate instars (fifth instar for *T. ni* and *H. virescens*, sixth instar for *D. pastinacella*) were fed an artificial diet containing

either 1% phytic acid or no additives as a control and were allowed to feed for 72 hr. After three days of feeding, the uneaten portions of diet were oven-dried and weighed. For sixth instar *D. pastinacella*, sample size for each diet type was 20; for fifth instar *T. ni*, sample size for each diet type was 10, and for fifth (ultimate) instar *H. virescens*, sample size for each diet type was 28. Sample size differed among species due to differences in availability at the time of experimental set-up and differences in synchrony of the colonies.

For all three species, gravimetric estimates of caterpillar performance were calculated by using parameters from Waldbauer (1968). One-way analyses of variance for relative growth rates (RGR), relative consumption rates (RCR), and efficiency of conversion of ingested material (ECI) were performed with phytic acid as the main effect. Use of this approach allows for comparisons with a vast literature of performance data on lepidopterans (Scriber and Slansky, 1981). However, Raubenheimer and Simpson (1992) have suggested that analysis of covariance is a more appropriate way to analyze growth and feeding measures as a result of difficulties in interpreting ratios used in conventional gravimetric indices of performance. The numerator and denominator of these ratios are not independent variables because elements of the numerator are included in the denominator. Accordingly, analysis of covariance was also performed for final larval mass and consumption with initial larval mass as the covariate; biomass gained (growth) was evaluated with amount of food consumed as the covariate. The analyses were first performed with a model statement including an interaction effect between the covariate and treatment to test for homogeneity of slopes. Homogeneity of slopes was confirmed ($P > 0.10$) in all analyses.

Xanthotoxin Metabolism. To determine if phytic acid interferes with cytochrome P450 function, xanthotoxin metabolism assays were performed for each species. For this assay, ultimate instars were raised on one of four artificial diets for 72 hours: control diet (lacking any additives), diet containing added phytic acid, diet containing added xanthotoxin, and diet containing both phytic acid and xanthotoxin. For all three species, the amount of phytic acid added, 1% fresh weight, was identical. The amount of xanthotoxin added to the experimental diets varied according to the known level of tolerance; whereas *T. ni* and *H. virescens*, polyphagous species that rarely encounter furanocoumarins, received diets amended with 0.05% xanthotoxin, commensurate with their sensitivity to this allelochemical, *D. pastinacella* received diet amended with 0.2% xanthotoxin, consistent with its level of resistance (Berenbaum, 1995). After 72 hours, the entire midgut of each caterpillar was removed and homogenized in cold phosphate buffer. These homogenates were incubated with a known concentration of xanthotoxin in a buffer containing a NADPH-generating system in order to measure cytochrome P450 activity. The disappearance of xanthotoxin over a 30-min period was measured by quantification of remaining xanthotoxin by HPLC. Details of the metabolism assay protocol are described in Zangerl and Berenbaum (1993). For *T. ni* and *H. virescens*, four

midguts were pooled for each replicate because cytochrome P450 activity in these insects is sufficiently low as to be undetectable with a single midgut. In the parsnip webworm, which specializes on furanocoumarin-containing plants, cytochrome P450 activity is measurable in a single midgut (Berenbaum and Zangerl, 1992). To prevent the total loss of xanthotoxin due to high metabolism rates in this insect, the reaction was allowed to run for 15 min instead of the standard 30 min. The rate of xanthotoxin metabolism was calculated as nanomoles per min per gram of fresh caterpillar mass. Differences between treatments were analyzed by two-way analysis of variance.

RESULTS

First Instar Survivorship. For the seed-feeding generalist, *H. virescens*, mortality after 120 hr was 0% and 10% on control and phytic acid diets, respectively (Table 1); no effect on survival could be discerned over this time interval. For the folivorous generalist *T. ni*, however, mortality was significantly higher on diet containing phytic acid for all time periods tested (Table 1). By 120 hours, all caterpillars in the phytic acid treatment had died, whereas mortality on control diet for this same time interval was 16% ($P < 0.0005$). For the oligophagous *D. pastinacella*, mortality was significantly greater after 120 hr on the diet containing phytic acid ($P < 0.0005$). By the end of one week, all webworms had died

TABLE 1. PERCENT MORTALITY OF NEONATE *Heliothis virescens*, *Trichoplusia ni*, AND *Depressaria pastinacella* FED AN ARTIFICIAL DIET WITH OR WITHOUT 1% FW PHYTIC ACID

	Control	Phytic acid	χ^2	P
48 Hours				
H. virescens	0%	5%	1.026	$0.4 > P > 0.3$
T. ni	8%	40%	5.373	$0.025 > P > 0.01$
D. pastinacella	5%	30%	2.771	$0.1 > P > 0.05$
72 Hours				
H. virescens	0%	5%	1.026	$0.4 > P > 0.3$
T. ni	16%	92%	26.087	$P < 0.0005$
D. pastinacella	5%	75%	17.604	$P < 0.0005$
96 Hours				
H. virescens	0%	10%	2.105	$0.2 > P > 0.1$
T. ni	16%	96%	29.302	$P < 0.0005$
D. pastinacella	5%	80%	20.051	$P < 0.0005$
120 Hours				
H. virescens	0%	10%	2.105	$0.2 > P > 0.1$
T. ni	16%	100%	32.841	$P < 0.0005$
D. pastinacella	15%	95%	22.727	$P < 0.0005$

TABLE 2. GRAVIMETRIC ESTIMATES OF PERFORMANCE (TREATMENT MEANS) OF ULTIMATE INSTAR *T. ni, H. virescens*, AND *D. pastinacella* FED AN ARTIFICIAL DIET WITH OR WITHOUT 1% PHYTIC ACID

	−Phytic acid	+Phytic acid	F^a	$P <$
Trichoplusia ni				
RGR (mg/mg/day)	0.870	0.437	7.44	0.01
RCR (mg/mg/day)	3.536	1.739	9.62	0.01
ECI (%)	12.78	20.64	0.41	0.53
Heliothis virescens				
RGR (mg/mg/day)	0.619	0.291	56.02	0.01
RCR (mg/mg/day)	2.858	1.542	69.55	0.01
ECI (%)	21.49	18.56	5.35	0.02
Depressaria pastinacella				
RGR (mg/mg/day)	0.752	0.066	45.36	0.01
RCR (mg/mg/day)	4.058	0.808	81.57	0.01
ECI (%)	17.17	12.86	0.54	0.47

[a] ANOVA

on the diet containing phytic acid, as compared to 15% mortality in the control treatment.

Gravimetric Estimates of Performance. In all three species, phytic acid significantly reduced relative growth rates (RGR) and relative consumption rates (RCR) (Table 2). For both *T. ni* and *H. virescens*, relative growth rate was reduced by

TABLE 3. COMPARISION OF GROWTH AND FEEDING MEASURES BY ANALYSIS OF COVERIANCE OF ULTIMATE INSTAR *Trichoplusia ni, Heliothis virescens* AND *Depressaria pastinacella* FED ARTIFICIAL DIETS WITH AND WITHOUT 1% PHYTIC ACID

	Treatment least square means				
	−Phytic acid	+Phytic acid	Covariate	F	$P <$
T. ni					
Final larval mass	53.56	35.62	Initial larval mass	8.25	0.009
Consumption (mg)	157.97	86.22	Initial larval mass	11.06	0.003
Growth (mg)	29.86	29.77	Consumption	0.00	0.974
H. virescens					
Final larval mass	63.86	45.43	Initial larval mass	111.28	0.001
Consumption (mg)	191.22	111.83	Initial larval mass	86.02	0.001
Growth (mg)	36.21	24.45	Consumption	25.24	0.001
D. pastinacella					
Final larval mass	19.66	6.29	Initial larval mass	47.20	0.001
Consumption (mg)	78.82	16.66	Initial larval mass	81.27	0.001
Growth (mg)	8.52	9.35	Consumption	0.16	0.693

approximately 50%; relative growth rate reduction in *D. pastinacella* exceeded 90%. Relative consumption rates for the two noctuid species were reduced by approximately 50% as well, and in *D. pastinacella*, relative consumption rate was reduced by approximately 80%. In *H. virescens*, phytic acid also reduced the efficiency of conversion of ingested food (ECI) (Table 2) (Figure 2). ANCOVA results are in agreement with conventional gravimetric estimates of performance (Table 3).

Xanthotoxin metabolism. In *T. ni* and *H. virescens*, phytic acid did not affect xanthotoxin metabolism (Table 4). In *D. pastinacella*, xanthotoxin metabolism was reduced by 60% in the presence of phytic acid (Table 4) (Figure 1).

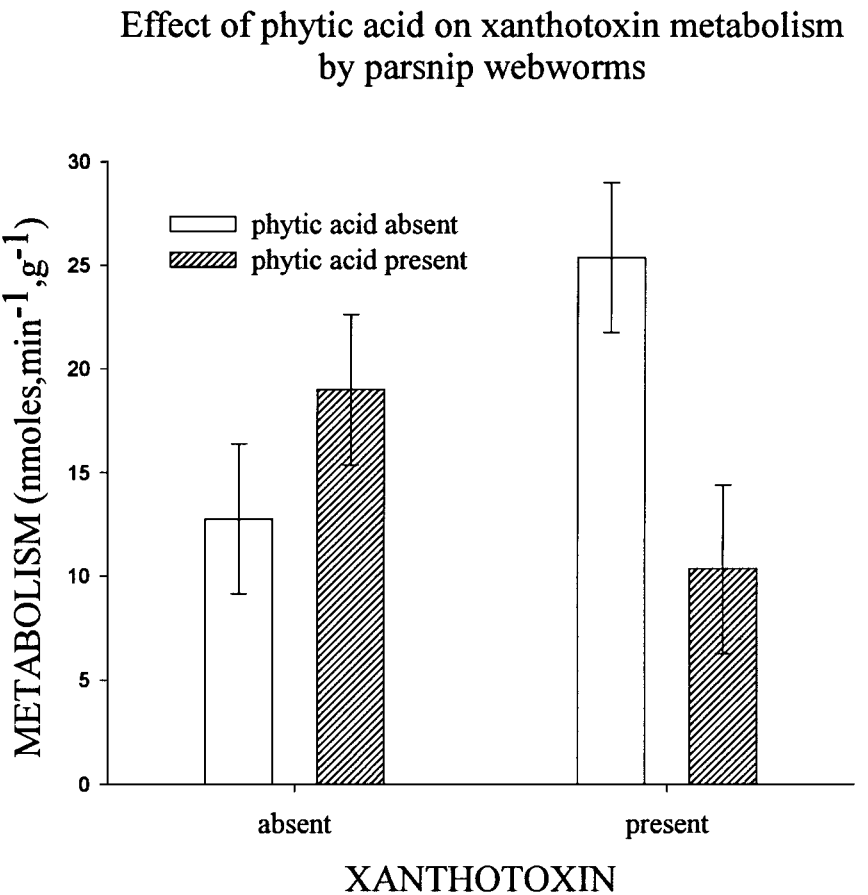


FIG. 1. Xanthotoxin metabolism in ultimate instar *D. pastinacella* in the presence and absence of pyhtic acid.

TABLE 4. XANTHOTOXIN METABOLISM (nmoles XAN/g CATERPILLAR/min) IN ULTIMATE INSTAR *Trichoplusia ni*, *Heliothis virescens*, AND *Depressaria pastinacella* FED ARTIFICIAL DIETS VARYING IN CONTENT OF PHYTIC ACID AND XANTHOTOXIN (SEE TEXT)

	Treatment LS means–metabolism (mg xan/mg caterpillar/min)						
	−P−X	−P+X	+P−X	+P+X	*p*(X)	*p*(P)	*p*(X×P)
H. virescens	0.12^{a}	n.d.^{b}	0.12^{a}	0.26^{a}	0.3032	0.0128	0.0134
T. ni	13.40^{a}	10.83^{a}	14.15^{a}	10.89^{a}	0.3340	0.8920	0.9074
D. pastinacella	12.75^{a}	25.37^{b}	18.99^{ab}	10.32^{a}	0.6064	0.2574	0.0124

Note. Means with the same letter are not significantly different by *t*-test, $p < 0.05$; P: phytic acid, X: xanthotoxin.

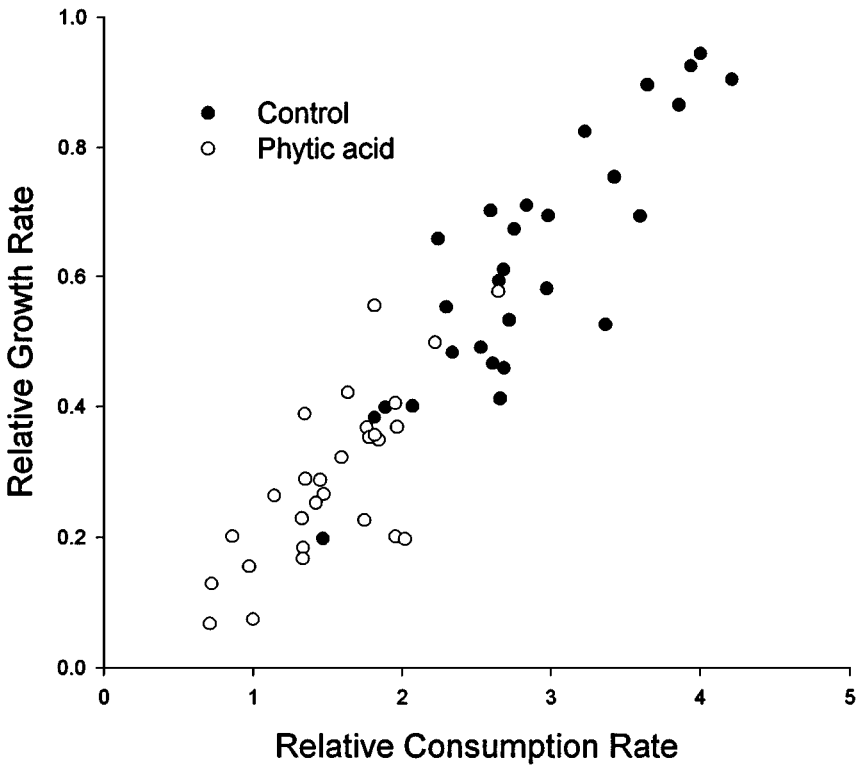


FIG. 2. Utilization plot of growth vs. consumption of ultimate instar *Heliothis virescens* fed artificial diet with or without phytic acid.

DISCUSSION

While there is a substantial amount of information regarding the effects of phytic acid on vertebrate nutrition (Reddy et al., 1989), literature regarding the influence of phytic acid on insect growth and detoxification enzymes is more limited. There is some correlative evidence of antifeedant effects in stored product insects (Jood et al., 1995; Marzo et al., 1997; Modgil and Mehta, 1997). We present here evidence for antifeedant effects of phytic acid on both folivorous and frugivorous insects that consume fresh tissues. Levels of 1% phytic acid, which are within the range encountered by fruit-feeding lepidopterans, can reduce consumption rates by 50% or greater. In the case of *H. virescens*, phytic acid slightly reduced the ability of this insect to convert ingested food into body mass. Moreover, we present evidence of outright toxicity of phytic acid to early stage caterpillars.

In vertebrates, phytic acid reacts with enzymes such as pepsin, α-amylase, β-galactosidase, and lipase, resulting in a decrease in their activities and concomitant reduction in digestive efficiency (Vaintraub and Bulmaga, 1991; Knuckles, 1998); it can also interfere with the activation of trypsinogen to trypsin and, thus, reduce the efficiency of protein utilization (Caldwell, 1992). Mechanisms thought to underlie these antinutritive activities include chelating essential minerals necessary for proper enzyme function and/or direct binding to enzymes, possibly at an allosteric site. Reduction in digestive efficiency in *H. virescens* may be the result of this form of interaction with digestive enzymes. In addition to possible effects on digestive enzymes, phytic acid had a demonstrable effect on the ability of *D. pastinacella* to metabolize xanthotoxin via cytochrome P450 monooxygenases (Table 4). This reduction in P450-mediated metabolism by phytic acid is most likely not due to reduced iron availability. In other studies (Green, 2000), P450-mediated metabolism of xanthotoxin in this species was not signficantly reduced when iron was omitted from the diet. In the case of *D. pastinacella*, which depends on cytochrome P450 monooxygenases to metabolize hostplant allelochemicals, phytic acid clearly inhibits P450-mediated metabolism of xanthotoxin (Table 4). Such P450 inhibition could contribute to reduced survival by exposing insects to the toxic effects of their hostplant allelochemicals. Indeed, survival of *D. pastinacella* on hostplants with above-average furanocoumarin concentrations is correlated with P450-mediated furanocoumarin metabolism rates (Berenbaum and Zangerl, 1998).

It is evident from these studies that phytic acid can interfere with growth and survival of caterpillars. In all three species examined, phytic acid reduced growth rates as compared to controls, and in two species caused significant mortality during the first five days of life. The general pattern of susceptibility is consistent with the ecological probability of exposure to phytic acid; the seed-feeding generalist, *H. virescens*, was largely unaffected, consistent with the widespread distribution

of phytic acid in seeds, whereas the foliage-feeding generalist, *T. ni*, and the bud-, flower-, and immature seed-feeding Apiaceae specialist, *D. pastinacella*, displayed substantial sensitivity to phytic acid, consistent with the fact that this compound is encountered rarely and at low concentrations. In its principal hostplant wild parsnip, *Pastinaca sativa* (A. Zangerl and M. Berenbaum, unpublished), as well as in other species in the family Apiaceae (Gupta et al., 1991) and other unrelated plants (Raboy and Dickinson, 1987), phytic acid does not appear in reproductive structures until 3–4 weeks after anthesis. Thus, the relative unsuitability of mature parsnip fruits for webworm growth (Zangerl et al., 1993) may be due in part to the accumulation of phytic acid with ripening. Date of first flowering in the field is negatively correlated with resistance to webworms (i.e., plants that flower early experience lower levels of herbivory) (Berenbaum et al., 1986); phytic acid accumulation in ripe seeds may contribute to this correlation.

Collectively, these experiments suggest that phytic acid in plant tissues may play a defensive function over and above its primary functions of phosphorus storage, energy storage, and cell wall precursor source (Reddy et al., 1989). In several crop species, phytic acid content varies among genotypes (Mebrahtu et al., 1997) and can be altered by artificial selection (Raboy et al., 1989). If this phytochemical trait is under genetic control under natural conditions, it may be available for selection by herbivorous insects such that the natural variation observed in nature may reflect past interactions with these herbivores.

Acknowledgments—This work was supported by NSF DEB 99 03867 to MRB and ARZ.

REFERENCES

BERENBAUM, M. R. 1991. Coumarins, pp. 221–249, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores, Their Interactions with Plant Secondary Metabolites. 2nd edn., Vol. 1. Academic Press, San Diego, CA.

BERENBAUM, M. R. 1995. Phototoxicity of plant secondary metabolites—insect and mammalian perspectives. *Arch. Insect Biochem. Physiol*. 29:119–134.

BERENBAUM, M. R., and ZANGERL, A. R. 1992. Genetics of physiological and behavioral resistance to host furanocoumanins in the parsnip webworm. *Evolution* 46:1373–1384.

BERENBAUM, M. R., and ZANGERL, A. R. 1998. Population-level adaptation to host-plant chemicals: The role of cytochrome P450 monooxygenases, pp. 91–112, *in* S. Mopper and S. Y. Strauss (eds.). Genetic Structure and Local Adaptation in Natural Insect Populations. Chapman & Hall, New York.

BERENBAUM, M. R., ZANGERL, A. R., and NITAO, J. K. 1986. Constraints on chemical co-evolution: Wild parsnips and the parsnip webworm. *Evolution* 40:1215–1228.

CALDWELL, R. A. 1992. Effect of calcium and phytic acid on the activation of trypsinogen and the stability of trypsin. *J. Agric. Food Chem.* 40:43–46.

EICHLIN, T. D., and CUNNINGHAM, H. B. 1978. The Plusiinae (Lepidoptera: Noctuidae) of America north of Mexico, emphasizing genitalia and larval morphology. *U.S. Dep. Agr. Techn. Bull*. 1567:14–15.

GREEN, E. S. 2000. Effects of nutrients and minerals on growth and detoxification in phytophagous insects. Ph.D. Thesis. University of Illinois at Urbana-Champaign.

GUPTA, K., THAKRAL, K. K., ARORA, S. K., and WAGLE, D. S. 1991. Studies on growth, stuctural carbohydrate, and phytate in coriander (*Coriandrum sativum*) during seed development. *J. Sci. Food Agric.* 54:43–46.

JOOD, S., KAPOOR, A. C., and SINGH, R. 1995. Polyphenol and phytic acid contents of cereal grains as affected by insect infestation. *J. Agric. Food. Chem.* 43:435–438.

KNUCKLES, B. E. 1998. Effect of phytate and other myo-inositol phosphate esters on lipase activity. *J. Food Sci.* 53:250–252.

LADEJI, O., OKOYE, Z. S. C., and OJOBE, T. 1995. Chemical evaluation of the nutritive value of leaf of fluted pumpkin *(Telferia occidentalis). Food Chem.* 53:353–355.

MARZO, F., AGUIRRE, A., CASTIELLA, M. V., and ALONZO, R. 1997. Fertilization effects of phosphorus and sulfur on chemical composition of seeds of *Pisum sativum* L. and relative infestation by *Bruchus pisorum* L. *J. Agric. Food Chem.* 45:1829–1833.

MEBRAHTU, T., MOHAMED, A., and ELMI, A. 1997. Accumulation of phytate in vegetable-type soybean genotypes harvested at four developmental stages. *Plant Foods Human Nutr.* 50:179–187.

METCALF, R. L., and METCALF, R. A. 1993. Destructive and Useful Insects: Their Habits and Control. McGraw-Hill, NY.

MODGIL, R., and MEHTA, U. 1997. Effect of *Callosobruchus chinensis* (bruchid) infestation on anti-nutritional factors in stored legumes. *Plant Foods for Human Nutr.* 50:317–323.

NITAO, J. K., and BERENBAUM, M. R. 1988. Laboratory rearing of the parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridae). *Ann. Ent. Soc. Am.* 81:485–487.

PILLAUF, J., and RIMBACH, G. 1997. Nutritional significance of phytic acid and phytase. *Arch. Anim. Nutr.* 50:301–319.

RABOY, V., and DICKINSON, D. B. 1987. The timing and rate of phytic acid accumulation in developing soybean seeds. *Plant Physiol.* 85:841–844.

RABOY, V., BELOW, F. E., and DICKINSON, D. B. 1989. Alteration of maize kernel phytic acid levels by recurrent selection for protein and oil. *J. Hered.* 80:311–315.

RAUBENHEIMER, D., and SIMPSON, S. J. 1992. Analysis of covariance: An alternative to nutritional indices. *Entomol. Exp. Appl.* 62:221–231.

REDDY, N. R., PIERSON, M. D., SATHE, S. K., and SALUNKHE, D. K. 1989. Phytates in Cereals and Legumes. CRC Press, Boca Raton, FL.

SCRIBER, J. M., and SLANSKY, F. 1981. The nutritional ecology of immature insects. *Annu. Rev. Entomol.* 26:183–211.

TIETZ, H. M. 1972. An Index to the Described Life Histories, Early Stages and Hosts of the Macrolepidoptera of the Continental United States and Canada. Vol. 1, Allyn AC, Sarasota, FL, pp. 467–468.

VAINTRAUB, I. A., and BULMAGA, V. P. 1991. Effect of phytate on the in vitro activity of digestive proteinases. *J. Agric. Food Chem.* 39:859–861.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

WALLACE, P. A., MARFO, E. K., and PLAHAR, W. A. 1998. Nutritional quality and antinutritional composition of four non-conventional leafy vegetables. *Food Chem.* 61:287–291.

ZANGERL, A. R., and BERENBAUM, M. R. 1993. Plant chemistry, insect adaptations to plant chemistry, and host plant utilization patterns. *Ecology* 74:47–54.

ZHOU, J. R., and ERDMAN, J. W. 1995. Phytic acid in health and disease. *Crit. Rev. Food Sci. Nutr.* 35:495–508.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

BROOK CHARR ALEVINS ALTER TIMING OF NEST EMERGENCE IN RESPONSE TO CHEMICAL CUES FROM FISH PREDATORS

REEHAN S. MIRZA,[1,*,†] DOUGLAS P. CHIVERS,[1,†] and JEAN-GUY J. GODIN[2]

[1]*Department of Biological Sciences*
University of Maine
5751 Murray Hall, Orono
Maine 04469-5751

[2]*Department of Biology*
Mount Allison University
Sackville, New Brunswick, E4L 1E4, Canada

(Received January 1, 2001; accepted May 14, 2001)

Abstract—Brook charr (*Salvelinus fontinalis*) lay their eggs in gravel nests called redds. The nests are not defended for extended periods and the eggs and young may be subject to predation from predators that travel through the substrate into the redd. In this study we tested whether long-term exposure of brook charr eggs to chemical cues from predatory sculpins (*Cottus cognatus*) resulted in an adaptive shift in the timing of emergence of charr alevins from artificial redds. We reared brook charr eggs in the presence of chemical cues from sculpins fed brook charr eggs, sculpins fed brine shrimp, and a well water control. Significantly more alevins emerged during the first week of the emergence period when exposed to chemical cues from sculpins fed eggs than from cues from sculpins fed brine shrimp or from well water. Alevins that emerged sooner were smaller and possessed larger yolk sacs than alevins that emerged later in the emergence period. Our results suggest that alevins that emerge sooner may escape predation in the redd, but may pay a cost in being under-developed upon emergence into the open water of the stream or lake.

Key Words—Alevin, redd, brook charr, slimy sculpin, emergence, predation, egg yolk.

*To whom correspondence should be addressed. e-mail: mirza@skyway.usask.ca
†Current address: Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, Saskatchewan S7N 5E2, Canada.

0098-0331/01/0900-1775$19.50/0

INTRODUCTION

Salmonid fishes (salmon, charr, and trout) lay their eggs within the substrate of streams and lakes by hollowing out spaces in the gravel and burying their eggs or by depositing their eggs into interstitial spaces between rocky cobble (Greely, 1932; Scott and Crossman, 1973). These 'nests' are termed redds. Eggs and newly-hatched young (alevins) remain in the redds for varying periods, where they develop by utilizing nutrients and energy from their yolk sacs. After a certain period alevins will begin to emerge from the gravel into the open-water habitats.

The mechanism that induces the migration of alevins out from the gravel is unclear but we do know that alevins possess the ability to alter their emergence behavior (Godin, 1982). Migration out of the gravel may be the result of environmental cues such as dropping dissolved oxygen levels in the gravel, changes in temperature, and/or the presence of predators. Moreover, emergence can be influenced by gravel size and sedimentation (Witzel and MacCrimmon, 1981).

Although burying eggs in the gravel affords some measure of protection from predation for the developing embryos, certain predators may still be able to enter the redd and prey upon developing embryos and newly-hatched alevins. For example, sculpins are small benthic fishes found in freshwater streams and lakes throughout North America (Van Vliet, 1964; Scott and Crossman, 1973). Sculpins typically reach lengths of 70–90 mm as adults and possess large flattened, shovel-shaped heads and large pectoral fins that may assist them in burrowing into the substrate. Several researchers have documented the presence of salmonid eggs in the stomachs of sculpins (e.g., Greely, 1932; Moyle, 1977; Hudson et al., 1995). Until recently, it was generally accepted that these were eggs that had been dislodged from redds and were consumed after they floated away from the redd. However, Dittman et al. (1998) have reported that sculpin enter redds and prey upon salmonid eggs.

Defenses of salmonid eggs and alevins towards in-gravel predators are presently unknown. However, we can predict that eggs may be able to hatch sooner and that alevins may leave the redd sooner in response to nest predators, thereby potentially avoiding predators within the redd. A few studies have documented that amphibians can alter hatching in response to predators. Sih and Moore (1993) report that amphibians can delay hatching in response to predators that forage on tadpoles. By delaying hatching the tadpoles reach a developmental stage where they are less vunerable to predators preying on larvae. Alternatively, Warkentin (1995, 1999) and Chivers et al. (2001) demonstrate that amphibians can hatch earlier in response to egg predators. It is not known whether charr possess the ability to alter their timing of hatching in response to predation risk, but even without this ability they may be able to alter their emergence behavior in response to perceived ambient risk of predation.

Aquatic animals can assess local predation risk by detecting chemical alarm cues released by nearby conspecifics (reviewed in Chivers and Smith, 1998), or by detecting chemical cues emanating from predators (reviewed in Kats and Dill, 1998). In many cases, prey animals need to be feeding on conspecifics of the prey in order for the latter to respond to the predator (reviewed in Chivers and Mirza, 2001). For example, Mathis and Smith (1993) found that fathead minnows (*Pimephales promelas*) responded to chemical cues from pike (*Esox lucius*) fed a diet of minnows, but not to cues from pike fed a diet of a different fish allopatric to minnows.

A common predator on brook charr, *Salvelinus fontinalis*, eggs is the slimy sculpin, *Cottus cognatus* (Greely, 1932). We (Mirza and Chivers unpubl. data) have recently observed that slimy sculpins are attracted to chemical cues of charr eggs emanating from the gravel. In the present study, we tested whether exposure to predator cues would cause brook charr alevins to alter their emergence patterns from artificial nests so as to reduce the risk of mortality within the redd. We predict that exposure to chemical cues from sculpins feeding on eggs would cause alevins to emerge earlier from the gravel. Furthermore, the individuals that emerge earlier should also be less well-developed (i.e., have larger yolk sacs at emergence). Individuals that emerge earlier may pay a fitness cost by emerging into an open water habitat in a less developed state (Gustafson-Greenwood and Moring, 1990).

MATERIALS AND METHODS

Our study was approved by the Institutional Animal Care and Use Committee of the University of Maine (Animal Research Protocol No. A98-10-03). Fertilized brook charr eggs (Maine wild strain) were supplied by the Enfield state hatchery in Enfield, ME in December of 1998. They were produced by mixing gametes from 1 large adult female and 5 large adult males. We obtained eggs immediately after fertilization. They were held in trays in flowing well water (9.5°C, 5 l/min) in the dark, in our lab at the University of Maine. Slimy sculpin were captured from Spruce Mountain Brook in central Maine using a backpack electroshocker and transported to our lab. Prior to the experimental trials sculpin were held in our lab in a 350-l artificial stream tank at 12°C under a 14:L 10:D photoperiod and fed frozen brine shrimp (*Artemia* spp.) ad libitum.

The experiment was conducted in a modified gravitational flow-through apparatus under a 12 L:12 D illumination regime. Well water was pumped into a large 890 l tank, chilled to 7.5°C, and then pumped into three separate header tanks at a rate of ~5 l/min. The header tanks contained either i) 10 sculpins (mean $\pm$ SD $=$ 68.2 $\pm$ 3.4 mm standard length) fed *ad libitum* brook charr eggs, ii) 10 sculpins (67.4 $\pm$ 2.1 mm standard length) fed *ad libitum* with brine shrimp

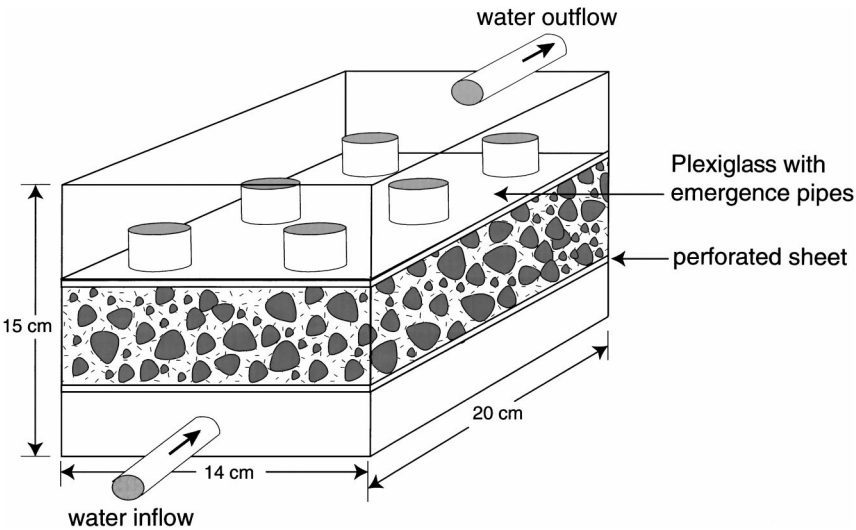


FIG. 1. Diagrammatic representation of an artificial redd.

or iii) well water only. Each header tank (76 × 38 × 36 cm) had nine outflows to artificial redd boxes, plus an overflow. Each outflow had a control valve for setting the flow rate to an artificial redd. Water flowed through each redd box and then was recaptured in catch basins which overflowed down the drain.

The type of artificial redd boxes we used (Figure 1) were modified from those used by Godin (1980). Each redd box consisted of a rectangular plastic box (20 × 15 × 14 cm) into which we placed a perforated Plexiglas sheet 3 cm above the bottom of the box. On top of this sheet we placed 5 cm of gravel (2.48 ± 0.63 cm diam.). The perforated plate allowed for water to flow up through the redd box without the inflow from the header tank being plugged by the gravel. A second Plexiglas sheet was placed on top of the gravel to stop alevins from re-entering the gravel once they emerged. Six holes (4.4 cm diam.) were cut into the sheet and each fitted with a 4-cm long piece of PVC pipe to allow alevins to swim up after emergence. Water flowed out the top of the redd box (Figure 1). This set-up allowed us to mimic the upwelling effect which occurs in natural brook charr redds (Greely, 1932). The sides of the redd boxes were wrapped in black plastic which would allow entry of light from the tops of the redd boxes only.

Charr eggs were placed within the gravel layer and covered the same day they were fertilized. We placed 150 eggs in each redd box. Redd boxes were randomly assigned one of the three treatments (9 redd boxes/treatment). Water flow and temperature were monitored daily in each individual redd box and the header tanks. The mean temperature (±SD) was 9.4 ± 0.19°C, and the mean flow-rate

($\pm$SD) was 344 ± 37 ml/min. There were no differences in temperature (ANOVA, $F_{2,14} = 2.37$, $P = 0.13$) or flow rate (ANOVA, $F_{2,14} = 0.06$ $P = 0.94$) among treatments.

Fresh eggs to be used for food were placed on a flat screen mesh tray (50×30 cm) in the catch basins. Because egg mortality was high due to fungal growth, eggs were frozen 14 days into the experiment. For the duration of the experiment, sculpins were fed previously frozen charr eggs *ad libitum*. Uneaten eggs were removed daily.

Redd boxes were monitored twice a day (05:00 hr and 17:00 hr) for emerging alevins. Early morning emergence checks corresponded with our simulated sunrise and the late afternoon check corresponded with sunset. By checking the redd boxes for emergence at sunrise and sunset we were able to determine whether the alevins emerged at night or during the day. Emergence from gravel nests in salmonids is largely nocturnal (Dill and Northcote, 1970; Godin, 1980;1982; Carey and Noakes, 1981). In the present study, the first alevin emergence was recorded 75 days after fertilization of eggs. This was designated as the first day of emergence. Emergence was recorded for five weeks after this date. Alevins were collected from the redd boxes using pasteur pipettes and euthanized with an overdose of methyl tricane sulphonate (MS-222). Alevins were then preserved in 5% buffered formalin, for at least 90 days, for morphometric analysis.

All emergent alevins were enumerated. For each redd box, in each of the three treatments, we calculated the percentage of alevins that emerged during each week. We compared the percentage of alevin emergence among the three treatments, using Kruskal-Wallis one-way ANOVAs for each week. This was followed by post-hoc non-parametric pair-wise comparisons where appropriate; comparing the sculpin-fed egg treatment to the other two treatments (Siegel and Castellan, 1988).

For the morphometric analysis we measured standard length and then weighed each specimen (after towel drying), removed the yolk plug, and re-weighed the specimen to determine the mass of the yolk plug. Preservation of the yolk plug in formalin caused it to harden and be easily removed from the yolk sac (Godin et al., 1977). We then conducted two-way ANOVAs on the standard length, yolk plug mass, and ratio of yolk plug mass to standard length, testing for treatment effects, time effects, and treatment $\times$ time interactions (Zar, 1996).

RESULTS

Exposure to chemical cues originating from sculpins had a marked effect on the pattern of emergence of brook charr alevins (Figure 2). For both the sculpin-fed brine shrimp treatment and the blank control treatment there was a steady increase in emergence that peaked in week 3, and then a steady decrease. In contrast, an initial pulse of emergence occurred in week 1 in the sculpin-fed egg treatment.

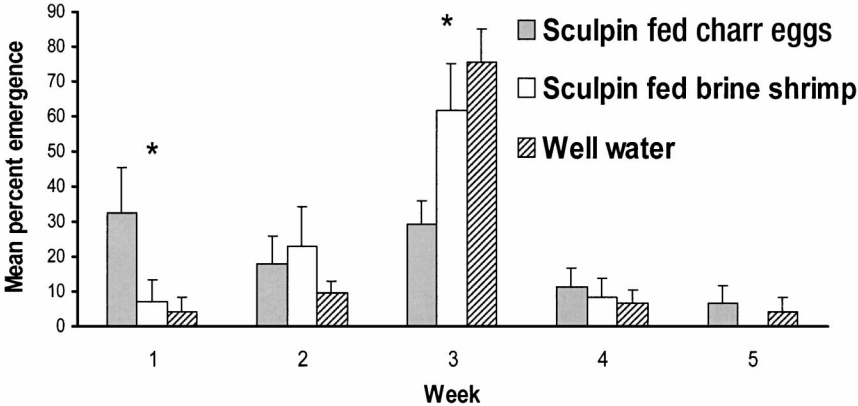


FIG. 2. Mean (+SE) percent emergence of alevins exposed to chemical cues from either sculpins fed charr eggs, sculpins fed brine shrimp or well water. Percent emergence was calculated as the number of alevins that emerged each week over the total number emerged after 5 weeks. All tests were conducted using non-parametric statistics (see text for details). *Denotes a significant difference at $P < 0.05$.

There was a general decline in emergence from week 1 through week 5. There was a difference in alevin emergence among the treatments during the first week ($X^2 = 7.85$, $df = 2$, $P = 0.01$, 1-tailed). Significantly more alevins emerged during week 1 in the sculpin-fed egg treatment than either of the other two treatments (non-parametric pair-wise comparison, $P < 0.05$). There was also a difference in alevin emergence among the treatments during week 3($X^2 = 7.39$, $df = 2$, $P = 0.013$, 1-tailed) as more alevins emerged from the blank control treatment than the sculpin-fed egg treatment (non-parametric pair-wise comparison, $P < 0.05$). There were no differences in the percentage of emergence among the treatments in weeks 2, 4, and 5 ($X^2 = 0.72$, $df = 2$, $P = 0.70$; $X^2 = 0.62$, $df = 2$, $P = 0.74$; $X^2 = 3.8$, $df = 2$, $P = 0.15$, respectively; Fig 2). Overall, we found that 61% of alevins emerged at night. However, there was no significant treatment difference in the overall numbers of alevins that emerged at night ($X^2 = 1.78$, $df = 2$, $P = 0.41$, 2-tailed).

There were effects of time since deposition into the redd on alevin length, yolk plug mass, and ratio of yolk plug mass to length (Table 1; Figure 3A). However, there were no treatment effects on length, yolk plug mass, or ratio of yolk plug mass to length; nor were there any interaction effects between treatment and time (Table 1). For both mass of yolk plug and mass to length ratio, individuals that emerged early had a larger yolk plug and larger mass to length ratio than those individuals that emerged later (Figure 3B,C). A comparison of the results of the morphometric analysis with the emergence data reveals that those individuals that

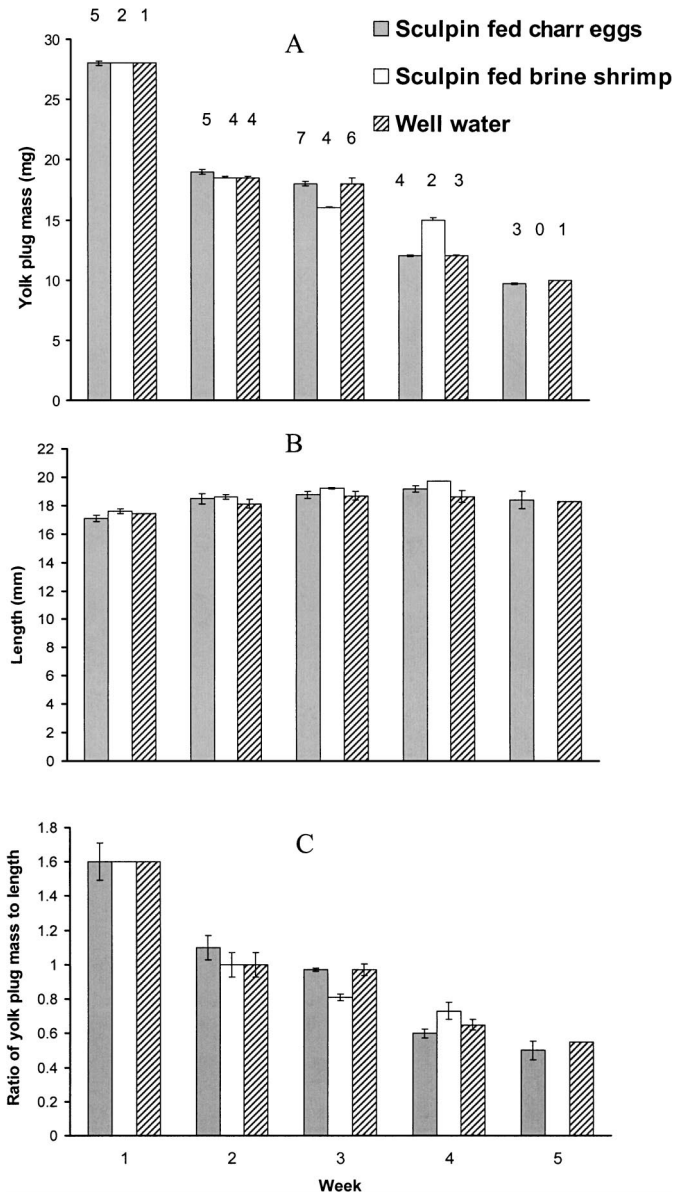


FIG. 3. Mean (±SE) of A) yolk plug mass (mg), B) standard length (mm) and C) ratio of yolk plug mass/length of alevins exposed to chemical cues from either sculpins fed charr eggs, sculpins fed brine shrimp or well water. Numbers above bars represent the number of redds in which emergences were recorded for that particular week (see text for details).

TABLE 1. RESULTS OF TWO-WAY ANOVAS TESTING FOR EFFECTS OF TREATMENT AND TIME (WEEK), AND THEIR INTERACTIONS ON ALEVIN LENGTH, YOLK PLUG MASS, AND RATIO OF YOLK PLUG MASS TO LENGTH

	F	*df*	*P*
Length			
Treatment	2.37	2, 36	0.108
Week	7.75	4, 36	<0.001
Treatment × Week	0.33	7, 36	0.938
Yolk Plug Mass			
Treatment	0.01	2, 36	0.996
Week	34.92	4, 36	<0.001
Treatment × Week	0.69	7, 36	0.697
Ratio of Yolk Plug Mass/Length			
Treatment	0.03	2, 36	0.998
Week	40.33	4, 36	<0.001
Treatment × Week	0.06	7, 36	0.753

emerged earlier had larger yolk sacs and that a larger proportion of those individuals emerged from the sculpin-fed egg treatment during the first week.

DISCUSSION

Our results demonstrate that brook charr alevins alter their pattern of emergence from the redd when exposed to chemical cues from sculpins fed brook charr eggs. By leaving the nest sooner, alevins may evade predation within the redd. However, escaping predation in the redd may have a cost associated with it, since individuals that emerged sooner were smaller and possessed larger yolk sacs than individuals that emerged later. Typically, alevins emerge from the redd after absorbing most of their yolk sac and then rest on the substrate outside the redd reabsorbing the remainder of the yolk sac before dispersing in the stream or lake (Godin, 1982). However, alevins may be vulnerable to predators outside the redd during this period. If alevins are emerging with large yolk sacs, it may hamper swimming ability making it harder to evade predators (Gustafson-Greenwood and Moring, 1990). Thus, individuals which emerge earlier may be more at risk to predation in the open-water habitat (Godin, 1982; Huntingford et al., 1988).

We also found that the pattern of alevin emergence differed between the sculpin-fed egg treatment and the other two treatments. In the sculpin-fed egg treatment, most of the emergence occurred in the first week and then slowly trailed off. By comparison, in the other two treatments there was a steady increase in emergence that peaked at week 3 and then steadily declined. Altering the natural

pattern of emergence may also increase susceptibility of emerging alevins to predators outside the redd (Godin 1980, 1982). Alevins that synchronize emergence may gain antipredator benefits from being in larger numbers through the predation risk dilution and confusion effects (Godin, 1982,1986).

Alevins exhibit a trade-off in predation risk. If they were to stay within the redd predation risk would be high. However, since alevins do not disperse right away after emergence the risk of predation outside the redd may also be high because other predators may be in the vicinity. Typically, most streams offer little shelter since the substrate consists mainly of cobble and gravel. Benthic predators, such as sculpins, would most likely be proficient in searching for prey in these habitats. Thus, it may be difficult for alevins which emerged with larger yolk sacs to hide and compensate for lost developmental time in the nest.

In addition to increased predation risk, there may be other costs associated with early emergence. Impaired swimming ability due to possessing a larger yolk sac may render alevins more prone to being displaced by water currents (Gustafson-Greenwood and Moring, 1990). Similarily, presence of predators outside the redd may induce downstream displacement which may be energetically costly (Bardonnet and Heland, 1994).

Our study is the first to demonstrate experimentally a shift in the timing of nest emergence behavior by alevins in response to chemical cues from predators. The response we observed was not a general response to sculpin chemicals, but rather was a response to cues of sculpins feeding on brook charr eggs. Cues from sculpins feeding on brine shrimp were not sufficient to elicit this change in emergence pattern. We do not know whether the observed shift in emergence would also occur in response to injured eggs alone. Is the response we observed a response to injured eggs or a response to injured eggs combined with predator odor? In either case, predation is the causal factor changing the emergence pattern.

We observed that brook charr alevins altered their nest emergence patterns in response to chemical cues associated with predation risk. The most likely explanation for the observed pattern is that alevins made behavioral decisions regarding when to emerge. However, it is possible that eggs detect chemical cues from predators and hence alter their development, which in turn influences timing of emergence. Blaustein (1997) hypothesizes that cladocerans and copepods were less abundant in the presence of fire salamander larvae (*Salamandra infraimmaculata*) because they exhibited hatching inhibition. Chivers et al. (2001) showed that both Pacific tree frog (*Hyla regilla*) and Cascades frog (*Rana cascadae*) eggs hatch faster in the presence of chemical cues from predatory leeches (families Glossiphonidae and Erpobdellidae), but not chemicals from non-predatory earthworms (*Lumbricus terrestris*). It is not known whether fish eggs detect chemical cues from predators and facultatively adjust timing of hatching in a similar manner. Further research should address this question.

Acknowledgements—We would like to thank Matt Puttlitz and Pam Bryer for technical assistance. We would also like to thank the Maine Department of Inland Fisheries and Wildlife and the Enfield State Hatchery for assistance and providing the trout eggs for this study. This study was funded by a grant to D.P. Chivers from the Maine Agriculture and Forest Experiment Station. The research presented here was described in Animal Research Protocol No. A98-10-03 by the Institutional Animal Care and Use Committee of the University of Maine, Orono.

REFERENCES

BARDONNET, A., and HELAND, M. 1994. The influence of predators on the habitat preferanda of emerging brown trout. *J. Fish Biol. A*45:131–142.

BLAUSTEIN, L. 1997. Non-consumptive effects of larval *Salamandra* on crustacean prey: Can eggs detect predators? *Oecologia* 110:212–217.

CAREY, W. E., and NOAKES, D. L. G. 1981. Development of photobehavioural responses in young rainbow trout, *Salmo gairdneri* Richardson. *J.Fish Biol.* 19:285–296.

CHIVERS, D. P., KIESECKER, J. M., MARCO, A., DEVITO, J., AANDERSON, M. T., and BLAUSTEIN, A. R. 2001. Predator-induced life history changes in amphibians: Egg predation induces hatching. *Oikos* 92:135–142.

CHIVERS, D. P., and MIRZA, R. S. 2001. Predator diet cues and the assessment of predation risk by aquatic vertebrates: A review and prospectus, *in* A. Marchlewska-Koj, J. J. Lepri, and D. Müller-Schwarze, (eds.) Chemical Signals in Vertebrates, Vol. 9. Plenum, New York.

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Écoscience* 5:338–352.

DILL, P. A., and NORTHCOTE, T. G. 1970. Effects of gravel size, egg depth and egg density on intragravel movement and emergence of coho salmon (*Oncorhynchus kisutch*) alevins. *J. Fish. Res. Bd. Can.* 27:1191–1199.

DITTMAN, A. H., BROWN, G. S., and FOOTE, C. J. 1998. The role of chemoreception in salmon-egg predation by coastrange (*Cottus aleuticus*) and slimy (*C. cognatus*) sculpins in Iliama Lake, Alaska. *Can. J. Zool.* 76:406–413.

GODIN, J.-G. J. 1980. Temporal aspects of juvenile pink salmon (*Oncorhynchus gorbuscha* Walbaum) emergence from a simulated redd. *Can. J. Zool.* 58:735–744.

GODIN, J.-G. J. 1982. Migrations of salmonid fishes during early life history phases: Daily and annual timing, pp. 22–50, *in* E. L. Brannon and E. O. Salo (eds.). Proceedings of the Salmon and Trout Migratory Behavior Symposium. University of Washington Press, Seattle, WA.

GODIN, J.-G. J. 1986. Antipredator function of shoaling in teleost fishes: A selective review. *Naturaliste Can. (Rev. Écol. Syst.)* 113:241–250.

GODIN, J.-G. J., DILL, P. A., and SANKURATHRI, M. 1977. A comparison of the relative variability of wet and dry weights of young pink salmon (*Oncorhynchus gorbuscha* Walbaum). *Fish. Mar. Serv. Res. Dev. Tech. Rep.* 702:1–8.

GREELY, J. R. 1932. The spawning habits of brook, brown and rainbow trout and the problem of egg predators. *Trans. Amer. Fish. Soc.* 62:239–248.

GUSTAFSON-GREENWOOD, K. I., and MORING, J.R. 1990. Territory size and distribution of newly-emerged Atlantic salmon (*Salmo salar). Hydrobiologia* 206:125–131.

HUDSON, P. L., SAVINO, J. F., and BRONTE, C. R. 1995. Predator–prey relations and competition for food between Age-0 lake trout and slimy sculpins in the Apostle Island Region of Lake Superior. *J. Great Lakes Res.* 21:445–457.

HUNTINGFROD, F. A., METCALFE, N. B., and THORPE, J. E. 1988. Choice of feeding station in Atlantic salmon, *Salmo salar*, parr: Effects of predation risk and life-history strategy. *J. Fish Biol.* 33: 917–24.

KATS, L. B., and DILL, L. M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Écoscience* 5:361–394.

MATHIS, A., and SMITH, R. J. F. 1993. Fathead minnows, *Pimephales promelas*, learn to recognize northern pike, *Esox lucius*, as predators based on chemical stimuli from minnows in the pike's diet. *Anim. Behav.* 46:645–656.

MOYLE, P. B. 1977. In defense of sculpins *Fisheries* 2:20–23.

SCOTT, W. B., and CROSSMAN, E. J. 1973. Freshwater Fishes of Canada. *Can. Bull. Fish. Aquat. Sci.* 184:1–966.

SIEGEL, S., and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral Sciences. 2nd edn. McGraw-Hill, New York.

SIH, A., and MOORE, R.D. 1993. Delayed hatching of salamander eggs in response to enhanced larval predation risk. *Am. Nat.* 142:947–960.

VAN VLIET, W. H. 1964. An ecological study of *Cottus cognatus* Richardson in northern Saskatchewan. M.Sc. Thesis. University of Saskatchewan, Saskatoon.

WARKENTIN, K. M. 1995. Adaptive plasticity in hatching age: A response to predation risk trade-offs. *Proc. Natl. Acad. Sci.* U.S.A. 92:3507–3510.

WARKENTIN, K. M. 1999. Effects of hatching age on development and hatchling morphology in the red-eyed tree frog, *Agalychnis callidryas. Biol. J. Linn. Soc.* 68:443–470.

WITZEL, L. D., and MACCRIMMON, H. R. 1981. Role of gravel substrate on ova survival and alevin emergence of rainbow trout. *Can. J. Zool.* 59:629–636.

ZAR, J. H. 1996. Biostatistical Analysis, 4th edn. Prentice-Hall, New York.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

CHANGES IN THE CUTICULAR HYDROCARBON PROFILE OF THE SLAVE-MAKER ANT QUEEN, *Polyergus breviceps* EMERY, AFTER KILLING A *Formica* HOST QUEEN (HYMENOPTERA: FORMICIDAE)

CHRISTINE A. JOHNSON,[1,*] ROBERT K. VANDER MEER,[2] and BARRY LAVINE[3]

[1]*Graduate School & University Center of the City University of New York Hunter College, New York, New York 10021*

[2]*United States Department of Agriculture Agricultural Research Service Medical & Veterinary Entomology Research Laboratory Gainesville, Florida, 32604*

[3]*Clarkson University Potsdam, New York, 13699*

(Received September 5, 2000; accepted May 6, 2001)

Abstract—Queens of the slave-maker ant, *Polyergus breviceps,* take over nests of their *Formica* host species by fatally attacking the resident queen. As workers only begin grooming the *P. breviceps* queen once she has ceased her attack, we investigated whether a change in parasite queen chemistry may account for the change in worker behavior. Cuticular hydrocarbon profiles of newly mated *P. breviceps* queens and of queens of their two *Formica* host species were found to be species-specific. Profiles of newly mated *P. breviceps* queens that had attacked a *Formica* queen, however, were virtually identical to the queen profile of the species killed. Mass spectral analysis revealed that the hydrocarbons on the cuticles of newly mated *P. breviceps* changed from primarily normal alkanes to methyl and di-methyl branched alkanes after attacks. The results suggest that cuticular compounds from the host queen were transferred to the parasite queen during their aggressive interaction.

Key Words—Cuticular hydrocarbons, Formicidae, slave-maker, *Polyergus breviceps, Formica gnava, Formica occulta,* colony takeover, queen killing, chemical camouflage.

*To whom correspondence should be addressed at Department of Ecology & Systematics, University of Helsinki, P.O. Box 17, FIN-00014 (Arkadiankatu 7), Helsinki, Finland. e-mail: christine.johnson@helsinki.fi

0098-0331/01/0900-1787$19.50/0

INTRODUCTION

Species of the ant genus, *Polyergus*, are all obligate social parasites, unable to survive without the foraging, feeding, and brood care behaviors of their *Formica* hosts. *Polyergus* workers acquire host workers ("slaves") by robbing host species nests of their pupae during summer months. The *Formica* pupae mature in the slave-maker's nest and become full-functioning colony members. Queens of *Polyergus* also depend on the behaviors of their slaves, and new queens require a slave supply to establish their colonies. However, rather than raiding a host nest for brood, a new queen secures her initial slave supply by taking over a host species nest. Frequently, an alate *Polyergus* gyne will mate while running alongside nestmates that are advancing towards a *Formica* nest to raid. By the time the newly mated queen reaches the targeted *Formica* nest, the inhabitants have already abandoned it (temporarily) and the raiders have all but completed their pillaging and are making their way back to their natal nest. The newly mated *Polyergus* gyne enters the nest, as do the *Formica* soon thereafter, and locates and fatally attacks the resident queen (Wheeler, 1906; Topoff et al., 1988; Mori et al., 1995). In laboratory nests, *Formica* workers at first will attack the invading queen, but attacks diminish once the *Polyergus* queen has begun her attack on the *Formica* queen. *Formica* workers during this time seem to be either appeased (Topoff et al., 1988; Mori et al., 2000a,b) or repelled (D'Ettorre et al., 2000) by a secretion from the Dufour's gland of the *Polyergus* queen. Immediately after the *Polyergus* queen ceases her attack on the host queen, approximately 25 min after attack onset, workers begin grooming the slave-maker queen. Results of laboratory experiments indicate that the aggressive interaction between the *Polyergus* and *Formica* queens is a key component in the ultimate acceptance of the *Polyergus* queen (Zaayer, 1967; Topoff et al., 1988, 1990; Topoff and Zimmerli, 1993; Mori et al., 1995). First, *Polyergus* queens are more successful in their attempts to take over a nest when a *Formica* queen is present (and the *Polyergus* queen kills her) than when the *Formica* nest is queenless. Worker attacks on an invading *Polyergus* queen in queenless colonies tend to be relentless and almost always result in her death (Zaayer, 1967; Topoff et al., 1988, 1990; Topoff and Zimmerli, 1993). Second, the associated change in worker behavior takes place immediately after attacks on the host queen cease, even if the *Formica* queen is still alive (although maimed, person. obs.). This is unlike the delayed and gradual decrease in worker aggression that is sometimes observed in other species when the resident queen is removed experimentally.

The prevailing hypothesis is that *Formica* workers adopt a *Polyergus* queen because she is "camouflaged" with the host queen cuticular chemicals, chemicals familiar to the workers (Topoff et al., 1988, 1990; Topoff and Zimmerli, 1993; Zimmerli and Topoff, 1994). The lipid layer of the insect cuticle is capable of absorbing other lipid soluble compounds (Soroker et al., 1994, 1995; Vienne et al., 1995), and it has been demonstrated that chemicals can be transferred among

individuals, even across higher level taxa, through social contact (see Vander Meer and Wojcik, 1982). Thus, while attacking a *Formica* queen, *Polyergus* queens may be absorbing chemicals involved in nestmate/queen recognition from the *Formica* queen. The recent results of Errard and D'Ettorre (1998) support this contention; after a *Polyergus rufescens* Latreille queen kills a *Formica cunicularia* Latreille queen, her cuticular hydrocarbon profile resembles the hydrocarbon profiles of *F. cunicularia* queens.

In this study, we examined whether a newly mated *Polyergus breviceps* Emery queen also undergoes changes in hydrocarbon profile after killing a queen of her host species, and whether the resulting profile is then similar to the queen profile of the host species. By comparing profiles of *P. breviceps* queens that had killed either a *Formica gnava* Buckley or *Formica occulta* Francoeur host queen, we were further able to ascertain whether the changes in hydrocarbon profiles were specific to the species of *Formica* queen killed. Of the lipids found on the cuticle, a significant percentage tends to be hydrocarbons (Jackson and Blomquist, 1976). Numerous studies have demonstrated a correlation between hydrocarbon patterns and nestmate recognition (e.g., Bonavita-Cougourdan et al., 1987; Vander Meer and Morel, 1998). Recently, the importance of hydrocarbons in nestmate recognition has been confirmed for some ant species (e.g., Lahav et al., 1999), although other classes of compounds are undoubtedly also involved (Obin, 1986). The particular pattern of hydrocarbons, frequently characteristic of a species (nest [Nowbahari et al., 1990] or caste [Wagner et al., 1998]), is likely to reflect similarities or differences in other non-polar and polar cuticular lipids among species. Hydrocarbon profiles can, therefore, be used to investigate the transfer of cuticular lipids, which are likely to contain nestmate recognition cues. Certainly, the strong correlation between hydrocarbon profiles shared by other parasites and predator myrmecophiles and their hosts and adoption by the host colony is suggestive of this (e.g., Vander Meer and Wojcik, 1982; Franks et al., 1990; Vander Meer et al., 1989).

METHODS AND MATERIALS

Ant Collections and Housing. Ant colonies were collected during June and July of 1997 and 1998 from the Chiricahua Mountains of southeastern Arizona, U.S.A.. Fifteen queenright colonies of *F. gnava* were collected from the Arizona oak-alligator juniper woodlands of the Southwestern Research Station (SWRS) of the American Museum of Natural History (el. 1646 m). Thirteen queenright colonies of *F. occulta* were collected from an area just east of the Barfoot Peak trailhead (el. 2750 m) in Coronado National Forest populated with ponderosa pine. Colonies were brought into the laboratory at SWRS and kept in large Tupperware® boxes lined with Fluon® (Northern Products, Woonsocket, RI) to prevent escape.

Newly mated *P. breviceps* queens from three nests with *F. gnava* slaves ($N = 49$) and from two nests with *F. occulta* slaves ($N = 13$) were collected as they approached the *Formica* nest being raided by their non-reproductive nestmates and placed in individual 4 dram vials that contained a cotton ball moistened with water.

Solvent Extraction of Queens. Twelve established (with colony) *F. gnava* queens and ten established *F. occulta* queens were removed from their nests and placed in individual Tupperware boxes ($20.5 \times 45 \times 3.5$ cm) lined with a thin layer of soil. A single newly mated *P. breviceps* queen was introduced into the box and allowed to attack the *Formica* queen. Immediately after attacks ceased, *P. breviceps* and *Formica* queens were placed in individual 7 ml scintillation vials and a quantity of high purity hexane (GC^2 Grade [B & J, Muskegon, MI]) sufficient to cover the entire body (approximately $0.3 - 0.5$ ml) was added to extract cuticular components (see Table 1). After 10 min, the solvent extract was transferred from the sample with a Pasteur pipette to a 2 ml scintillation vial and allowed to evaporate. In addition, cuticular chemicals from newly mated *P. breviceps* queens (that had not attacked a host queen) from nests containing *F. gnava* slaves and from nests containing *F. occulta* slaves, and from established *F. gnava* and *F. occulta* queens that had not been presented to *P. breviceps* queens for attack were extracted using the same protocol presented above (see Table 1). All specimens were preserved for voucher in 70% ethyl alcohol (maintained in personal collection—CAJ).

Chemical Analysis. The evaporated extracts were transported to the United States Department of Agriculture, Agricultural Research Service, Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, Florida where they were reconstituted in 0.2 ml hexane, vortexed for 2–3 sec, and applied to a small silicic acid (70–230 mesh 60 Å, [Aldrich Chemical Co., Inc.]) Pasteur pipette

TABLE 1. SPECIES AND CONDITIONS OF QUEENS FROM WHICH CUTICULAR HYDROCARBONS WERE ANALYZED

Species	Condition	Host species in nest	Number of individuals
Polyergus breviceps	Newly mated	*F. gnava*	42
		F. occulta	5
	Killed *Formica* queen	*F. gnava*	6
		F. occulta	6
Formica gnava	Killed by *Polyergus*	—	12
	No interactions with *Polyergus*	—	3
Formica occulta	Killed by *Polyergus*	—	10
	No interactions with *Polyergus*	—	3

column. Hydrocarbons were isolated from other lipids by eluting the column with hexane. The eluent, containing purified hydrocarbons (ca. 6–7 ml, which from previous experience eluted all hydrocarbons), was concentrated to ca. 10 μl under a stream of N_2. Samples were analyzed by gas chromatography (Varian 3700 [Varian Associates, Walnut Creek, CA] equipped with a split-splitless injector [Agilent Technologies, Palo Alto, CA], a capillary column [DB – 1, 30 m, 0.32 mm i.d., 0.25 μm film thickness; J & W Scientific, Inc., Folsom, CA] and flame ionization detector). The injector and detector were set at 300°C; the oven temperature was programmed from 190°C to 290°C at 5°C/min, and then held at 290°C for 5 min. Hydrogen was used as the carrier gas and nitrogen was used as the makeup gas. Data were analyzed using PE Nelson Turbochrom Navigator 6.1.0.1FO4 (Perkin Elmer Corp., Norwalk, CT). Hydrocarbon standards (C24, C26, C28, C30, C32, #NP-MIX-H [Alltech Associates, Inc., Deerfield, IL]) were injected at regular intervals during sample analysis. The standards were used to calculate Kovat Indices (KI).

Mass Spectrometry. Representative samples of (a) newly mated *P. breviceps* queens from nests containing *F. gnava* slaves, (b) newly mated *P. breviceps* queens that had killed a *F. gnava* queen, (c) *F. gnava* queens, (d) newly mated *P. breviceps* queens from nests containing *F. occulta* slaves, (e) newly mated *P. breviceps* queens that had killed an *F. occulta* queen, and (f) *F. occulta* queens were analyzed by gas chromatography/mass spectroscopy. Electron impact-mass spectra (EI-MS) were obtained with an HP 5890 Series II GC (Agilent Technologies, Palo Alto, CA) connected to an HP 5988A MS instrument (Agilent Technologies, Palo Alto, CA) operated at 70 eV and tuned to accentuate the high mass fragments. The GC, equipped with an on-column injector and a SPB1 capillary column (0.25 μm film; 30 m $\times$ 0.32 mm i.d. [Supelco, Inc., Bellefonte, PA]), was held at 60°C for 2 min and then increased to 200°C at 30°C/min, and then to 280°C at 2°C/min. The MS was controlled and the data analyzed using Vector/Two software (ProLab Resources, Inc., Madison, WI).

Data Analysis. Data were analyzed as two separate groups on the basis of the host species present in the nests of newly mated *P. breviceps* queens. Group 1 compared: (a) newly mated *P. breviceps* queens from nests containing *F. gnava* slaves, (b) newly mated *P. breviceps* queens that had killed a *F. gnava* queen, (c) *F. gnava* queens, (d) and *F. occulta* queens. Group 2 compared: (a) newly mated *P. breviceps* queens from nests containing *F. occulta slaves*, (b) newly mated *P. breviceps* queens that had killed an *F. occulta* queen, (c) *F. occulta* queens, and (d) *F. gnava* queens. For multivariate analysis, it was necessary to preprocess the data. The relative proportions of cuticular hydrocarbons were computed by dividing the area given for each cuticular hydrocarbon by the total integrated peak area of the profile, and then autoscaling each peak to ensure that it had equal weight in the analysis. Principal component analysis (Jolliffe, 1986) was then conducted on 45 normalized variables from Group 1, or on 48 normalized variables

from Group 2. Invariant features were excluded from the principal component analysis, therefore some GC peaks were excluded from the analysis of data sets 1 and 2. In data set 1, one newly mated *P. breviceps* sample and one *F. occulta* sample were deleted because the generalized distance test (Schwager and Margolin, 1982) determined them to be outliers at the 0.01 probability level. In data set 2, one newly mated *P. breviceps* sample, one *F. occulta* sample, and one *F. gnava* sample were also determined to be outliers and were subsequently deleted from the analysis. The multivariate analyses described here were performed by using Pirouette (Infometrix, Woodinville, WA).

RESULTS

Five of the six significant hydrocarbon peaks isolated from the cuticle of newly mated *P. breviceps* queens from nests containing *F. gnava* slaves were determined to be normal alkanes from C25 to C29 (trace of n-C24). The sixth peak was identified as 2-methyl hexacosane. KI data indicate that other 2-methyl even carbon backboned homologues may be present, however, their concentration was inadequate for mass spectral verification. In contrast to the predominance of normal alkanes on the cuticle of newly mated *P. breviceps* queens, the hydrocarbon components of *F. gnava* queens were all methyl or di-methyl branched hydrocarbons (see Table 2). No normal alkanes were detected. Soon after attacking a *F. gnava* queen, the hydrocarbon profile of the newly mated *P. breviceps* queen changed dramatically to one that approximated the profile of a *F. gnava* queen (Figure 1). Mass spectral analysis indicates that the hydrocarbon peaks found on the cuticle of a *P. breviceps* queen that had attacked a *F. gnava* queen contained hydrocarbon components that were identical to those found on host *F. gnava* queens (Table 2, Figure 1).

A plot of the two largest principal components, which account for 58% of the total cumulative variance, for Group 1 (*P. breviceps* associated with *F. gnava*, see Figure 3a) shows that newly mated *P. breviceps* queens were clearly separated from *P. breviceps* queens that had attacked a *F. gnava* queen, *F. gnava* queens, and *F. occulta* queens. *Polyergus breviceps* queens that had attacked a *F. gnava* queen, however, clustered with *F. gnava* queens, indicating hydrocarbon profiles of newly mated *P. breviceps* queens changed after attacks. *Formica gnava* queens killed by *P. breviceps* had variable amounts of the saturated hydrocarbons associated specifically with *P. breviceps* newly mated queens, an indication that there was some reciprocal transfer of cuticular compounds during the interactions between parasite and host queens. *Formica occulta* queens were distinctly separated from the three groups defined above on the second principal component.

The chromatograms of newly mated *P. breviceps* queens reared in nests with *F. occulta* host workers are qualitatively similar to those of queens reared in nests

TABLE 2. COMPOUND STRUCTURES OF PEAKS IN HYDROCARBON PROFILES OF *P. breviceps* QUEENS FROM NESTS WITH *F. gnava* HOST WORKERS THAT EITHER WERE NEWLY MATED OR KILLED A *F. gnava* QUEEN, AND OF *F. gnava* QUEENS

Peak number	Carbon number	Structures	Kovat index	Queens		
				Newly mated *P. breviceps*	*P. breviceps* that killed *F. gnava*	*F. gnava*
1	25	11-;12-MeC_{24}	2425		+[a]	+
2	25	2-MeC_{24}	2462		+	+
3	26	2,10-; 2,12-;2,14-DiMeC_{24}	2492		+	+
4	25	n-C_{25}:0	2500	+		
5	26	9-;11-;13-MeC_{25}	2528		+	+
6	27	9,13-;11,15-DiMeC_{25}	2561		+	+
7	27	5,11-;5,13-;5,15-DiMeC_{25}	2580		+	+
8	27	2,10-;2,12-;2,14-DiMeC_{25}	2595		+	+
9	26	n-C_{26}:0	2600	+		
10	27	10-;11-;12-;13-MeC_{26}	2625		+	+
11	27	2-MeC_{26}	2669	+	+	+
12	28	2,12-;2,14-;2,16-DiMeC_{26}	2697		+	+
13	27	n-C_{27}:0	2700	+		
14	28	9-;11-;13-MeC_{27}	2734		+	+
15	28	n-C_{28}:0	2800	+		+
16	29	n-C_{29}:0	2900	+		

[a] Indicates peak presence.

with *F. gnava* host workers, but the peaks differ in relative amounts (compare Figure 1a with Figure 2a). The cuticular hydrocarbons are composed primarily of normal hydrocarbons (C24 through C29). Minor components, 2-methyl branched hydrocarbons associated with even carbon backbones (C24, C26, C28), are also sometimes present in detectable amounts. The cuticular hydrocarbon profiles of *F. occulta* queens (Figure 2c) are more complex than those of *F. gnava* queens (Figure 1c). Like *F. gnava* queens, however, the cuticular hydrocarbons of *F. occulta* queens are composed exclusively of methyl and di-methyl branched compounds (Table 3). No normal alkanes were detected. Similar to *P. breviceps* queens that attacked a *F. gnava* queen, soon after attacking a *F. occulta* queen, the hydrocarbon profiles of the *P. breviceps* queens changed, mimicking the profile of *F. occulta* queens (Figure 2). Mass spectra of cuticular hydrocarbon peaks of the parasite *P. breviceps* queen after attacking a *F. occulta* queen were identical to

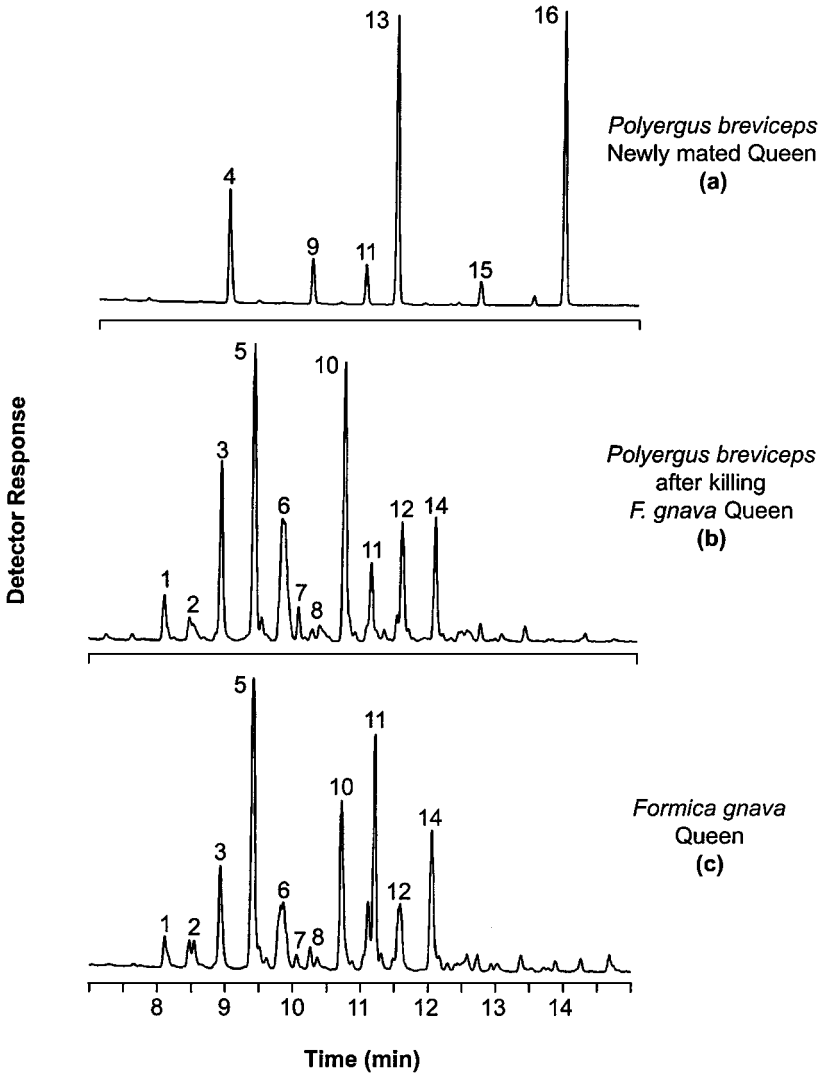


FIG. 1. Representative gas chromatographic profiles of cuticular hydrocarbons from *P. breviceps* and *F. gnava* queens. **(a)** Newly mated *P. breviceps* queen from nest containing *F. gnava* slaves. **(b)** *Polyergus breviceps* queen after attacking a *F. gnava* queen. Note the presence of chemical peaks that correspond to peaks in the profile of a *F. gnava* queen **(c)**. Compare with Figure 2.

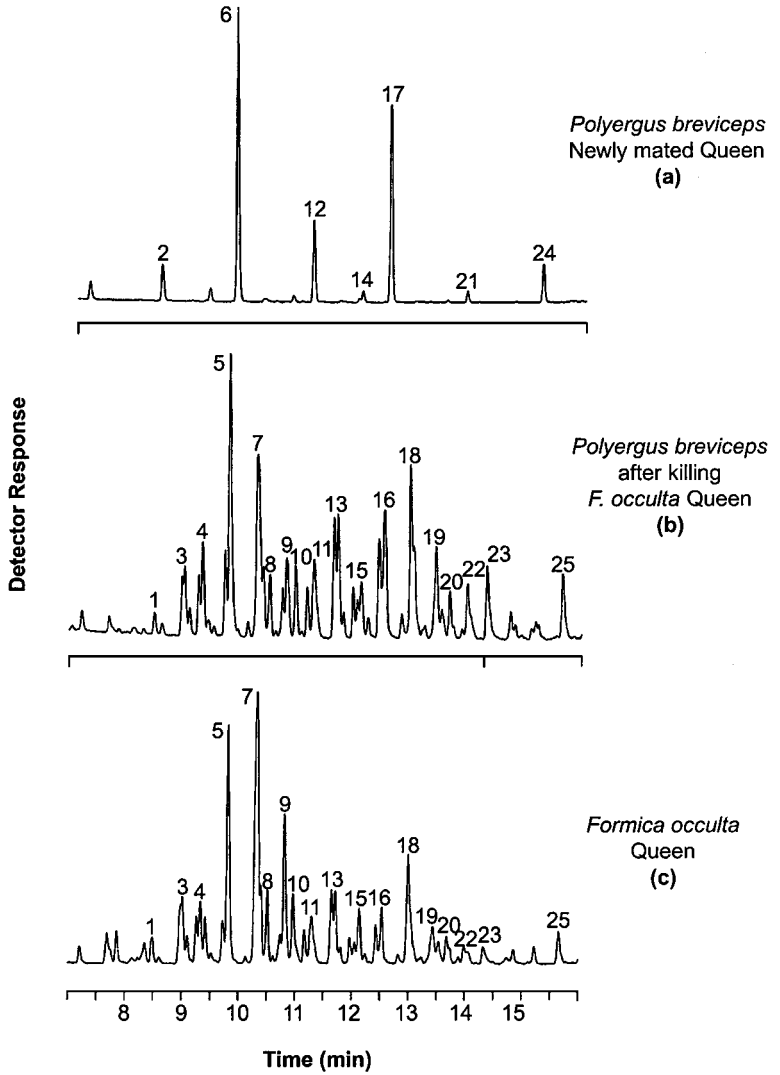


FIG. 2. Representative gas chromatographic profiles of cuticular hydrocarbons from *P. breviceps* and *F. occulta* queens. **(a)** Newly mated *P. breviceps* queen from nest containing *F. occulta* slaves. **(b)** *Polyergus breviceps* queen after attacking a *F. occulta* queen. Note the presence of chemical peaks that correspond to peaks in the profile of a *F. occulta* queen **(c)**. Compare with Figure 1.

TABLE 3. COMPOUND STRUCTURES OF PEAKS IN HYDROCARBON PROFILES OF *P. breviceps* QUEENS FROM NESTS WITH *F. occulta* HOST WORKERS THAT EITHER WERE NEWLY MATED OR KILLED A *F. occulta* QUEEN, AND OF *F. occulta* QUEENS

				Queens		
Peak number	Carbon number	Structures	Kovat index	Newly mated *P. breviceps*	*P. breviceps* that killed *F. occulta*	*F. occulta*
1	25	5,13-;5,15-; 5,17-DiMeC$_{23}$; 2,6-DiMeC$_{23}$	2380–2404		+[a]	+
2	24	n-C$_{24}$:0	2400	+		
3	25	6-;8-;10-;12-MeC$_{24}$	2425		+	+
4	25	4-;2-MeC$_{24}$	2462		+	+
5	26	2,10-; 2,12-;2,14-; 2,16-DiMeC$_{24}$	2492		+	+
6	25	n-C$_{25}$:0	2500	+		
7	26	9-;11-;13-MeC$_{25}$	2528		+	+
8	26	7-MeC$_{25}$	2545		+	+
9	27	7,11-;9,13-;11,15-DiMeC$_{25}$	2561		+	+
10	27	5,11-;5,13-;5,15-;5, 17-;5,19-DiMeC$_{25}$	2580			+
11	27	3,9-;3,11-;3,13-DiMeC$_{25}$	2595–2604		+	+
12	26	n-C$_{26:0}$	2600	+		
13	27	6-;8-;10-; 12-;14-MeC$_{26}$	2625–2631		+	+
14	27	2-MeC$_{26}$:0	2669	+	+	+
15	28	8,12-;10,14-DiMeC$_{26}$	2657–2676		+	+
16	28	2,6-;2,8-;2,10-;2,12-; 2,14-DiMeC$_{26}$	2690–2697		+	+
17	27	n-C$_{27}$:0	2700	+		
18	28	9-;11-;13-MeC$_{27}$	2734		+	+
19	29	7,11-;9,13-;11,15-DiMeC$_{27}$	2768–2776		+	+
20	29	5,12-;5,15-;5,17-; 5,19-DiMeC$_{27}$	2785		+	+
21	28	n-C$_{28}$:0	2800	+		
22	29	3,11-;3,13-;3,15-DiMeC$_{27}$	2802–2810		+	+
23	29	6-;10-;12-;14-MeC$_{28}$	2825		+	+
24	29	n-C$_{29}$:0	2900	+		
25	30	11-;13-;15-MeC$_{29}$	2930		+	+

[a] Indicates peak presence.

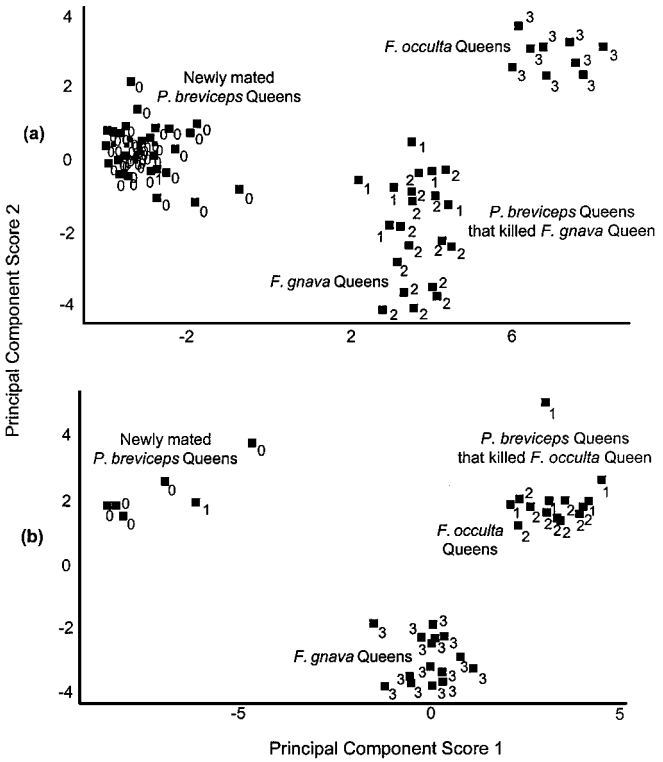


FIG. 3. Principal component maps of cuticular hydrocarbons from *P. breviceps*, *F. gnava*, and *F. occulta* queens. **(a)** 0 = newly mated *P. breviceps* queens from nests containing *F. gnava* slaves, 1 = killer newly mated *P. breviceps* queens from nests containing *F. gnava* slaves, 2 = *F. gnava* queens, 3 = *F. occulta* queens. **(b)** 0 = newly mated *P. breviceps* queens from nests containing *F. occulta* slaves, 1 = killer newly mated *P. breviceps* queens from nests containing *F. occulta* slaves, 2 = *F. occulta* queens, 3 = *F. gnava* queens.

those found on host *F. occulta* queens (Table 3, Figure 2). *Formica occulta* queens killed by *P. breviceps* queens seemed to acquire certain specific hydrocarbons associated with *P. breviceps* queens during the conflict, thus confirming again that there was some mutual transference of cuticular compounds.

A plot of the two largest principal components, which account for 61% of the total cumulative variance, for Group 2 (*P. breviceps* associated with *F. occulta*, see Figure 3b), shows that newly mated *P. breviceps* queens are well separated from newly mated *P. breviceps* queens that had just killed an *F. occulta* queen, *F. occulta* queens, and *F. gnava* queens. *Polyergus breviceps* queens that killed a *F. occulta* queen clustered among *F. occulta* queens. *Formica gnava* queens were distinctly separated from these groups.

DISCUSSION

Chemical Camouflage and Adoption of the Polyergus Queen. *Polyergus breviceps* queens, in taking over a *Formica* colony, must be accepted by adults that have not been exposed previously to her odor. It is clear from initial *Formica* worker aggression directed at *P. breviceps* queens invading a nest, that *P. breviceps* queens are not naturally candidates for adoption. The qualitative differences that we found in hydrocarbons between newly mated *P. breviceps* queens and host *Formica* queens was, therefore, none too surprising and indicate that *P. breviceps* and its host species have not co-evolved such that queen cuticular hydrocarbon profiles, and probably other relevant chemical profiles, are inherently similar. Instead, integration of parasite queen among host workers seems to require several diverse tactics, one of which is chemical camouflage. *Polyergus* queens first ward off attackers with secretions from the Dufour's gland while attacking the resident queen (Topoff et al., 1988; D'Ettorre et al., 2000; Mori et al., 2000 a,b). However, soon after the attack on the host queen ceases, host workers aggregate around the parasite queen and begin grooming her. Sampling of the cuticular hydrocarbons from newly mated queens and from attackers, just as attacks were completed, indicated that the newly mated queen profiles transformed dramatically, containing at first simple hydrocarbons and then harboring complex methyl and di-methyl branched hydrocarbons. These new profiles were virtually identical both in peak quality and relative proportion to the queen profiles of the species of queen attacked, a result similar to that discovered by Errard and D'Ettorre (1998) for *P. rufescens.* This, concomitant with the rapidity of the chemical change and modification in host worker behavior, suggests that the emergence of new chemical components (adoptive properties?) is not due to a biosynthetic change triggered by the aggressive interaction, at least initially, as has been proposed for other social parasites (Bagnères et al. 1996). Of course, only tracing the movement of labeled hydrocarbons could demonstrate chemicals were indeed transferred. It should be furthermore noted that, if this is a system of chemical transfer, it is not perfect. In two instances, profiles of *P. breviceps* queens that had killed a *Formica* queen (one *F. gnava* and one *F. occulta*) did not cluster with the respective *Formica* species, but clustered with newly mated *P. breviceps* (Figures 3a and b).

In the socially parasitic wasp, *Polistes atrimandibularis* Zimmermann, the profile change does undoubtedly seem to be the result of a change in metabolic pathway in addition to adsorption of host chemicals (Bagnères et al., 1996). Over a period of several months, unsaturated hydrocarbons of *P. atrimandibularis* queens that have invaded a nest of *Polistes biglumis bimaculatus* Geoffroy disappear from the parasite cuticle and are replaced with the saturated products of the host species. Generally, the invasion by *P. atrimandibularis* is a passive one and seems to be restricted to usurping incipient nests of its host. This is unlike the strategic invasion by queens of the socially parasitic wasp *Polistes sulcifer*

Zimmermann, which take over nests by expelling or killing the dominant queen of its host species, *Polistes dominulus* (Christ) (Turillazzi et al., 1990). Recently, it was demonstrated that *P. sulcifer* queens also change their chemical profile, resembling the host queen almost completely within 3 days (Turillazzi et al., 2000). The stroking and prolonged licking of host individuals suggest, furthermore, that *P. sulcifer* achieves host chemical similarity by obtaining chemicals directly from the host.

While the acquisition of new cuticular components by newly mated *P. breviceps* queens that have attacked a *Formica* queen is readily explainable, the apparent rapid loss of the n-alkanes in such a short time period is not. Most likely, the abundance of newly acquired material has overwhelmed the original components. Our data, however, were insufficient to determine if this indeed is the case. In the above mentioned species that undergo similar profile changes, pre-invasion or pre-attack profile components are also lost. Although the loss of particular components in these instances takes admittedly longer, one peak in the profile of *P. sulcifer* does show substantial decrease after only 90 min (Turillazzi et al., 2000).

Integration by Other Slave-Maker Species. Queens of other socially parasitic ant species also appear to infiltrate their host species colony by camouflaging themselves with host chemicals. For example, the slave-maker queen, *Leptothorax kutteri* Buschinger, aggressively grooms the host *Leptothorax acervorum* (Fabricius) queen and workers upon invading their nest, resulting in a chemical profile very similar to that of *L. acervorum*. Experimentally preventing a *L. kutteri* queen from grooming host individuals results in aggressive attacks, suggesting that a chemical transfer takes place and that this allows invading queens to become adopted by host workers (Franks et al., 1990). Some dependent colony founding queens, however, do not need to be adopted by adult host workers in order to establish a new colony. The facultative slave-maker, *Formica wheeleri* Creighton, usurps a *F. occulta* nest by invading a nest, inducing the adult inhabitants to flee, and appropriating the brood left behind (Topoff et al., 1990). The workers that emerge from the orphaned brood adopt the slave-maker queen and eventually rear her offspring. Presumably, these newly emerged workers, having been exposed to the *F. wheeleri* queen during early periods of development (Morel, 1983; Errard, 1984, Morel et al., 1988) or soon after eclosion (see Vander Meer and Morel, 1998), incorporate her odor into their nestmate recognition odor template and, thereby, accept her. A preliminary examination of cuticular hydrocarbon data revealed that the queen profiles of *F. wheeleri* and *F. occulta* were not similar, even after a new queen had been residing with *F. occulta* workers for one year (Johnson, 2000). These results promote the contention that the chemical change in *P. breviceps* is not fortuitous.

Newly Mated Queen Profiles. Acquisition of chemicals from a host species queen could facilitate integration of the *P. breviceps* queens among host workers, as well as allow the parasite queen to solicit the particular attention workers often display towards reproductive individuals. Nonetheless, the newly mated

P. breviceps profile should not be dismissed as inconsequential. The cuticular hydrocarbon profiles of *P. breviceps* queens here were clearly simple relative to the profiles of the host species queens. However, the parasite queen profiles were also qualitatively identical to the pupae hydrocarbon profiles of both *F. gnava* and *F. occulta* (Johnson, 2000; Johnson & Vander Meer unpublished data), but differed from pupae profiles of *P. breviceps*! The peaks in profiles of newly mated *P. breviceps* associated with *F. gnava* were, furthermore, of similar relative proportions. Others have noted previously that newly mated *Polyergus rufescens* queens (D'Ettorre et al., 2000) and callows belonging to other species (Lenoir et al., 1999) lack a chemical profile. This "chemical insignificance" is suggested to facilitate integration by minimizing aggression and allowing individuals to acquire the appropriate chemical suit (Lenoir et al., 2001). In the case of newly mated *P. breviceps* gynes, however, it seems that rather than being chemically insignificant, their pupa-like profile may be significant, having similar consequences as those gained by chemically insignificant profiles, such as diminishing aggressive reactions upon invading a nest and facilitating movement towards the host queen.

Visual inspection of hydrocarbon patterns of newly mated *P. breviceps* queens has revealed an additional interesting result. Hydrocarbon components of newly mated *P. breviceps* queens from nests with *F. gnava* slaves differed consistently in relative proportions from newly mated *P. breviceps* queens collected from nests with *F. occulta* slaves (compare Figure 1a with Figure 2a). Because *P. breviceps* are obligatorily dependent on their host species for survival, rearing *P. breviceps* without some chemical influence from their *Formica* host species seems unlikely. However, the clear differences in hydrocarbon profiles of *P. breviceps* female alates and their adult hosts are indicative of minimal chemical interaction. Thus, the consistent differences in cuticular hydrocarbon profiles of newly mated *P. breviceps* queens associated with the two host species could reflect (a) different environmental conditions in each host nest or (b) the emergence of two host races or species. It would be unduly worthwhile to clarify the relationship between these two populations of *P. breviceps.*

Previous Studies on Polyergus Worker Profiles. Not surprisingly, there are some similarities and differences in what we and Errard and D'Ettorre (1998) found with *Polyergus* queen profiles (and the respective changes) and what others have found examining worker profiles of *Polyergus* and other slave-makers and their hosts. The profile from workers of the Japanese slave-maker ant, *Polyergus samurai* Yano, was found to be qualitatively and quantitatively identical to the profile of their host species, *Formica japonica* Motschulsky (Yamaoko, 1990). Differences among colonies and sites for both parasite and host suggested that *P. samurai* synthesize very little of their own cuticular hydrocarbons, receiving the majority from the host species. Habersetzer and Bonavita-Cougourdan (1993) and Habersetzer (1993), on the other hand, found the European slave-maker, *Polyergus rufescens,* to maintain some species specificity in their cuticular

hydrocarbon profiles by retaining five major peaks not found in the profiles of their *Formica cunicularia* host. They also found that the *Formica* lost some of their colony characteristics, although this may be a confounding result of sampling workers from a mixed species nest. Bonavita-Cougourdan et al. (1996, 1997) also found *P. rufescens* to maintain some semblance of specificity by retaining compounds that were not on either of their two potential host species, *F. cunicularia* and *Formica rufibarbis* Fabricius. Their results, however, indicate a complex system of profile convergence that does not involve the transfer of chemicals across species. Instead, the proportions of cuticular hydrocarbons that are shared and normally synthesized by each species are adapted such that the other species profiles are rivaled. *Polyergus,* in the forefront, adjusted its profile the most, but the profile modification by both *Formica* species made them distinct from other monospecific, conspecific colonies.

One might expect similar results from another prominent slave-maker, *Harpagoxenus sublaevis* Nylander. However, *H. sublaevis,* which sometimes maintains two species of *Leptothorax* simultaneously, does not produce their own specific hydrocarbons (Kaib et al.,1993) but acquires constituents through allogrooming. Hence, they contribute nothing to the profiles of their host species. The two *Leptothorax* species, however, modify each other's signatures when both are in a single *H. sublaevis* nest (Kaib et al., 1993).

Differences in integration mechanisms of workers and slave-maker queens that usurp nests of worker adults are not surprising. Typically, both parasite and host workers in slave-maker nests are exposed to the nestmate recognition cues of the other species as immatures or callows, when much of the nestmate recognition template is being formed (see Jaisson 1975a,b; Le Moli and Passetti, 1977, 1978; Le Moli, 1980; Le Moli and Mori, 1982, 1987; Morel et al., 1988). They, therefore, need not rely on camouflage to be accepted by the other species. *Polyergus* queens as dependent colony founders, on the other hand, need an immediate chemical armor.

Acknowledgments—We are grateful to Michele Hosack (United States Department of Agriculture, Center for Medical and Veterinary Entomology) for her help with the gas chromatography and to the staff and volunteers of the Southwestern Research Station of the American Museum of Natural History where the fieldwork was conducted. Early versions of this manuscript were greatly improved by comments from Howard Topoff (Hunter College) and James Carpenter (American Museum of Natural History). We also thank two anonymous reviewers for their constructive comments. The research was aided by grants from the Animal Behaviour Society, Sigma Xi, and the Theodore Roosevelt Memorial Fund and by a fellowship from the Biopsychology subprogram at Hunter College to C.A. Johnson.

REFERENCES

BAGNÈRES, A. G., LORENZI, M. C., DUSTICIER, G., TURILLAZZI, S., and CLÈMENT, J. C. 1996. Chemical usurpation of a nest by paper wasp parasites. *Science* 272:889–892.

BONAVITA-COUGOURDON, A., BAGNÈRES, A. G., PROVOST, E., DUSTICIER, G., and CLÈMENT, J. L. 1997. Plasticity of the cuticular hydrocarbon profile of the slave-making ant *Polyergus rufescens* depending on the social environment. *Comp. Biochem. Physiol. B* 116:287–302.

BONAVITA-COUGOURDON, A., CLÈMENT, J. L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus* (Scop). *J. Entomol. Sci.* 22(1):1–10.

BONAVITA-COUGOURDAN, A., RIVIÈRE, G., PROVOST, E., BAGNÈRES, A. G., ROUX, M., DUSTICIER, G., and CLÈMENT, J. L. 1996. Selective adaptation of the cuticular hydrocarbon profiles of the slave-making ants *Polyergus rufescens* Latr. and their *Formica rufibarbis* Fab. and *F. cunicularia* Latr. slaves. *Comp. Biochem. Physiol. B* 113:313–329.

D'ETTORRE, P., ERRARD, C., IBARRA, F., FRANCKE, W., and HEFETZ, A. 2000. Sneak in or repel your enemy: Dufour's gland repellent as a strategy for successful usurpation in the slave-maker *Polyergus rufescens. Chemoecology* 10:135–142.

ERRARD, C. 1984. Evolution, en fonction de l'âge, des relations sociales dans les colonies mixtes heterospecifiques chez des fourmis des genres *Camponotus* et *Pseudomyrmex. Insectes Soc.* 31:185–194.

ERRARD, C., and D'ETTORRE, P. 1998. Camouflage chimique chez la reine de *Polyergus rufescens* lors de la fondation. *Actes Coll. Insectes. Soc.* 11:137–144.

FRANKS, N., BLUM, M., SMITH, R. K., and ALLIES, A. B. 1990. Behavior and chemical disguise of the cuckoo ant *Leptothorax kutteri* in relation to its host *Leptothorax acervorum. J. Chem. Ecol.* 16:1431–1444.

HABERSETZER, C. 1993. Cuticular spectra and inter-individual recognition in the slave-making ant *Polyergus rufescens* and the slave species *Formica rufibarbis. Physio. Entomol.* 18:167–175.

HABERSETZER, C., and BONAVITA-COUGOURDAN, A. 1993. Cuticular spectra in the slave-making ant *Polyergus rufescens* and the slave species *Formica rufibarbis. Physio. Entomol.* 18:160–166.

JACKSON, L., and BLOMOQUIST, G. 1976. Insect waxes, pp. 201–233, *in* P. E. Kolattuduy (ed.). Chemistry and Biochemistry of Natural Waxes. Elsevier, Amsterdam.

JAISSON, P. 1975a. Comment des formis apprennent à soigner leur progèniture. Recherche 6:680–683.

JAISSON, P. 1975b. L'impregnation dans l'ontogenèse du comportement de soins aux cocons chez la jeune Fourmi rousse (*Formica polyctena* Först.). Behaviour 52:1–37.

JOHNSON, C. A. 2000. Mechanisms of dependent colony founding in the slave-maker ant, *Polyergus breviceps* Emery (Hymenoptera: Formicidae). PhD Dissertation. The Graduate School and University Center of the City University of New York, New York.

JOLLIFFE, T. 1986. Principal Component Analysis. Springer-Verlag, New York.

KAIB, M., HEINZE, J., and ORTIUS, D. 1993. Cuticular hydrocarbon profiles in the slave-making ant *Harpagoxenus sublaevis* and its hosts. *Naturwissenschaften* 80:281–285.

LAHAV, S., SOROKER, V., and HEFETZ, A. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften* 86:246–249.

LE MOLI, F. 1980. On the origin of slaves in dulotic ant societies. *Boll. Zool.* 47:207–212.

LE MOLI, F., and MORI, A. 1982. Early learning and cocoon nursing behaviour in the red wood ant *Formica lugubris* Zett. (Hymenoptera: Formicidae). *Boll. Zoo.* 49:93–97.

LE MOLI, F., and MORI, A. 1987. Early social experience affects worker and cocoon discrimination in a slave ant species, p. 578, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, München.

LE MOLI, F., and PASSETTI, M. 1977. The effect of early learning on recognition, acceptance and care of cooons in the ant *Formia rufa L. Atti Soc. ital. Sci. nat. Museo civ. Stor. nat. Mil.* 118:49–64.

LE MOLI, F., and PASSETTI, M. 1978. Olfactory learning phenomena and cocoon nursing behavior in the ant *Formica rufa. Boll. Zoo.* 45:389–397.

LENOIR, A., D'ETTORRE, P., ERRARD, C., and HEFETZ, A. 2001. Chemical ecology and social parasitism in ants. *Ann. Rev. Entomol.* 46:573–599.

LENOIR, A., FRESNEAU, D., ERRARD, C., and HEFETZ, A. 1999. Individuality and colonial identity in ants: The emergence of the social representation concept, pp. 219–237, *in* C. Detrain, J. L. Deneubourg, and J. M. Pasteels (eds.). Information Processing in Social Insects. Birkhauser Verlag, Basel.

MOREL, L. 1983. Relation entre comportement agressif et privation sociale prècoce chez les jeunes immatures de la Fourmi *Camponotus vagus* Scop. *Comp. Ren. Acad. Sci. D*, (Paris) 296:449–452.

MOREL, L., VANDER MEER, R. K., and LAVINE, B. K. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*): Behavioral and chemical evidence for the role of age and social experience. *Behav. Ecol. Socio.* 22:175–183.

MORI, A., D'ETTORRE, P., and LE MOLI, F. 1995. Host nest usurpation and colony foundation in the European amazon ant, *Polyergus rufescens* Latr. (Hymenoptera: Formicidae). *Insectes Soc.* 42:279–286.

MORI, A., GRASSO, D., VISICCHIO, R., and LE MOLI, F. 2000a. Colony founding in *Polyergus rufescens:* The role of the Dufour's gland. *Insectes Soc.* 47:7–10.

MORI, A., VISICCHIO, R., SLEDGE, M. F., GRASSO, D. A., LE MOLI, F., TURILLAZZI, S., SPENCER, S., and JONES, G. R. 2000b. Behavioural assays testing the appeasement allomone of *Polyergus rufescens* queens during host-colony usurpation. *Ethol. Ecol. Evol.* 12:315–322.

NOWBAHARI, E., LENOIR, A., CLÈMENT, J. L., LANGE, C., BAGNÈRES, A. G., and JOULIE, C. 1990. Individual, geographical, and experimental variation of cuticular hydrocarbons of the ant *Cataglyphis cursor* (Hymenoptera: Formicidae): Their use in nest and subspecies recognition. *Biochem. Syst. Ecol.* 18:63–73.

OBIN, M.S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *J. Chem. Ecol.* 12:1965–1975.

SCHWAGER. S. J., and MARGOLIN, B. H. 1982. Detection of multivariate normal outliers. *Annu. Stat.* 10:943–953.

SOROKER, V., VIENNE, C., BILLEN, J., and HEFETZ, A. 1994. The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger. Naturwissenschaften* 81:510–513.

SOROKER, V., VIENNE, C., and HEFETZ, A. 1995. Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera: Formicidae). *J. Chem. Ecol.* 21:365–378.

TOPOFF, H., COVER, S., GREENBERG, L., GOODLOE, L., and SHERMAN, P. 1988. Colony founding by queens of the obligatory slave-making ant, *Polyergus breviceps:* The role of the Dufour's gland. *Ethology* 78:209–218.

TOPOFF, H., WEICKERT, T., and ZIMMERLI, E. 1990. A comparative study of colony takeover between queens of facultative and obligatory slave-making ants (Hymenoptera: Formicidae). *J. Insect Behav.* 3:813–817.

TOPOFF, H., and ZIMMERLI, E. 1993. Colony takeover by a socially parasitic ant, *Polyergus breviceps:* The role of chemicals obtained during host-queen killing. *Anim. Behav.* 46:479–486.

TURILLAZZI, S., CERVO, R., and CAVALLARI, I. 1990. Invasion of the nest of *Polistes dominulus* by the social parasite *Sulcopolistes sulcifer* (Hymenoptera, Vespidae). Ethology 84:47–59.

TURILLAZZI, S., SLEDGE, M. F., DANI, F. R., CERVO, R., MASSOLO, L., and FONDELLI, L. 2000. Social hackers: Integration in the host chemical recognition system by a paper wasp social parasite. *Naturwissenschaften* 87:172–176.

VANDER MEER, R. K., JOUVENAZ, D. P., and WOJCIK, D. P. 1989. Chemical mimicry in a parasitoid (Hymenoptera: Eucharitidae) of fire ants (Hymenoptera: Formicidae). *J. Chem. Ecol.* 15:2247–2261.

VANDER MEER, R. K., and MOREL, L. 1998. Nestmate recognition in ants, pp. 79–103, *in* R. K. Vander Meer, M. Breed, M. Winston, and K. E. Espelie (eds.). Pheromone Communication in Social Insects. Westview Press, Boulder.

VANDER MEER, R. K., and WOJCIK, D. P. 1982. Chemical mimicry in the myrmecophilous beetle *Myrmecaphodius excavaticollis. Science* 218:806–808.

VIENNE, C., SOROKER, V., and HEFETZ, A. 1995. Congruency of hydrocarbon patterns in heterospecific groups of ants: Transfer and/or biosynthesis? *Insectes Soc.* 42:267–277.

WAGNER, D., BROWN, M. F., BROUN, P., CUEVAS, W., MOSES, L. E., CHAO, D. L., and GORDON, D. M. 1998. Task-related differences in the cuticular hydrocarbon composition of harvester ants, *Pogonomyrmex barbatus. J. Chem. Ecol.* 24:2021–2037.

WHEELER, W. M. 1906. On the founding of colonies by queen ants, with special reference to the parasitic and slave-making species. *Bull. Amer. Mus. Nat. Hist.* 22:33–105.

YAMAOKA, R. 1990. Chemical approach to understanding interactions among organisms. *Physio. Ecol.* (Japan) 27:31–52.

ZAAYER, P. M. 1967. Paarung und koloniegründung von *Polyergus rufescens* Latr. im kunstnest (Hymenoptera: Formicidae). *Zeit. Arbeits. öster. Entom.* 19:1–9.

ZIMMERLI, E., and TOPOFF, H. 1994. Queens of the socially parasitic ant *Polyergus* do not kill queens of *Formica* that have not formed colonies (Hymenoptera: Formicidae). *J. Insect Behav.* 7:119–121.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

TASK-RELATED ENVIRONMENT ALTERS THE CUTICULAR HYDROCARBON COMPOSITION OF HARVESTER ANTS

DIANE WAGNER,[1,*] MADELEINE TISSOT,[2,†] and DEBORAH GORDON[2]

[1]*Department of Biological Sciences*
University of Nevada
Las Vegas, Nevada 89154-4004, USA

[2]*Department of Biological Sciences*
Stanford University
Stanford, California 94305-5020

(Received January 30, 2001; accepted May 6, 2001)

Abstract—Within a colony of harvester ants (*Pogonomyrmex barbatus*), workers in different task groups differ in the hydrocarbon composition of the cuticle. Foragers and patrollers, which spend extended periods of time outside the nest, have a higher proportion of saturated, unbranched hydrocarbons (*n*-alkanes) on the cuticle than nest maintenance workers, which spend only short periods of time outside the nest. We tested whether these task-related differences in ant cuticular chemistry arise from exposure to conditions outside the nest. Nest maintenance workers experiencing daily, short-term outside exposure developed a higher proportion of *n*-alkanes on the cuticle than workers kept inside the lab. Independent manipulations of ultraviolet radiation, relative humidity, and temperature revealed that only the combination of high temperature (ca. 38°C) and low relative humidity (ca. 8%) increased the proportion of cuticular *n*-alkanes. The results indicate that warm dry conditions, such as those encountered when an ant leaves the nest, trigger changes in cuticular chemistry.

Key Words—Cuticular hydrocarbons, Formicidae, *Pogonomyrmex barbatus*, *n*-alkanes, task.

INTRODUCTION

A social insect colony performs many tasks, including foraging, nest construction, and feeding the young. In many social insect species, workers are not specialized

*To whom correspondence should be addressed. E-mail: dwagner@ccmail.nevada.edu
†Current address: Department of Insect Biology, University of California, Berkeley, California 94720-5020.

0098-0331/01/0900-1805$19.50/0

to perform a particular task but change tasks throughout their lifetimes (Gordon, 1996; Franks et al., 1997). Typically, the tasks performed early in life take place entirely inside the nest, such as care of the queen and brood, while tasks that require leaving the nest are performed later in life (Wilson, 1971).

Workers of the harvester ant, *Pogonomyrmex barbatus* (F. Smith), perform four tasks outside the nest: nest maintenance, midden work, patrolling, and foraging (Gordon, 1986). Nest maintenance workers work mostly inside the nest, leaving the nest on brief trips lasting less than a minute (personal observation) to remove bits of soil and rock from the nest. Midden workers arrange organic debris on the nest mound. Patrollers choose the day's foraging location and respond to disturbances. Foragers find and retrieve food items. In contrast to nest maintenance, foraging and patrolling require a considerable amount of time outside the nest. A single trip by a forager averages 23 min, with a range of 2–165 min (Gordon and Kulig, 1996). It appears that a worker progresses from nest maintenance to foraging. Nest maintenance workers will switch to foraging when new food sources become available (Gordon, 1989). This transition occurs in one direction only: once a worker switches to foraging, it does not switch back to nest maintenance. New nest maintenance workers, if needed, are recruited from among the pool of interior workers (Gordon, 1989).

The transition to exterior work introduces harvester ants to new and extreme physiological challenges. *Pogonomyrmex barbatus* occupies arid regions of North America. Colonies are active during the warm months of the year, from March to November. Work outside the nest occurs in the daylight hours (Gordon, 1986), and soil temperatures during periods of activity are often very high (Whitford, 1976). In comparison to interior workers, exterior workers experience higher temperature, lower humidity, and direct solar radiation.

Caste differences in cuticular hydrocarbon composition have been reported for several social insect species (Howard et al., 1982; Smith and Taylor, 1990; Bonavita-Cougourdan et al., 1993; Haverty et al., 1996; Kaib et al., 2000). Previous work on *P. barbatus* demonstrated that foragers and patrollers have higher proportions of *n*-alkanes, relative to branched alkanes and alkenes, than nest maintenance workers (Wagner et al., 1998). Because harvester ant foragers and patrollers spend more time outside the nest than nest maintenance workers, the higher proportion of saturated, unbranched hydrocarbons on the cuticle might be a response to the warmer, drier conditions they face. Another possibility is that both task and cuticular composition change independently with age. The purpose of this study was to test the hypothesis that exposure to external conditions (ultraviolet light, warm temperature, low humidity, or some combination of these factors) causes an increase in the percentage of *n*-alkanes on the cuticle.

METHODS

Experiments were conducted at the Southwestern Research Station of the American Museum of Natural History in Portal, AZ, USA. In each experiment we exposed *P. barbatus* nest maintenance workers from 3 colonies to various combinations of the environmental conditions they would be likely to encounter outside the nest. Nest maintenance workers were collected as they left the nest carrying soil in their mandibles. The colonies from which we collected workers were large and appeared mature (5 years or older, Gordon, 1995); the nest mound of each was >1 m in diameter. In the laboratory, the workers from each colony were placed into a plastic box and provided with an aluminum foil shelter, a source of moisture, and an unlimited supply of 20% sucrose solution. On the day an experiment was to begin, a random sample of workers from each colony was killed by placing them in a freezer at -80°C. These ants were used to determine the initial cuticular hydrocarbon composition of the nest maintenance workers.

Exposure to Outside Conditions. Ants in this experiment were subjected to one of two treatments: (1) 50 min periods of exposure to outdoor conditions or (2) no exposure to outdoor conditions. Nest maintenance workers were collected from colonies S1, S6, and S7. Workers were randomly assigned to treatment and holding container. Holding containers were polystyrene dishes with plaster floors and gently sloping sides to prevent shadows on the floor of the dish when placed in the sunlight. The sides of the holding containers were coated with Fluon (Northern Products Inc., Woonsocket, RI) to prevent ants from escaping. There were 3 holding containers per treatment-colony combination, and 1–4 ants per holding container. The plaster was moistened daily with approximately 3–5 ml of water. Except during periods of active exposure, ants were provided with sucrose solution and each holding container was kept covered with a individual plastic plate.

Each morning thereafter between 1000 and 1130, food and shelters were removed from all containers. The lids were then replaced on the containers holding the indoor control ants. Ants to be exposed were carried outside in their open containers and placed on a level surface in direct sunlight for 50 min. The duration of exposure was chosen to be similar to that of two average foraging trips (about 23 min each, Gordon and Kulig, 1996). The positions of containers were rotated from day to day to prevent positional effects. After exposure, ants were returned to the lab and food and shelters were restored to all containers. Ants of the indoor control group were never put outside. Twenty days from the beginning of the experiment, all treatment and control ants were killed, by placing them into a -80°C freezer, and stored for later chemical analysis.

To measure the environmental conditions experienced by ants in the two treatments, we monitored temperature and relative humidity (RH) in holding containers that lacked ants, but that were treated in the same way as containers that

did hold ants (plaster was watered, food changed, and dishes covered as usual). We had to monitor empty containers because ants could escape by climbing onto the temperature probe. We placed two empty containers outdoors during each exposure trial and monitored temperature and humidity in them every few minutes. Temperature was measured on the surface of the plaster using a thermocouple thermometer equipped with a surface probe (Hanna Instruments, Woonsocket, RI). Temperature and RH 1 cm above the plaster surface were measured using a thermohygrometer (Oakton, Forestry Suppliers, Jackson MS #76001). Two additional empty containers were maintained inside the laboratory with the control ants. Once each day, we measured surface temperature, air temperature, and humidity inside the closed containers by inserting probes through doors in the plastic lids.

Ultraviolet Light Manipulation. We manipulated the level of exposure to ultraviolet (UV) light by placing ants outside under canopies constructed of filters that transmitted either low or high levels of UV, while monitoring temperature and humidity to assure that they did not vary among UV treatments. Low-UV canopies were constructed from plastic sheets with transmission of 0% at wavelengths less 380 nm (Lee Filters, Hampshire, UK #226). High-UV canopies were constructed of sheets with 90% transmission at 380 nm, 85% transmission at 350 nm, and 0% transmission at wavelengths less than 310 nm (Lee Filters #226). Canopies were constructed on $50 \times 50 \times 50$ cm frames made of dowels. The filter material was secured across the top and extended out 25 cm to each side. During exposure periods (1030 to 1200), the sun was high overhead and no direct sunlight reached the floor of the canopy without first passing through the filter. Reflected UV light from indirect sources could enter from the sides.

Nest maintenance workers from colonies I, K, and M were randomly assigned to high or low UV exposure, and to one of two holding containers per colony-treatment combination (1–4 ants per holding container). Each morning thereafter, ants were placed under the appropriate canopy for 50 min. All ants experienced a brief period of exposure to UV light, lasting approximately 5–10 sec, as they were carried from the laboratory to the canopies. When not underneath the canopies, ants were kept covered in the laboratory. After 11 days of daily treatments, ants were killed and stored at -80°C.

Temperature and humidity measurements were taken from two containers per treatment that lacked ants, as described above. These containers were moved outside and inside along with the ant containers. We took multiple measurements of temperature and RH from each dish while the ants were outside, and one measurement per day of conditions inside closed containers in the laboratory.

Temperature and Humidity Manipulations. We manipulated temperature and humidity in a 2×2 factorial experiment conducted in the laboratory. Each experimental treatment combination was administered in a plastic chamber, $50 \times 35 \times 20$ cm (length $\times$ width $\times$ depth) in size. Two chambers, one maintained at high

humidity and one at low, sat on a silicone rubber-laminated heating mat attached to a temperature controller (Thermolyne, Dubuque, IO), and two chambers sat on the desktop at room temperature. We defined high temperature as 35–40°C, moderate temperature as room temperature (25–30°C), high humidity as 95–100% RH, and low humidity as 10–30% RH. We created high RH in two chambers by sprinkling approximately 100 ml of water onto the chamber floors, and created low RH by sprinkling approximately 100 ml of desiccant crystals (Drierite, Fisher #07–577) on the floors in a similar manner. Desiccant crystals were regenerated and replaced daily. Chambers were cleaned and dried daily. We rotated chambers through treatments to prevent confounding chamber with treatment effects.

Ants from colonies I, J, and K were assigned randomly to treatment and holding containers. There were 1–5 ants per holding container and 2 holding containers for each treatment-colony combination. Chambers were prepared each morning and were considered ready to use when the temperature in the "warm" chambers was approximately 40°C, the relative humidity in the "dry" chambers was 10–20%, and the RH in the "moist" chambers was 90–100%. Containers holding ants were placed into the chambers between 1000 and 1130 hours. There was an equilibration period, lasting 5–10 min, during which chambers were returned to the target temperature and humidity. Ants were then kept in the chambers for another 50 min. Air temperature and RH inside the chambers were measured by inserting probes into small holes in the chamber walls. Whenever the probe was withdrawn, rubber stoppers were quickly inserted into the holes. The experiment continued for 11 days, after which all ants were killed and stored at −80°C.

Chemical Analysis. Chemical analyses were conducted at Stanford University. Frozen ants were transported to Stanford University on dry ice and stored at −80°C. Methods of extraction and analysis are described in detail in Wagner et al. (1998). We extracted the cuticular lipids from each ant separately by immersion in 1 ml of pentane. The mixture was gently shaken during the first minute of soaking. After 10 min soaking, the fluid was transferred to a clean vial and dried under a stream of N_2. The residue was dissolved in 50 μl of chloroform. Aliquots of 1 μl were introduced by splitless injection onto a SPB-1 fused silica capillary column (30 m, 0.25 mm ID, 0.5 μm film thickness; Supelco, Bellefonte, Pennsylvania); samples were purged after 1 min. The carrier gas was He, flowing at 1 ml/min. The injector was maintained at 300°C. The oven was set at 170°C during injection, raised quickly to 220°C at 25°C/min, then more slowly to 300°C at 3°C/min. Samples of a standardized mixture of hydrocarbons were interspersed among ant samples to confirm the consistency of elution times.

We identified unknown compounds, and confirmed the identity of peaks with retention times and relative positions known from previous work, by analyzing the mass spectra of peaks from a small sample of ants frozen at the time of collection. Lipids were extracted from 3–5 ants from each experiment and the extracts treated as described above. Approximately 2–4 μl were injected onto a capillary column

(DB-1, J&W Scientific, Folsom, CA, USA) and analyzed on an HP 5890/5970 GC/MS. Instrument temperatures and run times were the same as above, except the oven was raised to a final temperature of 310°C. We used Nelson et al. (1980 and *in press*) as references when identifying compounds. For each experiment, we used only those compounds that composed at least 0.5% of the total ion abundance in the data analysis.

Data Analysis. The dependent variable for all statistical analyses was the proportion of *n*-alkanes in the total ion abundance for each ant. Data were arcsine square root-transformed when necessary to meet the assumption of normality. For each experiment, we tested the effect of treatment and colony on proportional abundance of *n*-alkanes using a two-way factorial analysis of variance. For the outside exposure and UV manipulation experiments, we compared the average daily temperature and RH experienced by ants in the two treatments of each experiment using paired *t*-tests.

RESULTS

Chemical Analysis. We found between 21 and 30 cuticular lipid compounds on individual ants in the three experiments. All but two of these compounds had been identified in previous studies (Wagner et al., 1998; Wagner et al., 2000). Compounds included normal alkanes, mono- and di-methylbranched alkanes, alkenes, and esters. We were unable to identify two novel compounds found in trace amounts (each constituted $<1\%$ on average) on ants in the UV exposure experiment. The mass spectra of these unknown compounds indicated that they were not *n*-alkanes. No novel compounds were produced in response to experimental manipulation; rather, changes in *n*-alkane abundance reflected changes in the relative abundance of common compounds.

Exposure to Outside Conditions. During periods of exposure, ants placed outside experienced significantly higher temperatures and lower RH than those kept inside. The mean (range) conditions in containers during exposure and inside containers within the laboratory were as follows: air temperature 34.3°C (25.0–41.0) outside and 25.1°C (21.1–28.5) inside (t_{19}, paired by day, $= -11.6$, $P < 0.0001$); surface temperature 27.0°C (21.0–30.2) outside and 24.6°C (21.0–26.9) inside (paired $t_{19} = -6.2$, $P < 0.0001$); RH 42.4% (16.2–78.1) outside and 87.4% (75.4–97.0) inside (paired $t_{19} = 12.3$, $P < 0.0001$). The sun was obscured by clouds during 7 of the 20 days of exposure.

Mean percentages of cuticular hydrocarbon compounds in this experiment are listed in Table 1 (see Figure 1 for a representative chromatogram). Outside exposure led to an increase in the proportion of *n*-alkanes on the harvester ant cuticle (Figure 2, Table 1). The cuticular lipids of ants that were exposed to outside conditions for 50 min/day contained a significantly higher percentage of *n*-alkanes

TABLE 1. AVERAGE PERCENT COMPOSITION OF CUTICULAR HYDROCARBONS FROM EXPOSED AND CONTROL *P. barbatus* WORKERS

Compound	TIC Peak no.[a]	Colony S1		Colony S6		Colony S7	
		Control	Exposed	Control	Exposed	Control	Exposed
Alkanes							
n-Tricosane	1	6.9	9.1	5.7	6.7	4.2	7.2
n-Tetracosane	2	1.5	1.6	1.5	1.5	1.0	1.4
n-Pentacosane	3	30.7	33.5	24.2	26.9	20.6	27.9
n-Hexacosane	5	1.6	1.5	1.6	1.7	1.2	1.6
n-Heptacosane	7	5.0	5.1	4.2	3.8	3.7	4.5
n-Nonacosane	12	2.1	2.4	2.4	2.1	2.1	2.3
n-Hentriacontane	18	3.0	3.4	3.4	2.7	2.8	2.8
Total alkanes		50.8	56.6	43.0	45.5	35.7	47.6
Alkenes							
Heptacosene	6	2.2	1.8	2.2	2.0	3.4	2.6
Nonacosene	11	2.0	1.7	2.1	1.9	3.3	2.7
Hentriacontene	18	3.8	3.3	4.6	2.9	6.6	5.2
Total alkenes		8.1	6.8	9.0	6.8	13.3	10.5
Monomethylalkanes							
13-Methylpentacosane	4	2.7	2.3	2.9	3.3	4.3	3.0
13-Methylheptacosane	8	4.5	3.8	4.2	4.3	5.4	4.5
7-Methylheptacosane	9	4.2	3.9	3.4	3.2	4.5	4.3
15-Methylnonacosane	13	4.1	3.7	5.0	4.1	4.4	4.2
9-Methylnonacosane	14	1.5	1.4	2.8	1.8	1.8	1.8
7-Methylnonacosane	15	1.9	2.0	1.8	1.6	2.1	2.5
15-, 13-, 11-, 9-Methylhentriacontanes	19	5.8	5.5	6.8	6.0	6.1	5.1
7-Methylhentriacontane	20	1.6	1.2	2.0	1.8	1.9	0.2
17-, 15-, 13-, 11-, 9-, 7-Methyltritriacontanes	21	2.1	1.9	2.5	2.1	2.0	2.0
Total monomethylalkanes		28.3	25.7	31.3	28.2	32.5	27.6
Dimethylalkanes							
7,13-Dimethylheptacosane	10	2.2	1.9	1.9	2.0	3.1	2.0
7,13-Dimethylnonacosane	16	1.0	0.9	1.0	1.1	1.4	1.0
Total dimethylalkanes		3.3	2.7	2.9	3.0	4.5	3.0

Note: "Exposed" workers were placed outside daily; "control" workers were not placed outside.
[a] Total ion chromatograph (TIC) peak numbers correspond to those in Figure 1.

than those of ants kept indoors in covered containers ($F_{1,38} = 7.7$, $P < 0.01$). Colonies differed in the percentage of *n*-alkanes on the cuticle ($F_{2,38} = 9.9$, $P < 0.001$). There was no significant interaction between treatment and colony ($F_{2,38} = 0.7$, $P = 0.5$). There was a tendency for ants in both treatments to increase in the proportion of *n*-alkanes from the beginning of the experiment to the end. Ants

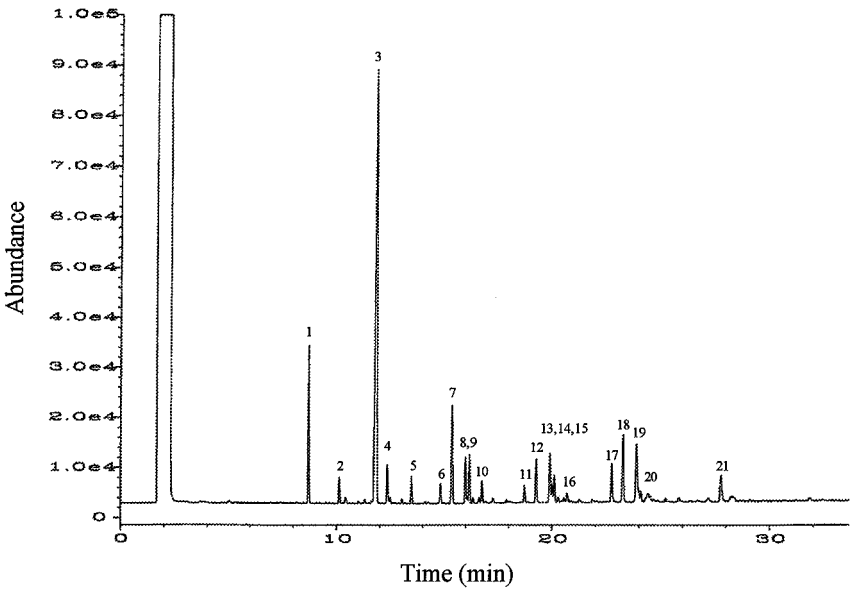


FIG. 1. Representative chromatogram of an ant in the exposure experiment. The large peak at 2 minutes is the solvent, chloroform. Numbers over peaks refer to the compounds listed in Table 1.

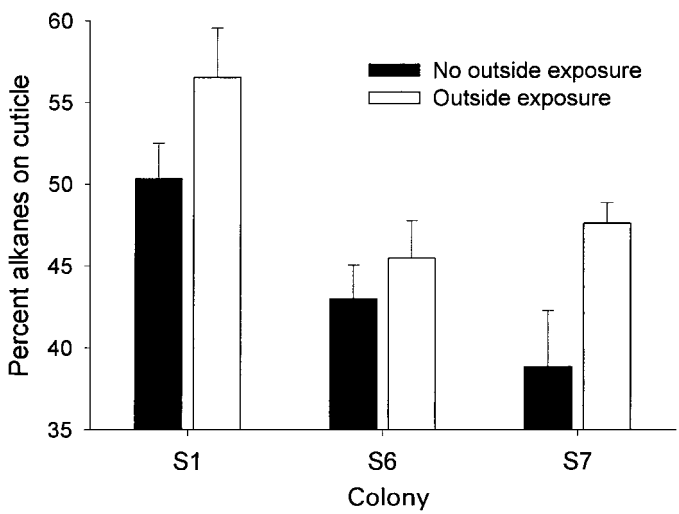


FIG. 2. Percentage of *P. barbatus* cuticular lipids composed of *n*-alkanes on ants exposed to outside conditions for 50 min/d and ants not exposed to outside conditions. Error bars show one mean standard error.

exposed to external conditions increased about 32% in *n*-alkanes over the initial state (overall mean initial % *n*-alkanes = 37.8, SE = 2.1, $N = 18$), while those kept indoors increased by about 16%.

Ultraviolet Light Manipulation. Temperature and humidity did not differ under canopies composed of UV-transmitting and UV-blocking material. Air temperature averaged 36.5°C (range 29.2–41.7) in high UV treatment containers and 37.6°C (30.5–41.7) in low UV treatment containers ($t_{10} = -0.7$, $P = 0.5$); surface temperature averaged 28.9°C (26.2–32.4) in high UV treatment containers and 28.7°C (26.3–32.8) in low - UV treatment containers ($t_{10} = 1.1$, $P = 0.3$); and RH averaged 37.7% (28.4–51.2) in high UV treatment containers and 36.6% (23.3–53.5) in low-UV treatment containers ($t_{10} = 1.1$, $P = 0.3$). Canopies trapped heat, so ants in this experiment experienced warmer conditions during periods outside than ants in the general exposure experiment. While inside the laboratory, within-container air temperature averaged 24.7°C (22.3– 26.5), surface temperature averaged 24.6°C (21.9–26.8), and RH averaged 88.6% (83.0–91.1).

Exposure to UV light had no effect on *n*-alkane abundance ($F_{1,28} = 1.8$, $P = 0.2$; Figure 3). As in the previous experiment, colonies differed in percent cuticular *n*-alkanes ($F_{2,28} = 19.3$, $P < 0.001$; Figure 3) and there was no significant interaction between treatment and colony ($F_{2,28} = 0.7$, $P = 0.5$). Cuticular *n*-alkanes increased about 40% over the course of the experiment, from a mean initial value of 29.8% alkanes (SE = 1.9, $N = 17$) to a final overall mean value of 41.6% (SE = 1.7, $N = 32$).

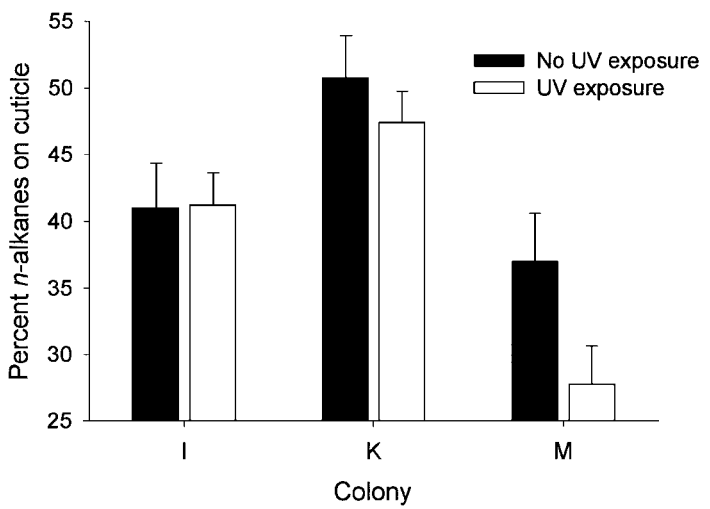


FIG. 3. Percentage of cuticular *n*-alkanes on harvester ants from different colonies exposed to sunlight that had passed through ultraviolet-transmitting and ultraviolet-blocking filters.

Temperature and Humidity Manipulation. The air temperatures and RH to which ants were exposed in this experiment averaged 38.7°C (range = 35.3–41.5) and 8.3% (4.5–12.1) in the high temperature–low RH chamber, 38.8°C (36.8–42.7) and 94.9% (78.9–99.9) in the high temperature–high RH chamber, 26.6°C (20.9–30.3) and 7.8% (4.0–18.2) in the moderate temperature–low RH chamber, and 26.2°C (21.8–28.9) and 86.0% (79.8–90.6) in the moderate temperature–high RH treatments.

The effects of temperature and humidity on cuticular composition were interdependent; in general, warm dry conditions lead to high *n*-alkane abundance (Figure 4). Humidity had a significant effect on *n*-alkane abundance, whereas temperature did not; however, the main effects must be interpreted cautiously because there was a significant interaction between temperature and humidity (Table 2). Inspection of Figure 4 indicates that low humidity affected cuticular composition only in combination with warm temperatures. Variation in humidity had little effect on *n*-alkane abundance at moderate temperatures. Colonies did not differ in *n*-alkane abundance, and there were no interactions involving colony (Table 2).

There was no consistent increase in *n*-alkanes over time in this experiment; rather, the magnitude and direction of change in percent *n*-alkanes depended on treatment. The average cuticular *n*-alkane abundance of ants exposed to warm, humid conditions was about 2% lower than that of the initial sample, whereas the

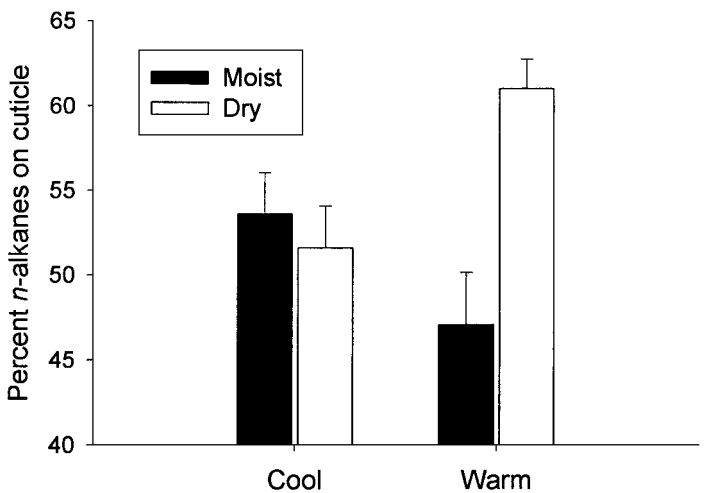


FIG. 4. Percentage of *n*-alkanes in the cuticular lipids of harvester ants subjected to combinations of temperature (warm and cool) and relative humidity (high and low). Colonies did not differ significantly in percent *n*-alkanes in this experiment and data from all colonies are combined for purposes of illustration.

TABLE 2. RESULTS OF ANALYSIS OF VARIANCE INVESTIGATING THE EFFECT OF TEMPERATURE, HUMIDITY, AND COLONY IDENTITY ON THE PERCENT *n*-ALKANES IN HARVESTER ANT CUTICULAR LIPID EXTRACTS

Source	*df*	*F*	*P*
Temperature (*T*)	1	0.1	0.8
Humidity (*H*)	1	6.6	0.01
$T * H$	1	12.6	<0.001
Colony (*C*)	2	1.6	0.2
$C * T$	2	0.1	0.9
$C * H$	2	0.6	0.6
$C * T * H$	2	1.9	0.2
Error	52		

n-alkane abundance of ants exposed to other treatments was 3–22% higher than the initial sample (initial average *n*-alkanes = 49.9%, SE = 2.4, $N = 25$).

DISCUSSION

The results indicate that environmental conditions affect the hydrocarbon composition of the harvester ant cuticle. Exposure to warm, dry conditions triggered an increase in the proportional abundance of cuticular *n*-alkanes. Exposure to UV radiation, in contrast, had little effect on cuticular *n*-alkane abundance. These results support the hypothesis that differences in exposure among task groups explains task-related differences in cuticular hydrocarbon composition. The total increase in *n*-alkane proportional abundance in exposed ants over 20 days (2–12%) was similar to differences between field-sampled nest maintenance workers and foragers reported previously (5–8%, Wagner et al., 1998). Our results corroborate those of previous studies investigating environmental effects on insect cuticular chemistry. Low RH lead to a similar increase in the relative abundance of *n*-alkanes in weevil larvae (*Oryzaephilus surinamensi*), although not in adults (Howard et al., 1995). Acclimation to warm temperatures increased the ratio of straight-chain relative to branched alkanes in the grasshopper *Melanoplus sanguinipes* (Gibbs and Mousseau, 1994).

The primary mechanism of water conservation in insects is a thin layer of lipids on the cuticle that forms a passive barrier to water loss through the integument (Hadley, 1994). In ants and many other arthropod taxa, cuticular lipid composition is dominated by various forms of hydrocarbons. The effectiveness of the cuticular lipid barrier at slowing water loss depends on its composition as well as its thickness. Hydrocarbon compounds differ in physical

properties that influence their effectiveness as waterproofing agents. Permeability to water increases sharply at compound-specific transition temperatures, probably the melting temperatures (Gibbs, 1998; Rourke and Gibbs, 1999). Double bonds and methylbranching dramatically reduce the melting point (Gibbs and Pomonis, 1995), so *n*-alkanes are thought to provide qualitatively better waterproofing than alkenes and branched alkanes of similar size. Studies of water loss in insects appear to support this hypothesis. Methylbranching and unsaturation are positively associated with water loss rate within several insect species (Hadley, 1978; Toolson, 1984). The proportional increase in cuticular *n*-alkanes detected in this study might serve to reduce water loss by ants exposed to warm dry conditions.

The transition from interior work to nest maintenance not only involves short bursts of exterior work, but brings ants closer to the surface while inside the nest, where they encounter warmer, drier internal conditions. Once a harvester ant begins to work outside the nest, it tends to stay in the upper chambers of the nest near the entrance (MacKay, 1983; Tschinkel, 1999). The entrance to a *P. barbatus* nest, which can be 1–4 cm wide, is connected by wide tunnels into one or more chambers just inside the nest entrance. Strong temperature and moisture gradients have been found in *Pogonomyrmex* nests during the summer months: areas near the surface are drier and warmer than deeper areas (MacKay, 1981). After performing nest maintenance work, ants move on to tasks that entail longer periods of external exposure, such as patrolling and foraging.

In addition to treatment effects, there was a general increase in the proportion of *n*-alkanes between the initial and final sampling times in both the outside exposure experiment and the UV exposure experiment, and an increase in three out of four treatments groups of the temperature and humidity experiment. There are two plausible explanations for this trend toward increasing *n*-alkane abundance over time. First, the conditions experienced by the ants in this study, including the indoor controls, may have been drier and warmer than those they experienced in the nest prior to collection, leading to the same sort of environmentally-triggered increase in *n*-alkanes that we detected in our experiments. Second, *n*-alkane abundance may increase as an ant ages, with environment acting to enhance or temper a general increase over time. We cannot reject either of these possibilities at the present time. Working with laboratory colonies of the ant *Myrmicaria eumenoides*, Kaib et al. (2000) found that the cuticular hydrocarbon composition of ants that switched from nest work to foraging changed with age over a period of 30–40 days, whereas the composition of workers that remained in the nest was unchanged over the same period. This result suggests either that task-related changes in cuticular chemistry are triggered by an ant's environment and not its age, or that only particular task groups undergo endogenous age-related changes in cuticular chemistry. Further work is underway to test the hypothesis that the relative abundance of *n*-alkanes is related to age, as well as environment, in *P. barbatus.*

We found that most colonies differed from one another in the proportion of *n*-alkanes in the cuticular lipids of nest maintenance workers. Colony-level differences in ant cuticular lipid composition have been detected in many ant species (Obin, 1986; Bonavita-Cougardan, 1987; Vander Meer et al., 1989; Nowbahari et al., 1990; Wagner et al., 1998; Nielsen, 1999). These differences may be due in part to genetic differences among colonies. There is some evidence of a heritable component to the cuticular hydrocarbon composition of fire ants and honeybees (Ross et al., 1987; Vander Meer et al., 1985; Page et al., 1991). Differences among the colonies we measured may also reflect how recently new nest maintenance workers were recruited from the younger workers inside the nest. For example, a colony whose nest was recently trampled by a cow will have recruited nest maintenance workers to repair the damage. Such a colony may have more new nest maintenance workers than one whose nest was undisturbed. Differences among colonies in cuticular *n*-alkane composition of their nest maintenance workers may reflect, in part, variation in the duration of the nest maintenance workers' tenure in that task, and thus, in how long the workers have been exposed to warm, dry conditions.

Rates of brief antennal contact, which involve the perception of odor, influence task decisions in *P. barbatus* (Gordon and Mehdiabadi, 1999). An ant's response depends on the task of the ant it meets (Gordon and Mehdiabadi, 1999). Cuticular hydrocarbons act as the cue for nestmate recognition in *P. barbatus* (Wagner et al., 2000), as in other ant species (Lahav et al., 1999; Thomas et al., 1999). We infer that task-specific differences in hydrocarbon profiles may also permit an ant to assess the task of a nestmate it meets. Our results show that the environmental conditions associated with a task, in particular exposure to warm temperature and low humidity, can shape task-specific differences in the cuticular hydrocarbon profile. When one ant contacts another, these task-related differences in hydrocarbon composition might provide information about the other's task. Similarly, we might recognize a carpenter by the calluses on her hands, whether or not she holds a hammer.

Acknowledgments—We are indebted to Chris Somerville at the Carnegie Institute of Washington and Martin Reinhard and Jean-Francois Debroux at the Civil and Environmental Engineering Department at Stanford University for the use of their laboratories. Gert-Jan de Boer and Pierre Broun gave us valuable technical assistance. David Millard kindly provided the UV filters. William Cuevas confirmed the identity of the cuticular compounds. The manuscript was improved by thoughtful comments from Nathan Sanders, Stephen Roberts, and Veronica Volny. Many thanks to Craig Heller for his assistance when samples from a earlier set of these experiments were destroyed. The research was supported by National Science Foundation grant IBN-9603639 to DMG.

REFERENCES

BONAVITA-COUGOURDAN, A., CLEMENT, J. L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus* Scop. *J. Entomol. Sci.* 22:1–10.

BONAVITA-COUGOURDAN, A., CLEMENT, J. L., and LANGE, C. 1993. Functional subcaste discrimination (foragers and brood-tenders) in the ant *Camponotus vagus* Scop.: Polymorphism of cuticular hydrocarbon patterns. *J. Chem. Ecol.* 19:1461–1477.

FRANKS, N. R., TOFTS, C., and SENDOVA-FRANKS, A. B. 1997. Studies in division of labor: Neither physics nor stamp collecting. *Anim. Behav.* 53:219–224.

GIBBS, A. G. 1998. The role of lipid physical properties in lipid barriers. *Am. Zool.* 38:268–279.

GIBBS, A., and MOUSSEAU, T. A. 1994. Thermal acclimation and genetic variation in cuticular lipids of the lesser migratory grasshopper (*Melanoplus sanguinipes*): Effects of lipid composition on biophysical properties. *Physiol. Zool.* 67:1523–1543.

GIBBS, A., and POMONIS, J. G. 1995. Physical properties of insect curticular hydrocarbons: Model mixtures and lipid interactions. *Comp. Biochem. Physiol. B*112:667–672.

GORDON, D. M. 1986. The dynamics of the daily round of the harvester ant colony (*Pogonomyrmex barbatus). Anim. Behav.* 34:1402–1419.

GORDON, D. M. 1989. Dynamics of task switching in harvester ants. *Anim. Behav.* 38:194–204.

GORDON, D. M. 1995. The development of an ant colony's foraging range. *Anim. Behav.* 49:649–659.

GORDON, D. M. 1996. The organization of work in social insect colonies. *Nature* 380:121–124.

GORDON, D. M., and KULIG, A. W. 1996. Founding, foraging, and fighting: Colony size and the spatial distribution of harvester ant nests. *Ecology* 77:2393–2409.

GORDON, D. M., and MEHDIABADI, N. J. 1999. Encounter rate and task allocation in harvester ants. *Behav. Ecol. Sociobiol.* 45:370–377.

HADLEY, N. F. 1978. Cuticular permeability of dessert tenebrionid beetles: correlations with epicuticular hydrocarbon composition. *Insect Biochem.* 8:17–22.

HADLEY, N. F. 1994. Water Relations of Terrestrial Arthropods. Academic Press, San Diego.

HAVERTY, M. I., GRACE, J. K., NELSON, L. J., and YAMAMOTO, R. T. 1996. Intercaste, intercolony, and temporal variation in cuticular hydrocarbons of *Coptermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 22:1813–1834.

HOWARD, R. W., HOWARD, C. D., and COLQUHOUN, S. 1995. Ontogenetic and envrionmentally induced changes in cuticular hydrocarbons of *Oryzaephilus surinamensis* (Coleoptera: Cucujidae). *Ann Entomol. Soci. Am.* 88:485–495.

HOWARD, R. W., MCDANIEL, C. A., NELSOM, D. R., BLOMQUIST, G. J., GELBAUM, L. T., and ZALKOW, L. H. 1982. Cuticular hydrocarbons of *Reticulitermes virginicus* (Banks) and their role as potential species and caste-recognition cues. *J. Chem. Ecol.* 8:1227–1239

KAIB, M., EISERMANN, B., SCHOETERS, E., BILLEN, J., FRANKE, S., and FRANCKE, W. 2000. Task-related variation of postpharyngeal and cuticular hydrocarbon compositions in the ant *Myrmicaria eumenoides. J. Comp. Physiol. A* 186:939–948.

LAHAV, S., SOROKER, V., and HEFETZ, A. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften* 86:246–249.

MACKAY, W. P. 1981. A comparison of the nest phenologies of three species of *Pogonomymex* harvester ants. *Psyche* 88:25–75.

MACKAY, W. P. 1983. Stratification of workers in harvester ant nests (Hymenoptera: Formicidae). *J. Kansas Entomol. Soc.* 56:538–542.

NELSON, D. R., FATLAND, C. L., HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, F. J. 1980. Re-analysis of the cuticular methylalkanes of *Solenopsis invicta* and *S. richteri. Insect Biochem.* 10:409–418.

NELSON, D. R., TISSOT, M., NELSON, L. J., FATLAND, C. L., and GORDON, D. M. In press. Novel esters and hydrocarbons in the cuticular surface lipids of the red harvester ant, *Pogonomyrmex barbatus*. *Comp. Biochem. Physiol. B.*

NIELSEN, J., BOOMSMA, J. J., OLDHAM, N. J., PETERSEN, H. C., and MORGAN, E. D. 1999. Colony-level and season-specific variation in cuticular hydrocarbon profiles of individual workers in the ant Formica truncorum. *Insectes Sociaux* 46:58–65.

NOWBAHARI, E., LENOIR, A., CLEMENT, J. L., LANGE, C., BAGNERES, A. G., and JOULIE, C. (1990) Individual, geographical and experimental variation of cuticular hydrocarbons of the ant *Cataglyphis cursor* (Hymenoptera: Formicidae): Their use in nest and subspecies recognition. *Biochem. Syst. Ecol.* 18:63–73.

OBIN, M. S. 1986, Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and role of cuticular hydrocarbons. *J. Chem.Ecol.* 12: 1965–1975.

PAGE, R. E., METCALF, R. A., METCALF, R. L., ERICKSON, E. H., and LAMPMAN, R. L. 1991. Extractable hydrocarbons and kin recognition in honeybee (*Apis mellifera). J. Chem. Ecol.* 17:745–756.

ROSS, K. G., VANDER MEER, R. K., FLETCHER, D. J. C., and VARGO, E. L. 1987. Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). *Evolution* 41:280–293.

ROURKE, B. C., and GIBBS, A. G. 1999. Effects of lipid phase transitions on cuticular permeability: Model membrane and in situ studies. *J. Exp. Biol.* 202:3255–3262.

SMITH, R. K., and TAYLOR, O. R. 1990. Unsaturated extracted hydrocarbon caste differences between European queen and worker honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *J. Kansas Entomol. Soc.* 63:369–374.

THOMAS, M. L., PARRY, L. J., ALLAN, R. A., and ELGAR, M. A. 1999. Geographic affinity, cuticular hydrocarbons and colony recognition in the Australian meat ant *Iridomyrmex purpureus. Naturwissenschaften* 86:87–92.

TOOLSON, E. C. 1984. Interindividual variation in epicuticular hydrocarbon composition and water loss rates of the cicada *Tibicen dealbatus* (Homoptera: Cicadidae). *Physiol. Zool.* 57:550–556.

TSCHINKEL, W. R. 1999. Sociometry and sociogenesis of colonies of the harvester ant, Pogonomyrmex badius: Distribution of workers, brood and seeds within the nest in relation to colony size and season. *Ecol. Entomol.* 24:222–230.

VANDER MEER, R. K., LOFGREN, C. S., and ALVAREZ, F. M. 1985. Biochmical evidence for hybridization in fire ants. *Florida Entomol.* 68:501–506.

VANDER MEER, R. K., SALIWANCHIK, D., and LAVINE, B. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta*: Implications for nestmate recognition. *J. Chem. Ecol.* 15:2115–2125.

WAGNER, D., BROWN, M. J. F., BROUN, P., CUEVAS, W., MOSES, L. E., CHAO, D. L., and GORDON, D. M. 1998. Task-related differences in the cuticular hydrocarbon composition of harvester ants, *Pogonomyrmex barbatus. J. Chem. Ecol.* 24:2021–2037.

WAGNER, D., TISSOT, M., CUEVAS, W., and GORDON, D. 2000. Harvester ants utilize cuticular hydrocarbons in nestmate recognition. *J. Chem. Ecol.* 26:2245–2257.

WHITFORD, W. G., JOHNSON, P., and RAMIREZ, J. 1976. Comparative ecology of the harvester ants *Pogonomyrmex barbatus* (F. Smith) and *Pogonomyrmex rugosus* (Emery). *Insectes Sociaux* 23:117–132.

WILSON, E. O. 1971. The Insect Societies. Belknap Press, Cambridge.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

MALE-PRODUCED SEX ATTRACTANT PHEROMONE OF THE GREEN STINK BUG, *Acrosternum hilare* (Say)

HEATHER L. McBRIEN,[1] JOCELYN G. MILLAR,[1,*] LEVI GOTTLIEB,[1,†] XIN CHEN,[1,‡] and RICHARD E. RICE[2]

[1]*Department of Entomology*
University of California
Riverside California 92521

[2]*Department of Entomology*
University of California
Davis, California 95616

(Received December 19, 2000; accepted May 12, 2001)

Abstract—Sexually mature virgin adult males of the green stink bug, *Acrosternum hilare* attracted sexually mature virgin adult females in laboratory bioassays using a vertical Y-tube. There was no indication that males attracted other males, or that females attracted either sex. These results suggested that *A. hilare* males produce a sex pheromone. Extracts of odors collected from sexually mature males contained compounds that were not present in extracts from females or sexually immature males. (4*S*)-*Cis*-(*Z*)-bisabolene epoxide ((4*S*)-*cis*-*Z*-BAE) was the major sex-specific component of the extract. The crude extract was attractive to female *A. hilare*, but when separated into four fractions, only the portion containing (4*S*)-*cis*-*Z*-BAE and the minor component (4*S*)-*trans*-*Z*-BAE was attractive to females. This fraction was as attractive as the crude extract, suggesting that the former contained all the pheromone components. Neither synthetic (4*S*)-*cis*-*Z*-BAE nor (4*S*)-*trans*-*Z*-BAE alone was attractive to females, but a 95:5 *cis:trans* blend, mimicing the ratio naturally produced by males, was attractive to females in Y-tube bioassays. Bioassays in a field cage showed that significantly more *A. hilare* females were attracted to cotton string lures treated with 1 mg of a 95:5 blend of (4*S*)-*cis*-*Z*-BAE and (4*S*)-*trans*-*Z*-BAE placed inside a bouquet of alfalfa than to an alfalfa bouquet containing a pentane-treated control. In field cage studies, attraction of females was greatest during the late afternoon and evening hours, and female *A. hilare* approached the synthetic pheromone source almost exclusively by walking.

*To whom correspondence should be sent. e-mail: jocelyn.millar@ucr.edu
†Current address: RAFAEL-Armament Developing Authority, Dep. M1, Haifa 31021, Israel.
‡Current address: Department of Chemistry, University of North Carolina, Durham, North Carolina 27705.

0098-0331/01/0900-1821$19.50/0

Key Words—*Acrosternum hilare,* sex pheromone, bisabolene epoxide, (*Z*)-(1*R*,2*S*,4*S*)-4-(1′,5′-dimethyl-1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane, (*Z*)-(1*S*,2*R*,4*S*)-4-(1′,5′-dimethyl-1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane, *Nezara viridula.*

INTRODUCTION

The green stink bug, *Acrosternum hilare* (Say) (Hemiptera: Pentatomidae) is a pest of a variety of crops in North America, including cotton, soybeans, pistachios, and pecans (Miner, 1966; Simmons and Yeargan, 1988; Rice et al., 1988; da Silva et al., 1996; Greene et al., 1999; reviews by McPherson, 1982 and Panizzi, 1997). In addition to direct damage caused by feeding, *A. hilare* vectors plant pathogens such as yeast-spot disease of soybeans (Daugherty, 1967; Clarke and Wilde, 1970) and the fungus *Botryosphaeria dothidea* in pistachios (Daane et al., 2000). Crop damage by these and related species of stink bugs may be exacerbated by recent fundamental changes in crop protection, especially the widespread deployment of genetically modified crop plants that express gene products from *Bacillus thurengiensis* (*B.t.*). Because the *B.t.* proteins expressed by a plant are active against only a limited number of insect families or even genera (e.g., some Lepidoptera and Coleoptera, depending on the *B.t.* strain), these plants are not protected from attack by other insects. For example, the deployment of transgenic cotton in the southeastern United States, and the consequent reduction in pesticide applications has been correlated with increased feeding damage by *A. hilare* and other species of pentatomid and mirid bugs (Turnipseed and Greene, 1996; Greene et al., 1999).

Typical methods for monitoring heteropteran species such as stink bugs include sampling with sweep nets or beating trays, and visual inspection of the crop for damage. These techniques are labor intensive and require multiple replication to generate realistic population assessments. Furthermore, adult bugs fly, and can rapidly migrate into a crop from surrounding fields as crops in those fields are harvested, or move into a crop from surrounding native vegetation as it dries during the summer. Consequently, frequent sampling is required to detect immigration of bug populations into crops, in order to apply control measures before significant damage occurs. Pheromone-baited traps might mitigate some of these problems (McBrien and Millar, 1999).

Previous studies have shown that sexually mature *A. hilare* males produce sex-specific compounds, and it was suggested that these compounds were sex or aggregation pheromones based on comparisons with the known pheromone compounds produced by males of a related species, the southern green stink bug *Nezara viridula* (L.) (Aldrich et al., 1987, 1989, 1993). In particular, the major component emitted by *N. viridula* males, *trans-Z*-bisabolene epoxide (*trans-Z*-BAE, Figure 1), is a minor component of the volatile chemicals produced

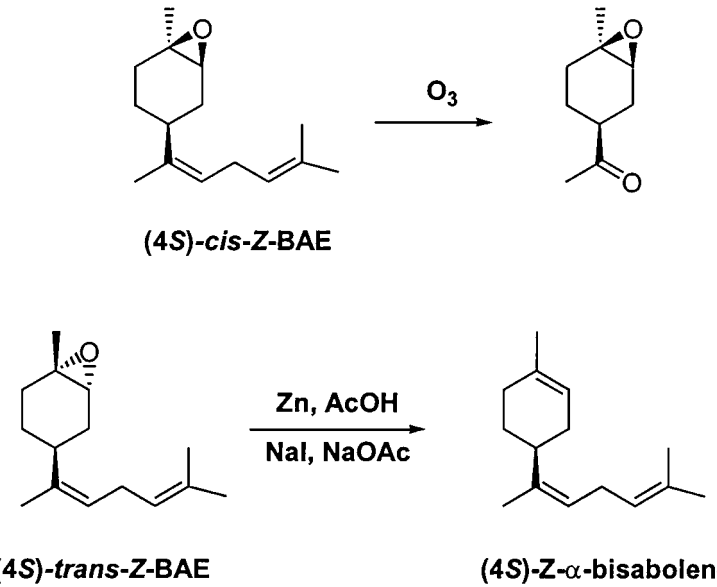


FIG. 1. Structures of (4*S*)-*cis*-*Z*-bisabolene epoxide, (4*S*)-*trans*-*Z*-bisabolene epoxide, and the products from their degradation by ozonolysis and deoxygenation, respectively.

by *A. hilare* males, whereas the major sex-specific component of extracts from *A. hilare* males, *cis*-*Z*-bisabolene epoxide (*cis*-*Z*-BAE, Figure 1), is a minor component in volatiles from *N. viridula* males. The absolute configurations of the BAEs produced by either species have not been determined, although only the (4*S*)-enantiomer of *trans*-*Z*-BAE was attractive to *N. viridula* females in bioassays (Baker et al., 1987). Further laboratory bioassays showed that a blend of (4*S*)-*trans*-*Z*-BAE and (4*S*)-*cis*-*Z*-BAE was more attractive to *N. viridula* females than (4*S*)-*trans*-*Z*-BAE alone (Brézot et al., 1994), suggesting that the *N. viridula* pheromone consists of at least two components. However, the possible function of the male-specific compounds as pheromone components for *A. hilare* has not been tested.

The objectives of the work described here were to:

1. Identify the sex-specific compounds produced by male *A. hilare*.
2. Determine whether the male-produced compounds constituted a sex pheromone only attractive to females, or an aggregation pheromone attractive to both sexes.
3. Fully characterize the pheromone, including determination of the number of components, and the complete identification of each component.
4. Synthesize and bioassay the pheromone components.

METHODS AND MATERIALS

Insect Colonies. Adults and nymphs of *A. hilare* were collected from an almond orchard near Fresno, CA in 1997 and 1998. Adults and nymphs of *N. viridula* were collected from an alfalfa plot at University of California, Riverside, Agricultural Operations. Rearing methods for both species are described in Čokl et al. (2001). Two days after the final moult, adults were separated by sex, and placed in paper ice cream cartons (20×18 cm) covered with cloth screening, with green beans and sunflower seeds supplied for food. These insects were used for collection of insect-produced volatiles or for bioassays, after which they were returned to the breeding colony.

Dissection of Adult Metathoracic Glands for Analysis of Defensive Compounds. Metathoracic glands (MTG) of 5 sexually mature virgin adults of both sexes were dissected following the method of Gilby and Waterhouse (1965), and the contents of each gland were analyzed. A drawn-out glass capillary tube was inserted into the MTG of each bug to collect the fluid contents, the fluid was dissolved in 200 μl pentane, and the pentane solution was analyzed by gas chromatography-mass spectrometry as described below.

Collection of Insect Volatiles. Volatile chemicals were collected from 5 separate groups of virgin adult male and female *A. hilare*. Sexes were aerated separately, and each cohort of bugs was aerated continuously for a period of 2–3 weeks to follow changes in the profile of compounds produced by the bugs as they became sexually mature. Analogous extracts were also prepared from sexually mature adult male *N. viridula* to provide authentic samples of the two BAE's.

Cohorts of 12–20 virgin adults were carefully coaxed (to minimize discharge of defensive secretions) into horizontal cylindrical glass aeration chambers (28×8.0 cm ID) as described in Millar and Sims (1998), with charcoal-filtered, humidified air pulled through the system at a flow rate of 1 liter/min. Each chamber was fitted with a cylinder of aluminum window screen for the bugs to walk and perch on, and 3 green beans and 5 sunflower seeds were provided as food. The aeration apparatus was set up next to a window, with additional light provided with a fluorescent light fixture (2 Sylvania Octron 32W light tubes) mounted above the aeration apparatus to provide a 16L:8D, long-day light cycle to ensure that bugs did not go into reproductive diapause. Ambient temperature during aerations was $\sim$24°C. Insect-produced volatiles were trapped with collectors made from 0.4 cm ID glass tubes containing a 2 mm long bed of activated charcoal (50–200 mesh, stripped of volatiles by heating at 200°C overnight while purging with activated charcoal-purified nitrogen) confined between two glass wool plugs. Collectors were changed every second day, at which time fresh food was also provided. Trapped volatiles were desorbed by rinsing the collectors with three 200 μl aliquots of pentane, followed by three 200 μl aliquots of ether. Extracts of volatiles were stored at -20°C in glass vials with Teflon cap-liners until used

for bioassays or analyses. Cohorts were 8–9 d old when aerations were started, except for one cohort in which aeration commenced with 0–2 d old bugs so that the onset of production of male-specific compounds could be determined. To provide sufficient materials for fractionation and bioassays, three larger cohorts of males (28–49 bugs) were aerated in a larger glass chamber (35 × 15 cm ID), with an air flow of 5 liter/min, and a larger collector (0.6 mm ID, 3 mm long).

Collection of Defensive Compounds. Groups of virgin adult male (10) and female bugs (25) were aerated separately in the glass aeration chamber for 1 hr as described above, except that the glass chamber was shaken vigourously for 3 min immediately before the aeration began, to induce discharge of the defensive compounds.

Analysis and Fractionation of Insect Extracts. Extracts were analyzed by gas chromatography (GC) with a Hewlett-Packard 5890 instrument equipped with DB-5 (30 m × 0.25 mm ID, J&W Scientific, Folsom CA) or DB-17 columns (30 m × 0.25 mm). Helium carrier gas was used, with analyses run in splitless mode with a temperature program of 50°C/1 min, 10°/min to 250°C for both columns. Injector and detector temperatures were 250 and 275°C respectively. Extracts were also analyzed by splitless coupled GC-mass spectrometry by using an HP 6890 GC equipped with an HP-5MS column (30 m × 0.25 mm id) and interfaced to an HP 5973 mass selective detector. All mass spectra were taken in electron impact mode, ionizing voltage 70 eV. The temperature program was the same as above.

Crude aeration extract (15,869 bug-hour equivalents) from sexually mature males was concentrated to 1.0 ml under a gentle stream of N_2. A 200 μl aliquot was set aside, and the remainder was fractionated on a solid phase extraction cartridge (Extract-Clean silica cartridge, 500 mg silica gel; Alltech #209250, Deerfield IL). The cartridge was preconditioned with 5 ml of 25% ether in pentane, then 5 ml of pentane, and the excess solvent was expressed from the cartridge with a pipette bulb. The combined crude *A. hilare* extract was loaded onto the cartridge, rinsing the walls with a few drops of clean pentane. The cartridge was then eluted with gravity flow with 3 × 1 ml pentane, 3 × 1 ml 10% ether in pentane, 3 × 1 ml 25% ether in pentane, and 2 ml ether, collecting each aliquot as a separate fraction. The fractions were analyzed by GC. The first two pentane fractions containing hydrocarbons were combined, the second two 10% ether fractions containing the BAE's were combined, and all the 25% ether in pentane and 100% ether fractions containing traces of more polar compounds were combined. These fractions were used for analyses and bioassays. The third pentane fraction and the first 10% ether in pentane fraction contained no detectable compounds by GC and so they were not combined with the other fractions to avoid unnecessary dilution.

Absolute Configuration of A. hilare *cis-BAE*. The absolute configuration of insect-produced *cis*-BAE was determined by oxidative degradation to *cis*-4-acetyl-1,2-epoxy-1-methylcyclohexane, followed by analysis on a chiral GC column, and comparison of retention times with those of standards of known absolute

configuration. (4*S*)- and (4*R*)-*cis*-4-acetyl-1,2-epoxy-1-methylcyclohexane standards were obtained by phase transfer catalyzed oxidation of commercial (−)- or (+)-limonene oxide with potassium permanganate (Chen et al., 2000). Separation of the resulting *cis*- and *trans*-4-acetyl-1,2-epoxy-1-methylcyclohexanes by flash chromatography on silica gel, eluting with 16% EtOAc in hexanes, yielded the chemically and stereoisomerically pure *cis*-(1*R*,2*S*,4*S*)- and *cis*-(1*S*,2*R*,4*R*)-enantiomers, respectively. ^{1}HNMR (300 MHz): *cis*: $\delta = 1.34$ (s, 3H), 1.40–1.78 (m, 3H), 2.03–2.12 (m, 3H), 2.13 (s, 3H), 2.24 (m, 1H), 3.05 (d, J = 4.9 Hz, 1H). *trans*: $\delta = 1.30$ (s, 3H), 1.32–1.46 (m, 2H), 1.64–2.12 (m, 4H), 2.14 (s, 3H), 2.57 (br. t, J ∼ 10Hz, 1H), 3.08 (t, J ∼ 2Hz, 1H). Spectroscopic data agreed with that previously reported (Delay and Ohloff, 1979; Chen et al., 2000). The enantiomers were resolved on a Cyclodex B GC column (20 m × 0.25 mm ID; J&W Scientific), run isothermally at 90°C in split injection mode with helium carrier gas and an injector temp of 250°C. The (1*R*,2*S*,4*S*)- and (1*S*,2*R*,4*R*)-enantiomers eluted at 20.79 and 21.03 min, respectively.

A total of 1,870 bug-hours of the BAE fraction of volatiles from mature males was concentrated to a few microliters in a conical-bottomed vial under a gentle stream of nitrogen, then taken up in ∼25 μl of CH_2Cl_2. The vial was chilled in a dry ice-acetone bath, and the chilled solution was subjected to microozonolysis for ∼20 sec (Beroza and Bierl, 1969). The solution was then warmed to room temperature and analyzed immediately on the Cyclodex B column, giving a single peak at 20.75 min. Aliquots of the ozonized extract also were coinjected with racemic and (1*S*,2*R*,4*R*)-*cis*-4-acetyl-1,2-epoxy-1-methylcyclohexane, respectively. The first injection resulted in two peaks of unequal size (larger, 20.61 min, smaller 20.88 min), and the second injection gave two peaks (20.64 and 20.82 min), proving that the insect-produced *cis*-*Z*-BAE starting material has the (4*S*)-configuration.

Absolute Configuration of A. hilare trans-*BAE*. The enantiomers of *trans*-4-acetyl-1,2-epoxy-1-methylcyclohexane were not separable on the Cyclodex B column, so a different method was used to determine the absolute configuration of the insect-produced *trans*-BAE. Both cis- and trans-BAE could be deoxygenated to (*Z*)-α-bisabolene, and racemic (*Z*)-α-bisabolene was resolved on the Cyclodex column at 90°C (85.70 and 86.36 min). To determine which enantiomer corresponded to which peak, a standard of (4*S*)-(+)-(*Z*)-α-bisabolene was isolated from *Opoponax* spp. oil (Umbelliferae) (Delay and Ohloff, 1979). Thus, *Opoponax* oil (820 mg; Berje Chemical Products, Bloomfield NJ) was Kugelrohr-distilled at room temperature under vacuum (0.02–0.04 mm Hg) to produce a fraction enriched in sesquiterpenes (680 mg, <10% monoterpenes by GC). This fraction was flash chromatographed on a column (∼25 × 2.5 cm id) of 230–400 mesh silica gel (Merck) that had been activated by heating overnight at 120°C and cooled under N_2 to prevent rehydration. The column was eluted sequentially with 400 ml of hexane, and 200 ml aliquots of 0.2, 0.4, 0.6, and 0.8% CH_2Cl_2 in hexane. A fraction

containing 87% (4*S*)-(*Z*)-α-bisabolene was obtained (88 mg). Spectroscopic and GC retention time data matched those of the racemic standard. On the chiral column, coinjection of this enantiomer with the racemic material determined that the (4*S*)-enantiomer corresponded to the earlier-eluting peak.

To deoxygenate the insect-produced BAE's to (*Z*)-α-bisabolene, an aliquot of the BAE-containing fraction of an aeration extract ($\sim$1500 bug-hours) was concentrated to a few μl under N_2, then taken up in 2 ml of CH_2Cl_2 and added to a mixture of Zn dust (200 mg), NaOAc (15 mg), and NaI (44 mg) in 0.5 ml of AcOH. The mixture was stirred under Ar for 4.5 hr, then filtered. The filtrate was diluted with water (1 ml) and extracted with CH_2Cl_2 (3 $\times$ 2 ml). The CH_2Cl_2 extract was washed sequentially with 1M NaOH (2 $\times$ 2 ml) and brine (2 $\times$ 2 ml), dried over Na_2SO_4, and concentrated by rotary evaporation at room temperature. The residue was taken up in hexane and analyzed on the Cyclodex B column, giving a single peak at 85.67 min. Coinjection of this material with the racemic standard resulted in enlargement of the earlier eluting peak, confirming that the insect-derived material was (4*S*)-(*Z*)-α-bisabolene.

Other Chemicals. (*Z*)-(1*R*,2*S*,4*S*)-4-(1′,5′-dimethyl 1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane ((4*S*)-*cis*-*Z*-BAE), (*Z*)-(1*S*,2*R*,4*S*)-4-(1′,5′-dimethyl 1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane ((4*S*)-*trans*-*Z*-BAE), (*E*)-(1*R*, 2*S*,4*S*)-4-(1′,5′-dimethyl 1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane ((4*S*)-*cis*-*E*-BAE), and (*E*)-(1*S*,2*R*,4*S*)-4-(1′,5′-dimethyl 1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane ((4*S*)-*trans*-*E*-BAE) were synthesized by previously described routes (Chen et al., 2000). (*E*)-2-Hexenal, (*E*)-2-hexenyl acetate, (*E*)-2-decenal, and dodecane were obtained from Aldrich Chemical (Milwaukee WI). Tridecane and pentadecane were obtained from ICN-K&K (Plainview, NY). Racemic (*Z*)-α-bisabolene was synthesized as previously described (Buss et al., 1987).

A mixture of 4 bisabolene epoxide isomers was generated to provide standards, by modification of a previously reported method (Tomioka and Mori, 1992). Thus, a solution of 5-bromo-2-methyl-2-pentene (1 g, 6.12 mmol; Aldrich), triphenylphosphine (1.61 g, 6.12 mmol) and diisopropylethylamine (53.3 μL, 0.306 mmol) in acetonitrile (12.5 ml) was refluxed under Ar for 24 hr. After cooling to room temperature and concentration, the residual viscous oil was heated (80°C) and stirred vigourously with benzene (10 ml) under argon. The mixture was cooled to room temperature and the upper benzene layer was pipetted off. After removal of residual benzene under vacuum, THF (22 ml) and DMPU (3 ml, 12.4 mmol) were added to the resulting gummy phosphonium bromide salt, and the mixture was warmed gently until the salt dissolved. $KN(TMS)_2$ (0.5M in toluene, 11 ml, 5.5 mmol) was then added dropwise, and the mixture was heated to 70°C for 15 min, then stirred at room temperature for 1 hr. The resulting deep red solution was cooled to -78°C and a solution of (4*S*)-*trans*-4-acetyl-1,2-epoxy-1-methylcyclohexane (702 mg, 4.56 mmol; Chen et al., 2000) in THF (10 ml) was added dropwise. The mixture was stirred at -78°C for 1 hr, then

warmed to room temperature over 3 hr and quenched with brine (40 ml). The THF was evaporated *in vacuo*, and the residue was extracted with petroleum ether (4×50 ml). The combined organic extracts were washed with water (4×50 ml) and brine (4×50 ml), dried over Na_2SO_4 and concentrated. Methanol (50 ml) was then added, and the extract was again concentrated to remove the residual toluene azeotropically. This procedure was repeated twice. The residue was distilled (Kugelrohr: room temperature to 100°C/0.03 mm Hg) to give a mixture of 4 diasteromeric bisabolene epoxide isomers, unreacted (−)-(4*S*)-*trans*-epoxyketone, and (4*R*)-*cis*-epoxyketone from epimerization of the (4*S*)-*trans*-isomer. The distillate was chromatographed (5% ether in hexane) to give 325 mg (35%) of a mixture of diasteromeric bisabolene epoxides containing 5% (4*S*)-*trans*-*Z*-BAE, 70% (4*R*)-*cis*-*Z*-BAE, 5% (4*S*)-*trans*-*E*-BAE, and 10% (4*R*)-*cis*-*E*-BAE. The *trans*-Z-α-BAE isomer was isolated in pure form from the mixture by HPLC (200×10 mm Econosil column, 10 μ irregular silica particles), eluting with 1% ether in hexane at 2 ml/min.

General Procedures for Y-tube Bioassays. Bioassays were conducted with a vertical glass Y-tube (5 cm ID; bottom arm = 18 cm long, top arms = 15 cm long, Y angle = 90°) with female ground glass joints, each fitted with male joints terminating in a hose nipple. The Y-tube was lit from above with a 1.2 m long light bank containing a Sylvania Octron 3500K F032/735 32 W fluorescent light and a GE F40PL/AQ Plant and Aquarium wide spectrum fluorescent light connected to a high frequency electronic ballast, providing light intensities of 350 lux at the bottom to 600 lux at the top of the Y-tube. Charcoal-purified air was pulled through the apparatus (2.5 liter/min) with house vacuum.

Virgin, sexually mature adults (16 to 18 d old) were tested individually and used only once. Each bug was gently placed inside the bottom male joint of the Y-tube, which was then inserted into the female joint. *A. hilare* are positively phototactic and walked up towards the light. A positive response was classified as a bug crossing a line 12 cm up either arm from the junction within 10 min of the start of the bioassay. Each Y-tube and joint was used once, then washed, acetone rinsed, and oven-dried at 140°C. Bioassays were conducted in a temperature (22–25°C) and humidity controlled room (50–60% RH) from ≈1100 to 1800 hr, 5 to 12 hr after the onset of photophase. Positions of treatments were alternated with each replicate.

Experiment 1. Live Insects as a Pheromone Source. The top joints of the Y-tube were connected to 125 ml sidearm flasks containing one sexually mature virgin bug and a green bean, or a green bean alone. Flasks were positioned so that their contents were not visible to a bug walking up the tube. Ten to 15 min after the flasks were set up, a test bug was introduced into the bottom of the Y-tube and its response was recorded. The flask and bug serving as a pheromone source was replaced every 2–3 replicates to minimize disturbance and production of defensive compounds, which may mask the effect of attractant pheromones. Four separate

experiments were conducted to test attraction of females to males, females to females, males to females, and males to males. Because males were attractive to females, and all other combinations of attractors and responders failed to demonstrate attraction, females were used as responders in all further experiments.

Experiment 2. Extract of Male-Produced Odors as a Pheromone Source. The numbers of virgin female *A. hilare* attracted to 10 bug-hour equivalents of crude extract in pentane (1 bug-hour equivalent = the volatiles produced by 1 bug in 1 hr) *versus* a pentane blank were compared. Extract or solvent controls were loaded on circular filter papers (1.5 cm diam) mounted on wires in the top of each arm. Ten bug-hours of extract contained ~4 μg of (4*S*)-*cis*-*Z*-BAE, the major component in the male extract, as determined by GC.

A portion of the male extract was separated into 4 fractions (see above), and the attractiveness of 10 bug-hour equivalents of each fraction was compared to a control consisting of the pentane: ether mixture in the fraction. Having determined that only the fraction containing (4*S*)-*cis*-*Z*-BAE and (4*S*)-*trans*-*Z*- BAE was attractive to females, this fraction was tested *versus* an equivalent amount of crude extract.

Experiment 3. Synthetic Chemicals as a Pheromone Source. (4*S*)-*Cis*-*Z*-BAE (chemical purity >92.6%; isomeric purity >97.8%, (4*S*)-*trans*-*Z*-BAE not detectable) and (4*S*)-*trans*-*Z*-BAE (chemical purity >88.6%; isomeric purity >97.8%; *cis*-(*Z*)-BAE not detectable) were synthesized as previously described (Chen et al., 2000). Three Y-tube bioassays were conducted, comparing the attraction of females to: 1) 4 μg of (4*S*)-*cis*-*Z*-BAE *vs* a hexane control; 2) 0.2 μg of (4*S*)-*trans*-*Z*-BAE *vs* a hexane control; and 3) a 95:5 blend (4 μg : 0.2 μg) of (4*S*)-*cis*-*Z*-BAE + (4*S*)-*trans*-*Z*-BAE *vs* a hexane control. This ratio approximates that in the natural extract (see below). Chemicals and solvent controls were loaded onto filter paper discs as described above.

Experiment 4. Field Cage Study Using Synthetic Pheromone Components. A 1 × 1 × 1 m field cage, covered on the top and three sides by aluminum screening and on the front by non-reflective cloth screening was set up at Agricultural Operations, University of California, Riverside. The cage was placed over a stiff cardboard platform covered with brown butcher paper. Cardboard strips (35 cm × 1.75 cm), covered on one side by Scotch® tape coated with Fluon®, were taped along the base of each cage wall to hinder bugs crawling up the walls of the cage. Fifteen min before the start of each bioassay, two freshly-cut bouquets of alfalfa (7 stalks each, 40–50 cm in height, stuck into a modeling clay base, also covered with alfalfa leaves and stalks), were placed 0.5 m apart, 0.25 m from two opposite side walls, and 0.5 m from the front and back walls. A 1-m cotton string was saturated with 3.5 ml of pentane containing 1.05 mg of a 95:5 blend of (4*S*)-*cis*-*Z*-BAE (chem. purity >97%, isomeric purity >98%, 1.9% (4*R*)-*trans*-*Z*-BAE present) and (4*S*)-*trans*-*Z*-BAE (chemical purity >98%, no isomeric impurities), and strung from the base of one bouquet to the cage roof immediately above the

bouquet. A string soaked in pentane was applied to the other bouquet. The positions of the pheromone-treated and control strings were interchanged for each replicate.

Thirty min before being introduced into the cage, 32–54 (mean 41, $N = 6$ trials) sexually mature virgin female *A. hilare* were placed with 2 green beans in a 25 × 15 × 5 cm cardboard box with a paper-lined bottom and Fluon®-coated walls. After 20 min, the beans were removed. Two to three min after positioning the pheromone lure, the paper lining with the bugs was lifted out of the box and placed just inside the front wall of the cage, equidistant from each bouquet. After 40 min, the number of females on each bouquet was counted. Trials were repeated on six separate evenings during August 1999, approximately 40 min before sunset.

Statistical Analyses. Y-tube bioassay data were analyzed using chi-square tests with one degree of freedom, using Yates' correction factor (Steel et al., 1997). Because different numbers of bugs were used for each replicate in the field cage study, the proportion of bugs on each bouquet, as a percentage of the total number of bugs on both bouquets, was calculated at the end of each trial. After arcsine transformation (Steel et al., 1997), treatment means were compared using a two-sample *t*-test. For all statistical tests, $\alpha = 0.05$.

RESULTS

In Y-tube bioassays, sexually mature females were attracted to odors from live males (Figure 2). Males were not attracted to odors from live males, nor were odors from live females more attractive than controls to bugs of either sex (Figure 2). Consequently, all subsequent bioassays used only female responders and extracts of male odors, or fractions thereof.

A crude extract of male-produced odors collected on activated charcoal was more attractive to female *A. hilare* than a solvent control (Figure 3). Bioassays using fractions of the extract indicated that only one of the four fractions, the fraction eluted from silica gel with 10% ether in pentane, was significantly attractive to females (Figure 3). In a two-choice assay, this fraction was as attractive as the crude extract (20 insects tested, 10 walked to each treatment, $\chi^2 = 0.025$, $P > 0.05$), suggesting that it contained all of the biologically active pheromone components.

Comparison of gas chromatograms of extracts of volatiles collected from sexually mature virgin males and females (>12 d old) revealed the presence of several male-specific compounds (Figure 4). These compounds were not found in aerations of sexually immature males, nor were they components of the defensive emissions or the metathoracic gland (MTG) contents (see below). Analysis of the biologically active fraction of the extract by GC and GC-MS determined that the

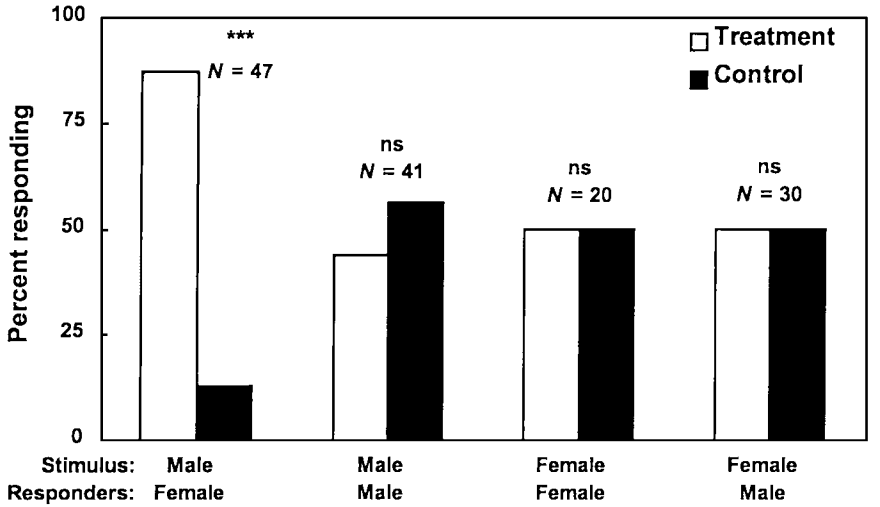


FIG. 2. Results of vertical Y-tube bioassays testing attraction of sexually mature virgin *A. hilare* to odors from sexually mature virgin conspecifics with a green bean *versus* a green bean control. The number of responders in each experiment (N) is indicated above each set of bars. For each experiment, results were analyzed with a χ^2-test. *** = $P < 0.001$, ns = not significant ($P > 0.05$).

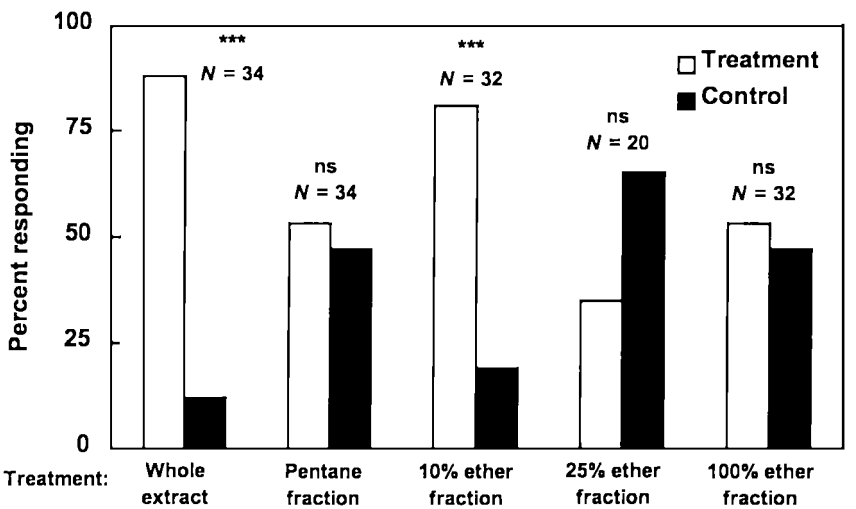


FIG. 3. Results of vertical Y-tube bioassays testing attraction of sexually mature virgin *A. hilare* to an extract or fractions of an extract of odors of *A. hilare* males, *versus* a solvent control. Data were analyzed as described in Fig. 2.

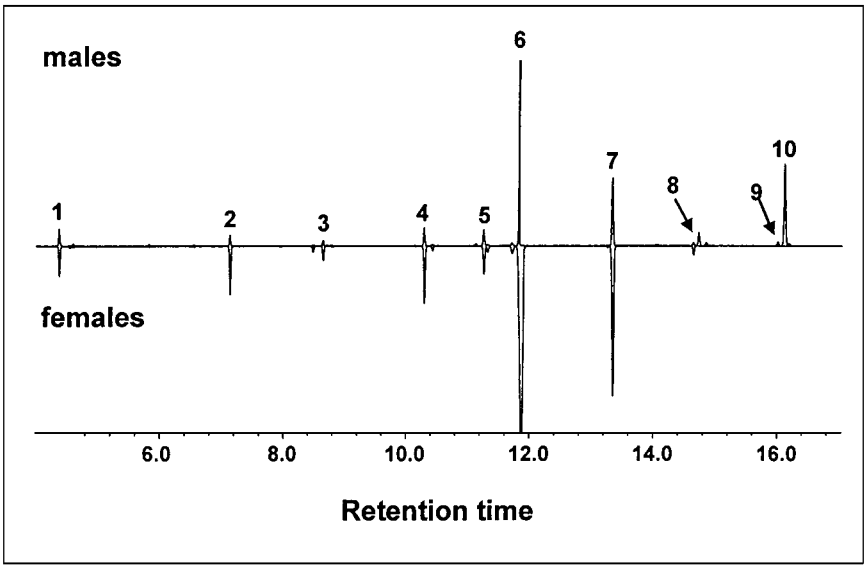


FIG. 4. Gas chromatograms of extracts of odors produced by sexually mature virgin male (top trace) or female (bottom, inverted trace) *A. hilare*, analyzed on a DB-5 column. GC conditions are described in Methods and Materials. Numbered peaks were identified as 1) (*E*)-2-hexenal, 2) (*E*)-2-hexenyl acetate, 3) undecane, 4) dodecane, 5) (*E*)-2-decenal, 6) tridecane, 7) (*E*)-2-decenyl acetate, 8) *Z*-α-bisabolene, 9) *trans*-*Z*-BAE, and 10) *cis*-*Z*-BAE.

major constituents were the two male-specific components, *cis*-*Z*-BAE (major) and *trans*-*Z*-BAE (minor), that had been identified previously by Aldrich et al. (1989). The identifications were confirmed by comparison with the authentic compounds in an extract from male *N. viridula* and finally, by comparisons with synthesized standards (GC retention time matches on DB-5, DB-17, Cyclodex B, and HP5-MS columns; mass spectral matches; GC retention time and mass spectral matches of the products of controlled degradation of the bug-produced BAEs with those of authentic standards, see below). The mean ratio of *cis*-(*Z*)-BAE to *trans*-(*Z*)-BAE in extracts from five cohorts of sexually mature male bugs (mean number of bugs per cohort $\pm$ SD $= 31 \pm 14$) was 95:5 (range = 93:7 to 96:4).

A third male-specific compound, which eluted in the pentane fraction of the crude extract, was identified as (*Z*)-α-bisabolene, as reported previously by Aldrich et al. (1989), by matchup of retention times and the mass spectrum with those of the known compound in the *N. viridula* extract, and confirmed by comparison with an authentic standard. The absolute configuration was determined to be (4*S*) by comparison of its retention time on a chiral Cyclodex B GC column with that of an authentic standard isolated from *Opoponax* oil (Delay and Ohloff, 1979).

Racemic (*Z*)-α-bisabolene gave two distinct but incompletely resolved peaks on this column, the earlier eluting of which was enlarged when the insect-derived and racemic material were coinjected.

The absolute configurations of the *cis*- and *trans*-*Z*-BAE's produced by *A. hilare* were determined indirectly because the enantiomers of *cis*- and *trans*-*Z*-BAE's were not resolved on the Cyclodex B column. *cis*-*Z*-BAE was cleaved by ozonolysis, and the absolute configuration of the resulting *cis*-4-acetyl-1,2-epoxy-1-methylcyclohexane product was determined to be (4*S*) by retention time comparisons on the Cyclodex B column with those of standards of known configuration.

Because the enantiomers of *trans*-4-acetyl-1,2-epoxy-1-methylcyclohexane were not resolved on the chiral column, a different strategy was used to determine the absolute configuration of the insect-produced *trans*-*Z*-BAE. Thus, the insect-produced BAEs were deoxygenated to *Z*-α-bisabolene by treatment with a mixture of Zn/AcOH/AcONa/NaI (Knoll and Tamm, 1975). Analysis of the products on the Cyclodex B column gave one symmetrical peak with no visible shoulder and a retention time matching that of the (4*S*)-enantiomer obtained from *Opoponax* oil (see above). This reconfirmed that the *cis*-*Z*-BAE (the major component in the fraction) had the (4*S*) configuration, and suggested a (4*S*) configuration for the minor *trans*-*Z*-isomer. However, assignment of the absolute configuration of the latter compound remains tentative because of the uncertainty of detecting a small amount ($\sim$5%) of the (4*R*)-enantiomer when the GC peaks from the two enantiomers were not completely resolved. The available evidence suggests that the insect-produced (*Z*)-α-bisabolene, *cis*-*Z*-BAE, and *trans*-*Z*-BAE all have the (4*S*) configuration, but the latter assignment should be confirmed by other methods.

A number of other compounds were identified in aeration extracts from undisturbed or shaken male and female *A. hilare*, and in MTG extracts (Table 1). Neither of the BAEs was detected in the defensive compounds released by disturbed bugs, nor were the BAEs found in the MTG extracts, suggesting that the BAEs are produced from glands other than those used for production and release of the defensive compounds.

Once the identities of the female-attracting male products were confirmed, Y-tube bioassays were continued with synthesized compounds. Neither (4*S*)-*cis*-*Z*-BAE nor (4*S*)-*trans*-*Z*-BAE alone were significantly more attractive than solvent controls. However, a 95:5 blend of the two BAEs, similar to that found in the extracts from males, was more attractive to females than controls (Figure 5), demonstrating that a blend of the two BAEs was essential for attraction.

These observations were verified in replicated field cage trials, in which the 95:5 pheromone blend dispensed from cotton string was more attractive to females than the control (treatment, mean $\pm$ SE, 80.5 $\pm$ 2.5% of the responding bugs, control 19.5 $\pm$ 2.5%, $t = 14.8$, $df = 10$, $P < 0.0001$). From all the bugs released,

TABLE 1. COMPOUNDS IDENTIFIED IN COMPOSITE AERATION EXTRACTS FROM SEXUALLY MATURE VIRGIN *Acrosternum hilare* ADULTS OF BOTH SEXES, AND METATHORACIC GLAND (MTG) EXTRACTS FROM INDIVIDUAL BUGS. AMOUNTS ARE EXPRESSED AS A PERCENTAGE OF THE MOST ABUNDANT COMPOUND, TRIDECANE

	Extract from undisturbed bugs		Extract from shaken bugs		Metathoracic gland extracts[a]	
Compound	Males	Females	Males	Females	Males	Females
(*E*)-2-Hexenal	3.2	2.6	2.7	—	5.6 ± 2.8	7.8 ± 8.3
(*E*)-2-4-Oxo-hexenal	—	—	—	—	10.8 ± 4.5	26.6 ± 16.9
(*E*)-2-Hexenyl acetate	2.2	4.5	1.8	—	1.7 ± 2.6	0.1 ± 0.2
(*E*)-2-Octenal	—	—	—	—	0.2 ± 0.1	0.3 ± 0.3
Undecane	1.1	1.4	—	—	0.3 ± 0.2	0.5 ± 0.6
Dodecane	3.7	5.7	2.1	2.7	2.1 ± 0.3	5.9 ± 6.8
(*E*)-2-Octenyl acetate	—	—	—	—	0.2 ± 0.2	0.4 ± 0.4
(*E*)-2-Decenal	3.4	2.9	4.7	31	9.9 ± 5.6	34.5 ± 27.7
Tridecane	100	100	100	100	100	100
(*E*)-2-Decenyl acetate	14.9	18.4	7.3	—	16.1 ± 3.4	43.4 ± 38.2
Pentadecane	Trace	1	—	—	0.2 ± 0.04	1.1 ± 2.1
Z-α-bisabolene	3.0	—	—	—	—	—
Trans-*Z*-BAE	1	—	—	—	—	—
Cis-*Z*-BAE	20.3	—	—	—	—	—

[a] Mean (±SD) of five separate gland extracts from each sex.

an average of 39.3 ± 5.4% were scattered randomly on the floor, walls, or ceiling of the cage at the end of the trial, and were classified as nonresponders. Bugs generally did not fly except immediately after being introduced into the cage, when a few females flew to the cage walls and ceiling. Most of the remaining females walked to the alfalfa bouquets and climbed up the stalks, where many remained for the duration of the trial.

DISCUSSION

In laboratory bioassays, only female bugs were attracted to odors from live males, indicating that mature males produce a sex pheromone, as was observed for the phytophagous stink bug species *Thyanta pallidovirens* (Millar, 1997; McBrien et al., 1998). In contrast, a number of other pentatomid species (*Nezara viridula*, Harris and Todd, 1980; several *Euschistus* spp., Aldrich et al., 1991; *Biprorulus bibax*, James et al., 1994; *Plautia stali*, Moriya and Shiga, 1984 and Sugie et al., 1996; *Piezodorus hybneri*, Leal et al., 1998) are reported to produce aggregation pheromones attractive to both sexes.

We were able to confirm previous suggestions (Aldrich et al., 1989) that the bisabolene epoxides produced by sexually mature male *A. hilare* were indeed

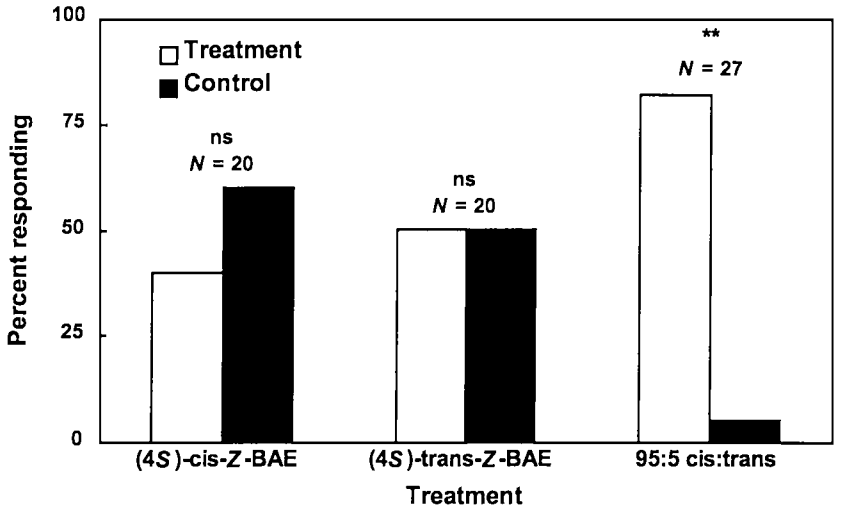


FIG. 5. Results of vertical Y-tube bioassays testing attraction of sexually mature virgin female *A. hilare* to the synthetic pheromone components, (4*S*)-*cis*-*Z*-BAE and (4*S*)-*trans*-*Z*-BAE, or a 95:5 blend of the two components, *versus* solvent controls. Data were analyzed as described in Fig. 2. ** = $P < 0.01$.

the sex pheromone components. Neither (4*S*)-*cis*-*Z*-BAE nor (4*S*)-*trans*-*Z*-BAE alone were attractive to female *A. hilare*, but when combined in the 95:5 ratio naturally produced by males in our Californian population, the blend was attractive to females in both laboratory and field bioassays, demonstrating that the active pheromone consists of at least two components. However, the importance of the specific blend ratio is not clear. The 95:5 ratio of (4*S*)-*cis*-*Z*-BAE to (4*S*)-*trans*-*Z*-BAE produced by Californian males is similar to that reported for male *A. hilare* from Georgia, USA, and also to the ratio produced by males of *A. aseadum* and *A. marginatum* (Aldrich et al., 1989, 1993). In contrast, *A. pennsylvanicum* males produce the two components in an approximately 1:1 ratio (Aldrich et al., 1989). Furthermore, these two components also constitute the sex pheromone of *Nezara viridula*, but the effects of the ratio of the two components produced by males from different populations remains controversial (Baker et al., 1987; Aldrich et al., 1987, 1993; Brézot et al., 1994). To date, large variations in the ratio of the two BAEs between individuals within single populations have confounded attempts to determine the importance of the ratio to *N. viridula* adults (Brézot et al., 1994; Ryan et al., 1995).

The major component produced by *A. hilare* males, *cis*-*Z*-BAE, has the (4*S*) configuration, with indications that the minor *trans*-*Z*-BAE also has the (4*S*) absolute configuration. These compounds have been reported in extracts from several

Nezara and *Acrosternum* species (Baker et al., 1987; Aldrich et al., 1989, 1993), but no attempts to determine the absolute configurations of the insect-produced compounds have been reported previously. There was no evidence to indicate that the third male-produced compound, (4*S*)-(*Z*)-α-bisabolene, is a pheromone component, and the fraction containing this compound was not attractive to female bugs. In addition, the fraction containing the two BAE's was as attractive as the whole extract, indicating that the (4*S*)-(*Z*)-α-bisabolene (and any components in other fractions) had no discernable role as attractant pheromone components. Because many species that produce (*Z*)-α-bisabolene also produce BAEs (Aldrich et al., 1989, 1993), it is unlikely that (*Z*)-α-bisabolene has a role in inhibiting cross-attraction of related species. The obvious structural resemblance between (4*S*)-(*Z*)-α-bisabolene and the BAE's, and the fact that they all have the same configuration at the (4*S*) center suggests that the bisabolene may be a precursor to the pheromone components.

Although the site of production of the BAEs has not been determined for *A. hilare*, they are not produced in the metathoracic glands (MTG), the site of production of the bugs' defensive secretions. The BAEs also are clearly distinct in structure and function from the defensive compounds. The MTG gland contents were not sexually dimorphic, and the compounds were all well-known, common components of hemipteran defensive secretions (Gilby and Waterhouse, 1965; Staddon, 1979; Aldrich, 1988). Significant quantities of these compounds were present in aeration extracts, indicating that they may be released sporadically or continuously at low levels. Alternatively, the relatively crowded conditions of the aeration chambers may have induced low levels of release.

Field cage bioassays using the synthetic pheromone demonstrated that female *A. hilare* approached the pheromone source almost exclusively by walking (McBrien and Millar, 1999). At the time of this study, in late summer, female responses to pheromone were strongest in late afternoon or evening, as has been noted for other pentatomid spp. (Harris and Todd, 1980; Moriya and Shiga, 1984). A significant proportion of the females tested did not appear to respond to the pheromone, but because of the small size of the cage, these females may have already reached their distance of closest approach to the pheromone source. For example, it has been demonstrated that *N. viridula* uses attractant pheromones to bring males and females together on the same plant or substrate. However, once on the same plant, the insects initiate a dialog consisting of substrate-borne vibrational signals, with the male tracking the female's signal along the plant surfaces until he locates her. Periodic vibrational responses by the male while he is searching for the female apparently induce her to continue signalling until she is located by the male (Ota and Čokl, 1991; Miklas et al., 1999). A second *Nezara* species, *N. antennata*, produces analogous signals (Kon et al., 1988), suggesting that this form of short-range communication may be common in the Pentatomidae. Thus, pentatomid sex pheromones may act to attract insects to the vicinity of a

pheromone source, but in the absence of the secondary vibrational cues for short range guidance, insects responding to the pheromone may not actually walk up to and contact the pheromone source.

The presence of host plants or host plant mimics (e.g., vertical silhouettes) also may be important both in attraction of females, and in inducing females that have been attracted to remain near the pheromone source by providing food or shelter. Further studies are needed to clarify possible interactions between host plant cues and the pheromones, and to clarify the relative roles of pheromones and shorter-range substrate-borne vibrational signals in the reproductive behavior of *A. hilare*.

Acknowledgments—We gratefully acknowledge financial support from the California Pistachio Commission, the Washington State Tree Fruit Commission, the California Pear Pest Management Fund, the University of California IPM project, and the USDA Areawide Codling Moth Project. We also thank J. S. McElfresh for assistance in the laboratory, J. R. Aldrich for archived chromatograms and mass spectra of *A. hilare* extracts, and Berje Chemical Products Inc. (Bloomfield NJ) for a gift of *Opoponax* oil.

REFERENCES

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Ann. Rev. Entomol.* 33:211–238.

ALDRICH, J. A., LUSBY, W. R., MARRON, B. E., NICOLAOU, K. C., HOFFMANN, M. P., and WILSON, L. T. 1989. Pheromone blends of green stink bugs and possible parasitoid selection. *Naturwissen.* 76:173–175.

ALDRICH, J. R., HOFFMANN, M. P., KOCHANSKY, J. P., LUSBY, W. R., EGER, J. E., and PAYNE, J. A. 1991. Identification and attractiveness of a major pheromone component for Nearctic *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae). *Environ. Entomol.* 20:477–483.

ALDRICH, J. R., NUMATA, H., BORGES, M., BIN, F., WAITE, G. K., and LUSBY, W. R. 1993. Artifacts and pheromone blends from *Nezara* spp. and other stink bugs (Heteroptera: Pentatomidae). *Zeit. fur Naturforschung* 48C:73–79.

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and LOCKWOOD, J. A. 1987. Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteroptera: Pentatomidae). *J. Exp. Zool.* 244:171–175.

BAKER, R., BORGES, M., COOKE, N. G., and HERBERT, R. H. 1987. Identification and synthesis of (*Z*)-(1′*S*,3′*R*,4′*S*)(−)-2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methylhepta-2,5-diene, the sex pheromone of the Southern Green Stink Bug, *Nezara viridula* (L.). *J. Chem. Soc. Chem. Comm.* 1987:414–416.

BEROZA, M., and BIERL, B. A. 1969. Ozone generator for microozonolysis. *Mikrochim. Acta* 1969: 720–723.

BRÉZOT, P., MALOSSE, C., MORI, K., and RENOU, M. 1994. Bisabolene epoxides in sex pheromone in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Role of *cis* isomer and relation to specificity of pheromone. *J. Chem. Ecol.* 20:3133–3147.

BUSS, A. D., GREEVES, N., MASON, R., and WARREN, S. 1987. Applications of the stereochemically-controlled Horner-Wittig reaction: Synthesis of feniculin, (*E*)-non-6-en-1-ol, a pheromone of the Mediterranean fruit fly, (*E*)- and (*Z*)-dec-5-en-1-ol, trisubstituted alkenes, and (*Z*)-α-bisabolene. *J. Chem. Soc. Perkin Trans. I* 1987:2569–2577.

CHEN, X., GOTTLIEB, L., and MILLAR J. G. 2000. Highly stereoselective syntheses of the sex pheromone components of the southern green stink bug *Nezara viridula* (L.) and the green stink bug *Acrosternum hilare* Say. *Synthesis* 2000:269–272.

CLARKE, R. G., and WILDE, G. E. 1970. Association of the green stink bug and the yeast-spot disease organism of soybeans. II. Frequency of transmission to soybeans, transmission from insect to insect, isolation from field population. *J. Econ. Entomol.* 63:355–357.

ČOKL, A., MCBRIEN H. L., and MILLAR, J. G. 2001. Comparison of substrate-borne vibrational signals of two stink bug species, *Acrosternum hilare* and *Nezara viridula* (Heteroptera: Pentatomidae). *Ann. Ent. Soc. Am.* 94:471–479.

DAANE, K. M., STEFFAN, S. A., YOKOTA, G. Y., and MICHAILIDES, T. J. 2000. Biological investigations of hemipteran pests to improve control and reduce the spread of the fungus *Botryosphaeria dothidea*, pp. 119–120. California Pistachio Industry Annual Report. Crop Year 1999–2000.

da SILVA, P. G., DAANE, K. M., BEEDE, R. H., HOLTZ, B. A., and BENTLEY, W. J. 1996. Biological control of hemipteran pests of pistachio: Appearance, activity and movement of insects in the orchard, pp. 61–66. California Pistachio Industry Annual Report. Crop Year 1995–1996.

DAUGHERTY, D. M. 1967. Pentatomidae as vectors of yeast-spot disease of soybeans. *J. Econ. Entomol.* 60:147–152.

DELAY, F., and OHLOFF, G. 1979. Syntheses and absolute configuration of (*E*)- and (*Z*)-α-bisabolenes. *Helv. Chim. Acta* 62:369–377.

GILBY, A. R., and WATERHOUSE, D. F. 1965. The composition of the scent of the green vegetable bug, *Nezara viridula. Proc. Royal Soc. London. Ser. (B).* 162:105–120.

GREENE, J. K., TURNIPSEED, S. G., SULLIVAN, M. J., and HERZOG, G. A. 1999. Boll damage by southern green stink bug (Hemiptera: Pentatomidae) and tarnished plant bug (Hemiptera: Miridae) caged on transgenic *Bacillus thuringiensis* cotton. *J. Econ. Entomol.* 92:941–944.

HARRIS, V. E., and TODD, J. W. 1980. Male-mediated aggregation of male, female and 5th-instar southern green stink bugs and concomitant attraction of a tachinid parasite, *Trichopoda pennipes. Entomol. Exp. et App.* 27:117–126.

JAMES, D. G., MORI, K., ALDRICH, J. R., and OLIVER, J. E. 1994. Flight-mediated attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to natural and synthetic aggregation pheromone. *J. Chem. Ecol.* 20:71–80.

KNOLL, W., and TAMM, C. 1975. 122. Biosynthese der verrucarine und roridine. Teil 5. Synthese von zwei diastereoisomerenpaaren des [1,8-14C]-α-bisabolols und versuche zu deren einbau in verrucarol. *Helv. Chim. Acta* 58:1162–1171.

KON, M., OE, A., NUMATA, H., and HIDAKA, T. 1988. Comparison of the mating behavior between two sympatric species, *Nezara antennata* and *N. viridula* (Heteroptera: Pentatomidae), with special reference to sound emission. *J. Ethol.* 2:91–98.

LEAL, W. S., KUWAHARA, S., SHI, X., HIGUCHI, H., MARINO, C. E. B., ONO, M., and MEINWALD, J. 1998. Male-released sex pheromone of the stink bug *Piezodorus hybneri. J. Chem. Ecol.* 24:1817–1829.

MCBRIEN, H. L., and MILLAR, J. G. 1999. Phytophagous bugs, pp. 277–304, *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Crops. CAB International, Wallingford, England.

MCBRIEN, H. L., RICE, R. E., MILLAR, J. G., and MCELFRESH, J. S. 1998. Male-produced sex attractant pheromone of the stink bug *Thyanta pallidovirens*, *in* Annual Meeting of the Entomological Society of America, Las Vegas, NV, Nov. 8–12, 1998. Paper 743.

MCPHERSON, J. E. 1982. The Pentatomoidea (Hemiptera) of Northeastern North America with Emphasis on the Fauna of Illinois. Southern Illinois University Press, Carbondale, IL.

MIKLAS, N., RENOU, M., ČOKL, A., and VIRANT-DOBERLET, M. 1999. Songs and pheromone interplay in sexual communication in *Nezara viridula* (Heteroptera: Pentatomidae), *in* 16th Annual Meeting of the International Society of Chemical Ecology, Marseille, Nov. 13–17, 1999. Poster # 111.

MILLAR, J. G. 1997. Methyl (2*E*,4*Z*,6*Z*)-deca-2,4,6-trienoate, a thermally unstable, sex-specific compound from the stink bug *Thyanta pallidovirens. Tetrahedron Lett.* 38:7971–7972.

MILLAR, J. G., and SIMS, J. J. 1998. Preparation, cleanup, and preliminary fractionation of extracts, pp. 1–37, *in* J. G. Millar and K. F. Haynes (eds.). Methods in Chemical Ecology, Vol. 1: Chemical Methods. Chapman and Hall, NY.

MINER, F. D. 1966. Biology and control of stink bugs on soybeans. *Arkansas Ag. Exper. Station Bull.* 708:1–40.

MORIYA, S., and SHIGA, M. 1984. Attraction of the male brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae) for males and females of the same species. *Appl. Entomol. Zool.* 19:317–322.

OTA, D., and ČOKL, A. 1991. Mate location in the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae), mediated through substrate-borne signals on ivy. *J. Insect Behav.* 4:441–447.

PANIZZI, A. R. 1997. Wild hosts of pentatomids: Ecological significance and role in their pest status on crops. *Annu. Rev. Entomol.* 42:99–122.

RICE, R. E., BENTLEY, W. J., and BEEDE, R. H. 1988. Insect and mite pests of pistachios in California. University of California. Division of Agriculture and Natural Resources Publication 21452.

RYAN, M. A., MOORE, C. J., and WALTER, G. H. 1995. Individual variation in pheromone composition in *Nezara viridula* (Heteroptera: Pentatomidae): How valid is the basis for designating "pheromone strains"? *Comp. Biochem. Physiol B* 111:189–193.

SIMMONS, A. M., and YEARGAN, K. V. 1988. Development and survivorship of the green stink bug, *Acrosternum hilare* (Hemiptera: Pentatomidae) on soybean. *Environ. Entomol.* 17:527–532.

STADDON, B. W. 1979. The scent glands of Heteroptera. *Advanced Insect Physiol.* 14:351–418.

STEEL, R. G. D., TORRIE, J. H., and DICKEY, D. A. 1997. Principles and Procedures of Statistics. A Biometrical Approach, 3rd edn. McGraw-Hill, New York.

SUGIE, H., YOSHIDA, M., KAWASAKI, K., NOGUCHI, H., MORIYA, S., TAKAGI, K., FUKUDA, H., FUJIIE, A., YAMANAKA, M., OHIRA, Y., TSUTSUMI, T., TSUDA, K., FUKUMOTO, K., YAMASHITA, M., and SUZUKI, H. 1996. Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Hemiptera: Pentatomidae). *Appl. Entomol. Zool*. 31:427–431.

TOMIOKA, H., and MORI, K. 1992. Simple synthesis of a diastereomeric mixture of 2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methyl-2,5-heptadiene, the pheromone of the green stink bug. *Biosci. Biotech. Biochem.* 56:1001.

TURNIPSEED, S. G., and GREENE, J. K. 1996. Strategies for managing stink bugs in transgenic B.T. cotton, pp. 935–936, *in* Proc. 1996 Beltwide Cotton Conference, National Cotton Council of America, Memphis, TN.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

IDENTIFICATION OF A FEMALE-SPECIFIC, ANTENNALLY ACTIVE VOLATILE COMPOUND OF THE CURRANT STEM GIRDLER*

ALLARD A. COSSÉ,[1,†] ROBERT J. BARTELT,[1] DAVID G. JAMES,[2] and RICHARD J. PETROSKI[1]

[1]*USDA Agricultural Research Service*
National Center for Agricultural Utilization Research
Bioactive Agents Research Unit
1815 N. University Street, Peoria, Illinois 61604

[2]*Irrigated Agriculture Research and Extension Center*
Washington State University
24106 North Bunn Road
Prosser, Washington 99350

(Received November 30, 2000; accepted May 12, 2001)

Abstract—We identified (*Z*)-9-octadecen-4-olide as a female-specific, antennally active compound from the currant stem girdler *Janus integer* Norton. Female specificity was demonstrated by gas chromatographic comparison of liquid chromatography fractions of male and female volatile emissions and whole body extracts. The γ-lactone was identified by coupled gas chromatographic-electroantennographic detection (GC-EAD), coupled gas chromatographic-mass spectrometric (GC-MS) analysis, microchemical reactions, and GC and MS comparison with authentic standards. GC-EAD analysis of female volatile emissions and cuticular extracts showed a single peak of activity on male antennae, which was not present in male-derived materials. Female antennae did not respond to any of the tested materials. The hydrogenation product of the natural EAD-active material was a known saturated γ-lactone. The mass spectrum of the dimethyl disulfide derivative of the natural γ-lactone was consistent with a double bond present in the 9 position. Comparison of the natural γ-lactone and a synthesized racemic mixture of (*Z*)-9-octadecen-4-olide on a chiral GC column showed the presence of a single enantiomer in the natural material.

*Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

†To whom correspondence should be addressed. e-mail: cosseaa@mail.ncaur.usda.gov

0098-0331/01/0900-1841$19.50/0

Key Words—*Janus integer*, sawfly, Hymenoptera, Cephidae, sex pheromone, (*Z*)-9-octadecen-4-olide, coupled gas chromatographic-electroantennographic detection (GC-EAD).

INTRODUCTION

The currant stem girdler, *Janus integer* Norton (Hymenoptera: Cephidae), is an occasional pest of red currant (*Ribes* spp.) in North America (Crassweller et al., 1997). In spring, egg laying female *J. integer* can make numerous punctures in canes, resulting in drooping and wilting of new shoots. Further damage occurs as emerging larvae tunnel within the canes. This insect also attacks poplar and willow trees, and damage to currants usually is more severe near stands of these trees (Crassweller et al., 1997).

Preliminary field observations indicated that caged virgin females attracted male *J. integer* (D. G. James, unpublished data), thus *J. integer* could utilize a female-produced sex pheromone for mate location. Currently, the only studied cephid pheromone is that of the wheat stem sawfly, *Cephus cinctus* Norton (Cossé et al., unpublished data). The pheromone may be beneficial to current pest control practices, especially since insecticides are ineffective against larvae inside the currant stems.

This paper describes the isolation, structure determination, and electroantennographic activity of a female-specific volatile compound and compares this with other sawfly pheromones.

MATERIALS AND METHODS

Insects. Overwintering currant stem girdler larvae present in dormant red currant stems were collected near Prosser, Washington, during January 2000. Larva-containing stems were placed individually into glass vials, capped with a moist piece of cloth-covered cotton, and sent to NCAUR. Upon arrival in Peoria, Illinois, the vials were kept at 25°C under a 16:8 (L:D) hour photoperiod regime. Adults emerged about three weeks later. Adults used in this study were 1- to 4-days old.

Volatile Collections and Cuticular Extracts. One- to three-day volatile collections were made from individuals or groups of 4 male or female insects. Volatiles were trapped on Super Q porous polymer (80–100 mesh, Alltech, Deerfield, IL) or on solid-phase microextraction (SPME) fibers (100 μm poly(dimethyl)siloxane, Supelco, Bellefonte, PA). Volatile collections were made by methods and equipment generally described by Cossé and Bartelt (2000). Cuticular extracts were obtained by a single rub of the abdomen of individual male or female insects with a SPME fiber. In addition, individuals or groups of 8 male or female insects were killed by freezing when 1–4 days old, and soaked overnight in 1 ml hexane.

Hexane extracts were stored at −20°C. Extracts were concentrated under a gentle stream of nitrogen and fractionated on open columns of silica gel (0.5 × 3 cm). Two-milliliter fractions were collected for each of the following elution solvents: hexane; 5%, 10%, and 25% ether in hexane (by volume); and ether. Fractions were concentrated to a volume similar to the non-fractionated samples prior to any further analysis.

Electrophysiology. Coupled gas chromatographic-electroantennographic (GC-EAD) analyses were made by methods and equipment generally described by Cossé and Bartelt (2000). GC-EAD connections were made by inserting a glass-pipette Ag-AgCl-grounding electrode into the back of an excised sawfly head. A second glass-pipette Ag-AgCl-recording probe was placed in contact with the distal end of one antenna. Both pipettes were filled with Beadle-Ephrussi (Ephrussi and Beadle, 1936) saline.

Instrumentation. Volatile collections, extracts, and liquid chromatography (LC) fractions were analyzed by gas chromatography with flame-ionization detection (GC-FID) and coupled GC-mass spectrometry (GC-MS, electron impact and chemical ionization). Samples were injected in splitless mode using Hewlett Packard 6890 instruments fitted with 30 meter EC-1 capillary columns (0.25 mm I.D., 0.25 μm film thickness, Alltech, Deerfield, IL). Temperature programs were from 50°C to 275°C at 10°C per min (GC-MS) or at 15°C per min (GC-EAD). Injector temperatures were maintained at 250°C, and GC-EAD effluent interface from post-column splitter was kept at 275°C. Injectors were fitted with SPME injector liners (Supelco, Bellefonte, PA) for SPME analysis. Mass spectrometry was performed with a Hewlett Packard 5973 instrument (electron impact, 70 eV). Chemical ionization spectra (isobutane) were obtained on a Thermo Quest Trace GC 2000 quadrupole mass spectrometer. The Wiley mass spectral library, with 275,821 spectra, was available on the MS data system (Wiley, 1995). Chiral resolution was achieved on a capillary column having a diacetyl-derivatized β-cyclodextrin phase, β-DEX 225 (30 m length, 0.25 mm ID, 0.25 μm film thickness; Supelco, Bellefonte, PA) operated at 200°C.

Microchemical Reactions. Hydrogenation of GC-EAD-active LC fractions was used to confirm the number of carbon-carbon double bonds. Samples (100 μl) were stripped to dryness under a stream of nitrogen and resuspended in 100 μl ethanol to which was added ∼0.5 mg of 10% Pd on charcoal. Reduction was accomplished by bubbling a gentle steam of hydrogen through the sample for 5 min at room temperature. The reduced sample was filtered and analyzed by GC-MS and GC-EAD.

Dimethyl disulfide (DMDS) derivatives were prepared for determination of double bond locations of EAD-active material (Carlson et al., 1989). Samples (100 μl) were stripped to dryness under a stream of nitrogen, and DMDS and 5% iodine in ether were added (equal volumes, about 25 μl each). The samples were heated at 45°C for 1–2 hr, then diluted with hexane and worked up with

aqueous sodium thiosulfate to destroy the iodine. The organic layer was dried over sodium sulfate, evaporated under nitrogen, resuspended in about 10 μl hexane, and analyzed by GC-MS. Oven temperature was programmed up to 300°C, and MS scanning range was 40–600 amu.

The antenally active 10% ether/hexane LC fractions contained two major free fatty acids, linoleic- and oleic acid, which were methylated to the corresponding methyl esters with diazomethane. Approximately 5 mg of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald, Aldrich, Milwaukee, WI) were added to a 1:1 mixture of ether and a mixture of 5% aqueous KOH and methanol (1:1). Ten micro liters of the yellow ether layer were added to 100 μl of the EAD-active LC fraction. After 5 min, the mixture was evaporated and the residue dissolved in hexane for GC-MS and GC-EAD analysis.

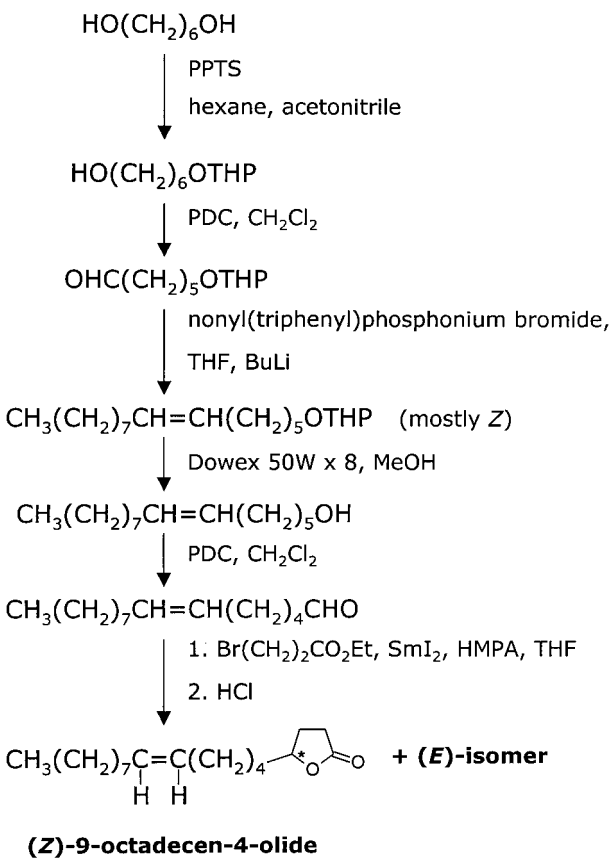


FIG. 1. Synthesis of (*Z*)-9-octadecen-4-olide (see text for abbreviations).

Synthesis of (Z)-9-octadecen-4-olide. A preliminary synthesis to confirm structure was conducted by one of us (RJP), based on well-known reactions (Figure 1). One hydroxy group of 1,6-hexanediol (Aldrich, Milwaukee, WI) was protected as a tetrahydropyranyl (THP) ether by using pyridinium *p*-toluenesulfonate (PPTS) as catalyst (Miyashita et al., 1977). Monoprotection of the diol was accomplished by modification of the published procedure (Miyashita et al., 1977). In a biphasic mixture of acetonitrile and hexane, the reactants were primarily in the more polar (acetonitrile) phase, but the mono ether migrated to the hexane phase, reducing the chance of further derivatization to the diether. The free hydroxyl was oxidized with pyridinium dichromate (PDC) in CH_2Cl_2 to give the aldehyde (Corey and Schmidt, 1979). The aldehyde was reacted with nonyl(triphenyl)phosphonium bromide to give, predominantly, the *Z*-olefin (Sonnet, 1974). The THP protective group was easily removed by acid catalysis (Dowex 50 W $\times$ 8) in MeOH (Beier and Mundy, 1979). The alcohol was oxidized to the aldehyde as described above. Samarium iodide mediated the coupling of $Br(CH_2)_2CO_2Et$ with the aldehyde, to form the 4-hydroxy ester. This cyclized to the desired γ-lactone during the acidic workup (Otsubo et al., 1987). The SmI_2-induced coupling of an unactivated organic halide with an aldehyde is promoted by hexamethylphosphoric triamide (HMPA). Both enantiomers are formed.

RESULTS AND DISCUSSION

Cuticular Extracts and Volatile Collections. Comparisons of the GC profiles of extracted materials obtained by SPME rub and hexane soak showed a high degree of similarity (Figure 2). GC-EAD analyses ($N = 15$) of cuticular extracts (SPME rub and hexane soak) from individuals or groups of male or female insects were obtained by using male and female antennae. EAD activity occurred consistently at one GC retention time (21.8 min) when cuticular extracts from female insects ($N = 6$) (Figure 2) were tested on male antennae, but not on female antennae. Male and female antennae showed no EAD activity with cuticular extracts derived from males ($N = 9$). Similarly, GC-EAD analyses ($N = 10$) of volatiles collected (Super Q and SPME) from individuals or groups of female insects ($N = 5$) consistently indicated the same GC retention time, with EAD activity only on male antennae. Male and female antennae showed no EAD activity with volatiles derived from males ($N = 5$).

Liquid Chromatography. The EAD-activity of female cuticular extracts eluted from silica gel with 10% ether in hexane, suggested an oxygenated compound for the EAD-active material. No other antennal activity was noted for any of the other silica gel fractions (Figure 3).

Comparison of GC-MS analyses of 10% ether/hexane fractions of male versus female cuticular extracts demonstrated a female specific compound, as shown in

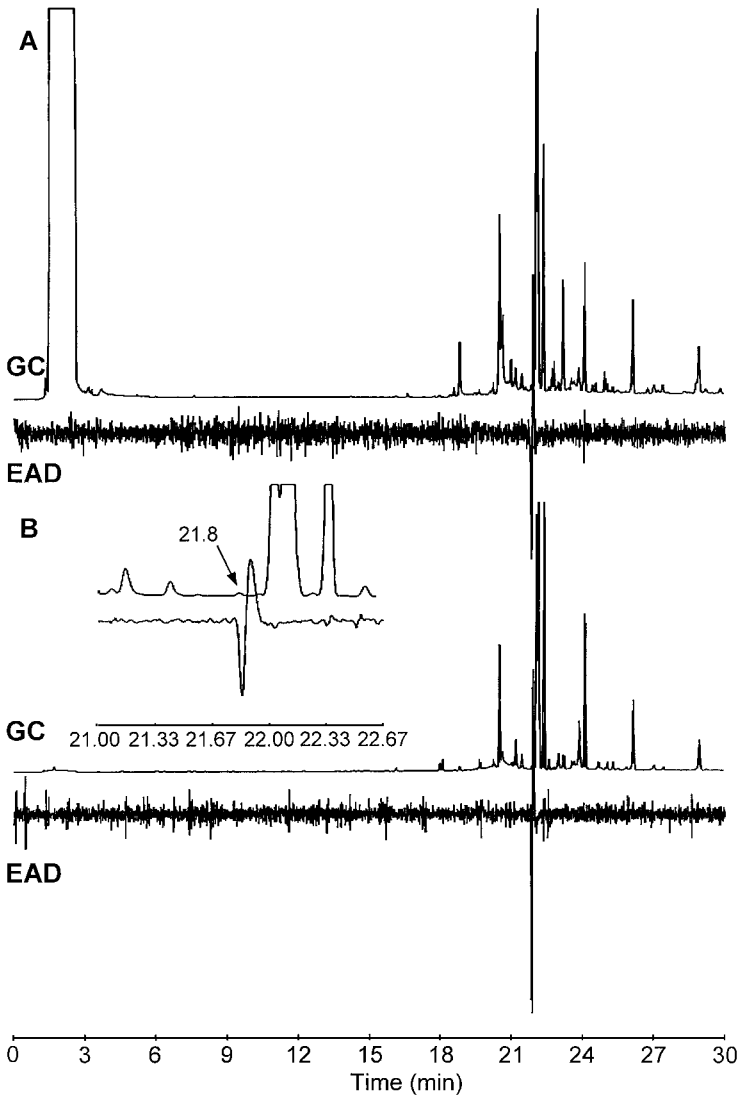


FIG. 2. Simultaneously recorded gas chromatographic (GC) and electroantennographic detection (EAD) of typical male antennae to a hexane extract of female *J. integer* (A) and a SPME cuticular rub of female *J. integer* (B). Insert shows the GC retention time of EAD-activity.

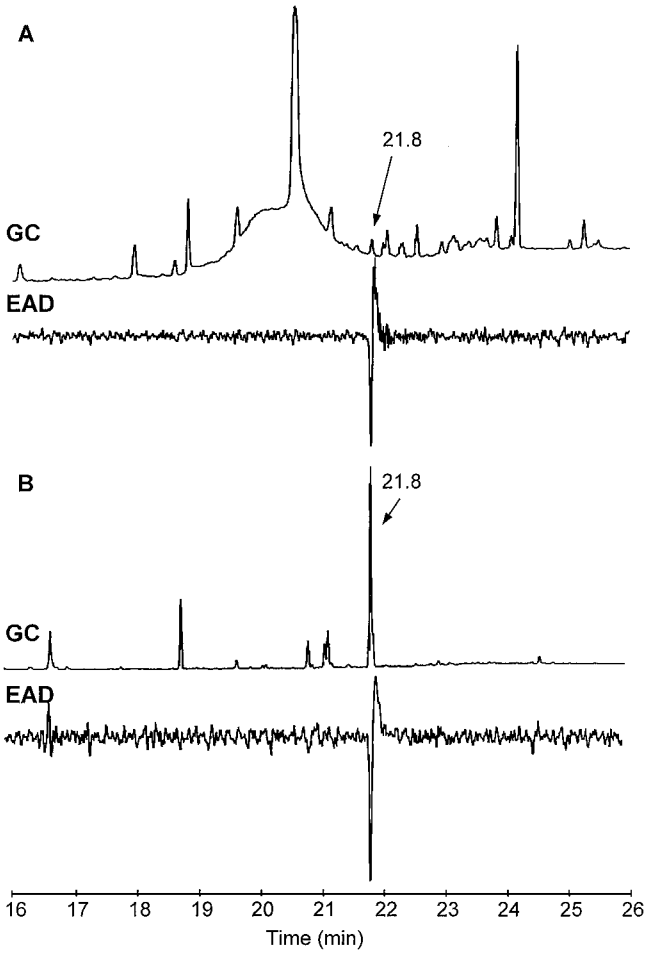


FIG. 3. Simultaneously recorded gas chromatographic (GC) and electroantennographic detection (EAD) of typical male antennae to liquid chromatography fraction (10% ether in hexane) of female *J. integer* hexane extract (A), and synthetic (*Z*)-9-octadecen-4-olide (B). Arrows denotes the GC retention times of EAD-activity.

Figure 4. This compound corresponded to the EAD-active material, based on comparisons of GC-MS and GC-EAD elution profiles (same column, but different temperature ramps).

The mass spectrum of the EAD-active compound indicated a molecular weight of 280 (Figure 5), which was confirmed by the chemical ionization mass spectrum (base peak at m/z 281), but its EI spectrum did not match any of the spectra in the MS library.

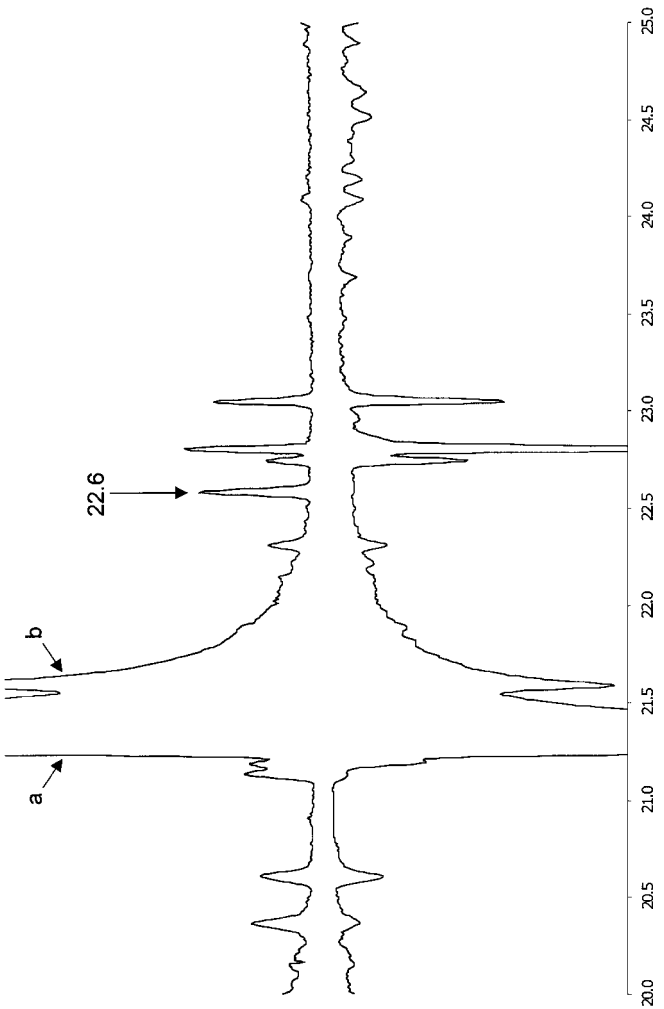


FIG. 4. The gas chromatographic comparison of liquid chromatography fractions (10% ether in hexane) of female (upper) and male (lower) *J. integer* hexane extracts. Arrows denotes the presence of linoleic (a) and oleic acids (b).

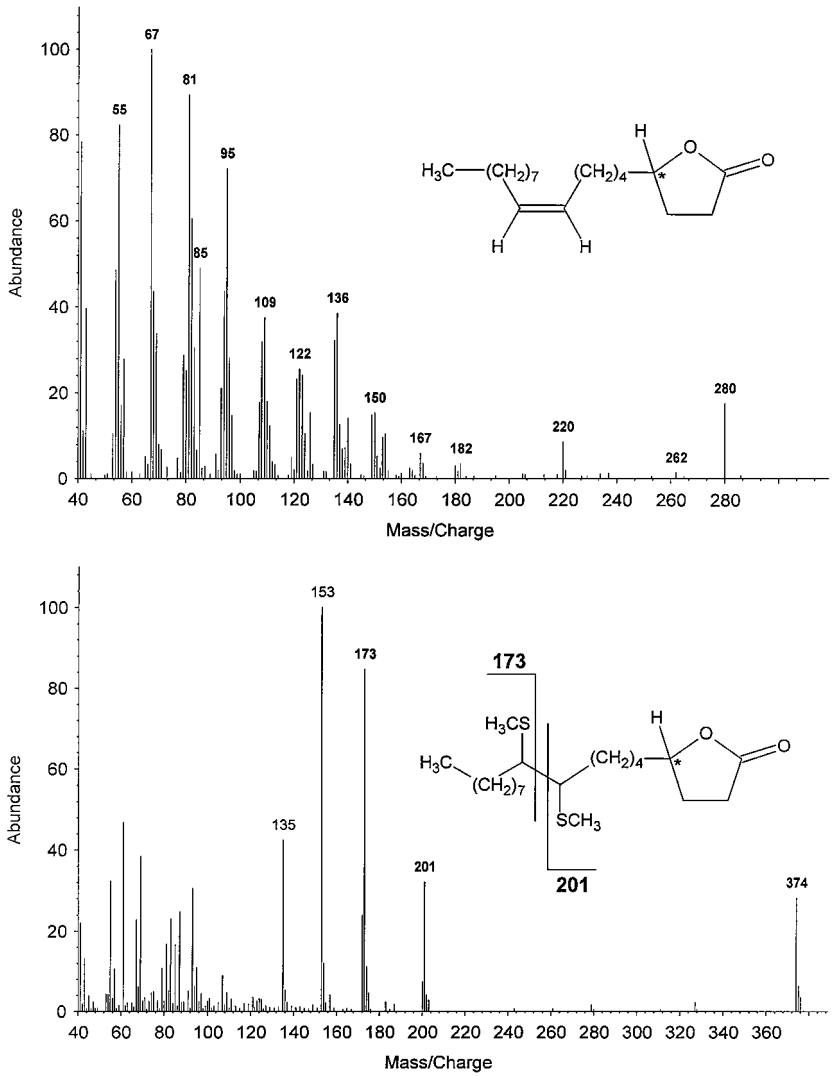


FIG. 5. EI-Mass spectrum of (Z)-9-octadecen-4-olide from female *J. integer* (upper) and EI-mass spectrum of the DMDS derivative of this compound, showing origins of major fragments (lower).

A molecular weight of 280 suggested $C_{19}H_{36}O$ (2 double bonds or ring equivalents) or $C_{18}H_{32}O_2$ (3 double bonds or ring equivalents) as likely molecular formulas. In fact, the silica gel fraction with the female-specific compound contained two relatively abundant fatty acids, which were identified by MS as

linoleic- and oleic acid (Figure 5). The female 10% ether/hexane fraction was treated with several chemical reagents to further elucidate the chemical composition of this EAD-active material.

Methylation. The addition of diazomethane to the female 10% ether/hexane fraction converted the carboxylic acids to the corresponding methyl esters, but did not affect the EAD-active compound, indicating it did not have a free carboxyl group.

Hydrogenation. The mass spectrum of the EAD-active compound after hydrogenation showed a molecular ion at m/z 282, indicating the presence of one carbon-carbon double bond. Furthermore, the MS library picked γ-stearolactone as a likely match for the hydrogenation product. This MS library match was confirmed by comparison of the mass spectra and GC retention times of the hydrogenation product and an authentic sample of γ-stearolactone (Cermak and Isbell, 2000). Similar to the natural product, this synthetic standard also had a fragment ion at m/z 85, which would be expected for a γ-lactone (cleavage where side chain joins ring), suggesting that the natural compound was probably a γ-lactone with one carbon-carbon double bond in the side chain; the fragment ion at m/z 85 would not be present if the carbon-carbon double bond was residing in a ring. The presence of a double bond might also be important for antennal activity, because GC-EAD analysis of the hydrogenation product showed a complete loss of antennal activity. In addition, GC-EAD analysis prior to the diazomethane treatment showed no antennal activity for the oleic acid; surprisingly however, the methyl ester of oleic acid was antennally active.

DMDS Derivatization. The mass spectrum of the DMDS (MW = 94) derivative of the EAD-active compound is presented in Figure 5. The readily formed derivative showed a molecular ion at m/z 374 (= 280 + 94), and the fragment ions (m/z 173 and 201) were consistent with a double bond being present in the 9 position.

Synthetic (Z)-9-octadecen-4-olide. The synthetic γ-lactone matched the natural female-derived *J. integer* compound exactly by mass spectrum, mass spectrum of DMDS derivative, and by GC-EAD analyses (Figure 3). The Wittig synthesis (Figure 1) created both the (*Z*) and (*E*) configurations at the double bond. The *Z*-isomer was recognized because it eluted slightly earlier by GC on the non-polar column (*Z*-isomer at 21.82 min, *E*-isomer at 21.87 min) and because it was more abundant (ca. 70:30), as expected for the Wittig reaction conditions (Sonnet, 1974). GC analysis established that the natural material contained only the earlier-eluting *Z*-isomer, and by GC-EAD the synthetic *Z*-isomer was antennally active (Figure 6). The synthetic γ-lactone was synthesized as a racemic mixture and its two enantiomers showed base-line separation on the chiral GC column (Figure 7). The earlier eluting enantiomer had the same GC retention time as the natural compound. Future chiral synthesis of the γ-lactone will determine the absolute configuration of the natural γ-lactone.

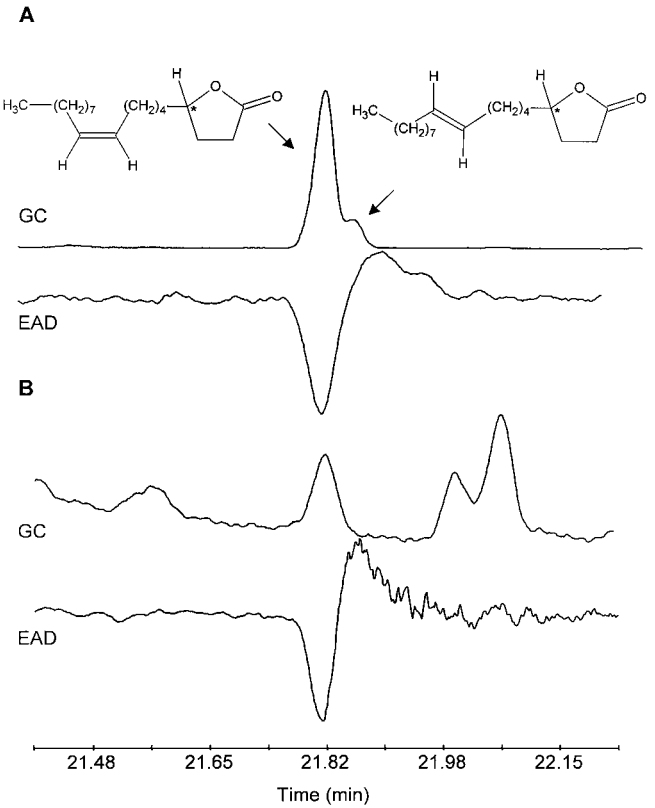


FIG. 6. Simultaneously recorded gas chromatographic (GC) and electroantennographic detection (EAD) of typical male antennae to an isomer mixture of synthetic (*Z*)-, and (*E*)-9-octadecen-4-olide (A) and liquid chromatography fraction (10% ether in hexane) of female *J. integer* hexane extract (B).

Based on GC-FID peak areas and fraction volumes, the estimated maximum release rate of natural γ-lactone was 10 ng/female/day.

Sawfly Sex Pheromones. Forthcoming behavioral tests will determine whether this female-specific γ-lactone of *J. integer* is capable of attracting male sawflies and, thus, be confirmed as a sex pheromone.

Little is published on pheromone systems of sawflies belonging to the family Cephidae. The only studied cephid pheromone is that of the wheat stem sawfly *Cephus cinctus* Norton (Cossé et al., unpublished data).

Insect Specific γ-Lactones. Sex pheromones of sawflies belonging to the family Diprionidae and to one species belonging to the family of Tenthredinidae are well-studied (Bartelt and Jones, 1983; Bartelt et al., 1983; Anderbrant, 1999). However, no γ-lactone containing compounds have been reported as sawfly

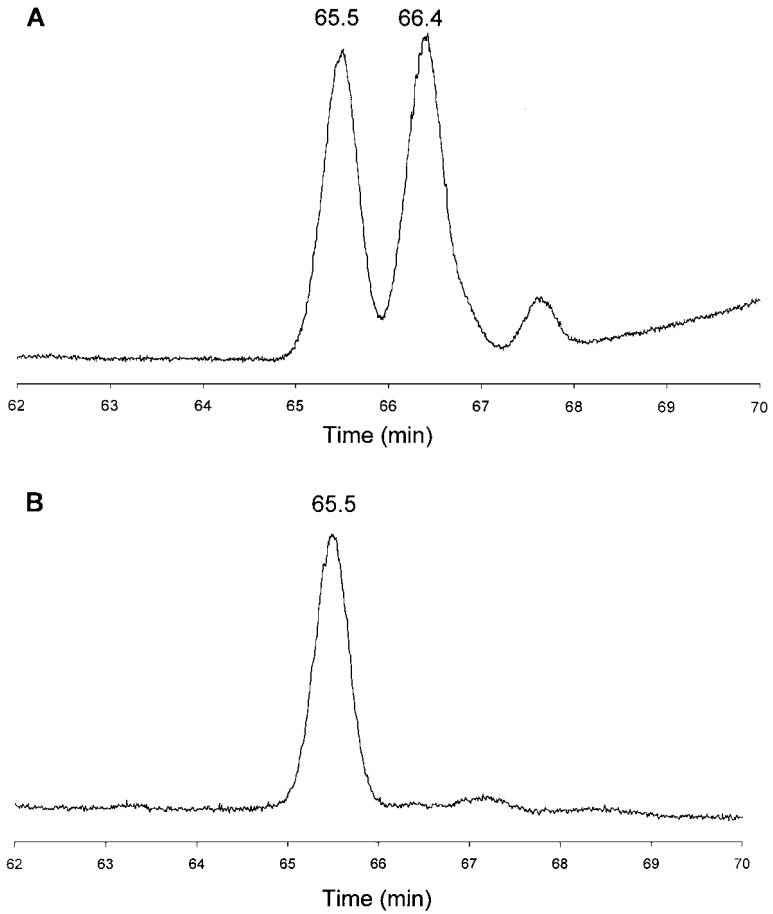


FIG. 7. Chiral separation by gas chromatography of racemic (*Z*)-9-octadecen-4-olide (A) and (*Z*)-9-octadecen-4-olide (B) from female *J. integer*.

pheromones, although related γ-lactones are known as scarab beetle pheromones (Tumlinson et al., 1977; Leal, 1999). For example, the sex pheromone of the yellowish elongate chafer, *Heptophylla picea* Motschulsky was identified as (*R*,*Z*)-7,15-hexadecadien-4-olide and is probably biosynthesized from stearic acid (Leal et al., 1996). Similarly, fatty acid derivatives might be involved in the proposed sex pheromone of currant stem girdler.

Acknowledgments—We thank the following colleagues at NCAUR, Peoria, Illinois: Ronald D. Plattner for assistance in obtaining CI mass spectra and Terry Isbell for the sample of γ-stearolactone.

REFERENCES

ANDERBRANT, O. 1999. Sawflies and seed wasps, pp. 199–226 *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated With Agricultural Plants. CABI Publishing, Wallingford, U.K.

BARTELT, R. J., and JONES, R. L. 1983. (*Z*)-10-Nonadecenal: A pheromonally active air oxidation product of (*Z,Z*)-9-19 dienes in yellowheaded spruce sawfly. *J. Chem. Ecol.* 9:1333–1341.

BARTELT, R. J., JONES, R. L., and KRICK, T. P. 1983. (*Z*)-5-Tetradecen-1-ol: A secondary pheromone of the yellowheaded spruce sawfly, and its relationship to (*Z*)-10-Nonadecenal. *J. Chem. Ecol.* 9:1343–1352.

BEIER, R., and MUNDY, B. P. 1979. A facile removal of the tetrahydopyranyl proctecting group from alcohol derivatives. *Synth. Comm.* 9:271–273.

CARLSON, D. A., ROAN, C.-S., YOST, R. A., and HECTOR, J. 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* 61:1564–1571.

CERMAK, S. C., and ISBELL, T. A. 2000. Synthesis of δ-stearolactone from oleic acid. *JAOCS* 77:243–248.

COREY, E. J., and SCHMIDT, G. 1979. Useful procedures for the oxidation of alcohols involving pyridinium dichromate in aprotic media. *Tetrahed. Lett.* 5:399–402.

COSSÉ, A. A., and BARTELT, R. J. 2000. Male-produced aggregation pheromone of *Colopterus truncatus*: Structure, electrophysiological and behavioral activity. *J. Chem. Ecol.* 26:1735–1748.

CRASSWELLER, R. M., GOULART, B., FELLAND, C., HULL, L., SAUNDERS, M., THURSTON, S., BRITTINGHAM, M., TRAVIS, J., RYTTER, J., HALBRENDT, J., HOCK, W., MUZA, A., and DAUM, D. 1997. Small-scale fruit production: A comprehensive guide. Pennsylvania State University, University Park, Pennsylvania. Pennsylvania State University Cooperative Extension, Publication AGRS-60.

EPHRUSSI, B., and BEADLE, G. W. 1936. A technique of transplantation for *Drosophila*. *Am. Nat.* 70:218–225.

LEAL, W. S. 1999. Scarab beetles, pp. 51–68 *in* J. Hardie and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated With Agricultural Plants. CABI Publishing, Wallingford, U.K.

LEAL, W. S., KUWAHARA, S., ONO, M., and KUBOTA, S. 1996. (*R,Z*)-7,15-Hexadecadien-4-olide, sex pheromone of the yellowish elongate chafer, *Heptophylla picea*. *Bioorg. Med. Chem.* 4:315–321.

MIYASHITA, N., YOSHIKOSHI, A., and GRIECO, P. A. 1977. Pyridinium *p*-toluenesulfonate. A mild and efficient catalyst for the tetrahydropyranylation of alcohols. *J. Org. Chem.* 42:3772–3775.

OTSUBO, K. KAWAMURA, K., INANAGA, J., and YAMAGUCHI, M. 1987. A direct synthesis of γ-, δ-, and ϵ-lactones utilizing SmI_2-induced Barbier-type reaction in the presence of hexamethylphosphoric triamide (HMPA). *Chem. Lett.* 7:1487–1490.

SONNET, P. E. 1974. *cis*-Olefins from the Wittig reaction. *Org. Prep. Proced. Int.* 6:269–273.

TUMLINSON, J. H., KLEIN, M. G., DOOLITTLE, R. E., LADD, R. E., and PROVEAUX, A. T. 1977. Identification of the Japanese beetle sex pheromone: Inhibition of male response by an enantiomer. *Science* 197:789–792.

WILEY Spectral Library [CD-ROM], 6th edn. 1995. Wiley, New York.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

ISOLATION, IDENTIFICATION AND FIELD TESTS OF THE SEX PHEROMONE OF THE CARAMBOLA FRUIT BORER, *Eucosma notanthes*

C. C. HUNG,[1,2] J. S. HWANG,[2] M. D. HUNG,[3] Y. P. YEN,[4] and R. F. HOU[1,*]

[1]*Department of Entomology*
National Chung Hsing University
Taichung, Taiwan, R.O.C.

[2]*Department of Biopesticide*
Taiwan Agricultural Chemicals and Toxic
Substances Research Institute (TACTRI)
Wufeng, Taichung, Taiwan, R.O.C.

[3]*Department of Agricultural Chemistry, TACTRI*

[4]*Department of Applied Chemistry*
Providence University
Shalu, Taichung, Taiwan, R.O.C.

(Received June 11, 1999; accepted April 25, 2001)

Abstract—Two components, (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac) and (*Z*)-8-dodecenol (*Z*8-12:OH), were isolated from sex pheromone glands of the carambola fruit borer, *Eucosma notanthes*, and were identified by GC, and GC-MS, chemical derivatization, and comparison of retention times. The ratio of the alcohol to acetate in the sex pheromone extracts was 2.7. However, synthetic mixtures (1 mg) in ratios ranging from 0.5 to 1.5 were more effective than other blends in trapping male moths in field tests.

Key Words—Carambola fruit borer, *Eucosma notanthes*, sex pheromone, (*Z*)-8-dodecenyl acetate, (*Z*)-8-dodecenol.

INTRODUCTION

The carambola fruit borer, *Eucosma notanthes* Meyrick (Lepidoptera: Tortricidae), is a key pest of carambola (also known as star fruit), *Averrhoa carambola* L., in Taiwan (Ho, 1985). This insect has 8 generations per year and may attack carambola fruits all year. Its population density remains high from July to

*To whom correspondence should be addressed. e-mail: rhou@dragon.nchu.edu.tw

0098-0331/01/0900-1855$19.50/0

November. At fruiting time, the moths lay eggs on the surface, and the larvae penetrate into the fruits, causing severe damage. Damaged fruits drop or become unedible and unmarketable. Although farmers usually apply insecticides or bag the fruits to protect them from attack by fruit flies and borers, the fruit damage rate varies from 29 to 77% (Ho, 1988a,b).

Recently, (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac) was found to be an effective sex attractant for this borer in field tests and laboratory bioassays (Hwang et al., 1987, 1996; Hwang and Hung, 1994). High concentrations of this component used as a mating disruptant resulted in significant control of the borer (Hwang and Hung, 1997). Therefore, *Z*8-12:Ac was suspected to be one of the sex pheromone components of the carambola fruit borer. However, the natural sex pheromone components and their blend for this borer were still not yet known. In this study, two components of the sex pheromone were extracted from calling virgin females and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Attraction of the moth to different blend ratios of the sex pheromone components was determined in field tests.

METHODS AND MATERIALS

Insects. Experimental colonies of *E. notanthes* were initially collected from carambola orchards at Changhua, Taiwan. Larvae were mass-reared on a diet at $25 \pm 1^{\circ}$C, $70 \pm 5\%$ relative humidity, and 12 hr photoperiod (Hung and Hwang, 1991). Sexes were identified at the pupal stage and maintained separately in 200-ml plastic jars until eclosion. Adult moths were supplied with 5% honey in water and maintained in plastic bags (30×40 cm).

Preparation of Sex Pheromone Gland Extracts. At the maximum calling and mating time between 1–3 hr after lights on, ovipositors of 1–4-day-old virgin females were excised (Hung et al., 1997). Pheromone glands were immersed in *n*-hexane for 5 sec and the extract was stored at -19°C.

Sources and Purity of Authentic Samples. Authentic samples used for comparison and bioassay were as follows: decanol (Lancaster, 98%), dodecyl acetate (Aldrich, 97%), (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) (Instituut voor Planteziektenkundig Onderzoek, the Netherlands, >99%), (*Z*)-8-dodecenyl acetate (*Z*8-12:Ac) (Sigma, 96.8%), (*Z*)-8-dodecenyl acetate (Instituut voor Planteziektenkundig Onderzoek, >99%), (*Z*)-9-dodecenyl acetate (*Z*9-12:Ac) (Instituut voor Planteziektenkundig Onderzoek, >99%), (*E*)-8-dodecenyl acetate (*E*8-12:Ac) (Sigma), dodecanol (12:OH) (Lancaster, 97%), (*E*)-8-dodecenol (*E*8-12:OH) (Chemtech B. V., the Netherlands), (*Z*)-8-dodecenol (*Z*8-12:OH) (Instituut voor Planteziektenkundig Onderzoek, >99%), 1-tetradecanol (Fluka, 97%), 1-hexadecanol (Aldrich, 99%), 1-octadecyl acetate (TCI, 99%) and 1-octadecanol (Lancaster,

97%). Three additional authentic samples, decyl acetate, 1-tetradecyl acetate and 1-hexadecyl acetate, were synthesized from decanol, 1-tetradecanol, and 1-hexadecanol, respectively.

Chemical Analysis. Gas chromatography analyses were performed on a Hewlett-Packard 5890A GC (Avondale, Pennsylvania) with a capillary DB-Wax column (30 m $\times$ 0.25 mm; 0.25-μm; J&W Scientific, Folsum, CA). The oven temperature was set at 150°C for 20 min, then increased at 8°C/min to final temperature, 240°C. GC-MS spectra were recorded on a Finnigan MAT GCQ system equipped with an ion trap mass analyzer and a DB-Wax capillary column. The temperature program was the same as above. Electron impact (EI) and chemical ionization (CI) mass spectral data of natural and authentic compounds were recorded at 70 eV with the transfer line at 230°C and the ion source at 200°C. Methane was used as a reagent gas for CI analysis. The extract solution of 52,820 ovipositors prepared as described above was subsequently concentrated to 1 ml, and analyzed with the external standard method.

Derivatization. Retention times and retention indices were determined with (*Z*)- and (*E*)-8-12:Ac, and (*Z*)- and (*E*)-8-12:OH as described above in GC analysis. Double bond position and configuration were determined by the mass spectral fragmentation patterns of their dimethyl disulfide (DMDS) derivatives, which were synthesized following the procedure of Buser et al. (1983) and Leonhardt and DeVilbiss (1985). GC-MS was equipped with a CP-Sil 8 CB fused silica capillary column (Chrompack, the Netherlands) (30 m $\times$ 0.25 mm; 0.25 μm). Chromatography was carried out with a temperature program initially at 80°C for 2 min, then increasing at 20°C/min to 140°C, followed by increasing at 4°C/min to 240°C.

Field Tests. To verify the attractiveness of the pheromone components to males of *E. notanthes* and to develop an optimized trap lure, seven field experiments were conducted. The numbers of males captured in traps baited with different components or blend ratios of two authentic compounds were calculated and evaluated. In an attempt to realize the relationship between the attractiveness and the double bond position in the components, three analogs, *Z*7-12:Ac, *Z*8-12:Ac and *Z*9-12:Ac, were used for attraction tests. In addition, both *Z*8-12:Ac and *Z*8-12:OH were suspected to be the main pheromone components; therefore, various blends of these two components were also tested. Since the synthetic *Z*8-12:Ac always contains an impurity of *E*8-12:Ac, which could affect the attractiveness of *Z*8-12:Ac, 7 blends of *Z*8-12:Ac and *E*8-12:Ac were examined.

Field tests were conducted in carambola orchards at Changhua, Taiwan, between August 1996 and October 1997. Each lure comprising 1 mg of content was absorbed on a rubber septa (Aldrich, *Z*-12435-4) and glued to a wing sticky trap (Taiwan Jia-Fu Co.) (Hung et al. 1999). Each trap was hung at 180–200 cm above the ground, and spaced 1–2 trees (10 $\sim$ 15 m) between traps. A randomized

complete block design was used for the field tests. Four orchards spaced 100 ~ 200 m apart were used as blocks. The area of each orchard was between 0.2 and 0.5 ha. Each orchard had all treatments including pheromone formulations and blank. Treatments within a block were randomized. Traps were checked weekly for 4 ~ 8 weeks and treatments within a block were rotated one position each time (Hwang et al., 1987; Hwang and Hung, 1994).

Statistical analysis was conducted with the SAS system. Trap catch data were subjected to an analysis of variance, and treatment means were separated by Duncan's multiple range test.

In the first experiment, each of the three isomers, i.e., *Z*7-12:Ac, *Z*8-12:Ac and *Z*9-12:Ac, was tested. Each isomer was mixed individually with *Z*8-12:OH in a ratio of 1 : 1 and tested for the second experiment. In the third experiment, *Z*8-12:Ac and *Z*8-12:OH were assayed individually in three orchards. In the fourth experiment, five different ratios of *Z*8-12:Ac to *Z*8-12:OH were tested, namely, 100 : 0, 100 : 1, 100 : 100, 100 : 200, and 0 : 100. In the fifth and sixth experiments, ratios of *Z*8-12:Ac and *Z*8-12:OH were reevaluated in order to obtain the optimum ratio of these two components for maximum capture of males in pheromone-baited traps. Additionally, five and three ratios of *Z*8-12:Ac and *Z*8-12:OH in 100 : 5, 100 : 10, 100 : 50, 100 : 100, 100 : 150, and 100 : 100, 100 : 150, 100 : 270, respectively, were assayed. In the last experiment, seven ratios of *Z*8-12:Ac and *E*8-12:Ac in 100 : 0, 99.5 : 0.5, 98 : 2, 96 : 4, 94 : 6, 92 : 8, or 0 : 100 were tested.

RESULTS

Chemical Analysis. A small amount of sex pheromone was isolated from a total of 4,043 females. Only four peaks were found in the gas chromatogram; two major peaks, 1 and 4, and two minor peaks, 2 and 3 (Figure 1B). The retention times of these four peaks matched those of 4 authentic samples; peaks 1 and 4 gave the same retention times as *Z*8-12:Ac and *Z*8-12:OH, respectively, while peaks 2 and 3 were also coincided with 12:OH and *E*8-12:OH, respectively (Figure 1A, B). The gland extracts and 14 authentic samples were analyzed by GC-MS (Figure 1C, D). The retention times of 14 authentic samples are shown as peak 1 to 14 in Figure 1C. Each peak was identified as follows: 1 (10:Ac, 5.02), 2 (10:OH, 6.38), 3 (12:Ac, 10.47), 4 (*E*8-12:Ac , 11.82), 5 (*Z*8-12:Ac, 12.35), 6 (12:OH, 13.98), 7 (*E*8-12:OH, 16.02), 8 (*Z*8-12:OH, 16.72), 9(14:Ac, 22.37), 10 (14:OH, 24.87), 11 (16:Ac, 27.72), 12 (16:OH, 28.95), 13 (18:Ac, 30.85) and 14 (18:OH, 31.75 min). The sex pheromone extracts show 12 peaks in the gas chromatogram (Figure 1D). Their retention times were as follows: 1(9.52), 2(10.13), 3(11.30), 4(12.33), 5(16.83), 6(25.88), 7(29.77), 8(30.15), 9(31.50), 10(31.73), 11(31.83), and 12(33.08 min). Comparison of the retention times and EI and CI mass spectra of the fourth and fifth peaks of the extracts with those of synthetic *Z*8-12:Ac and

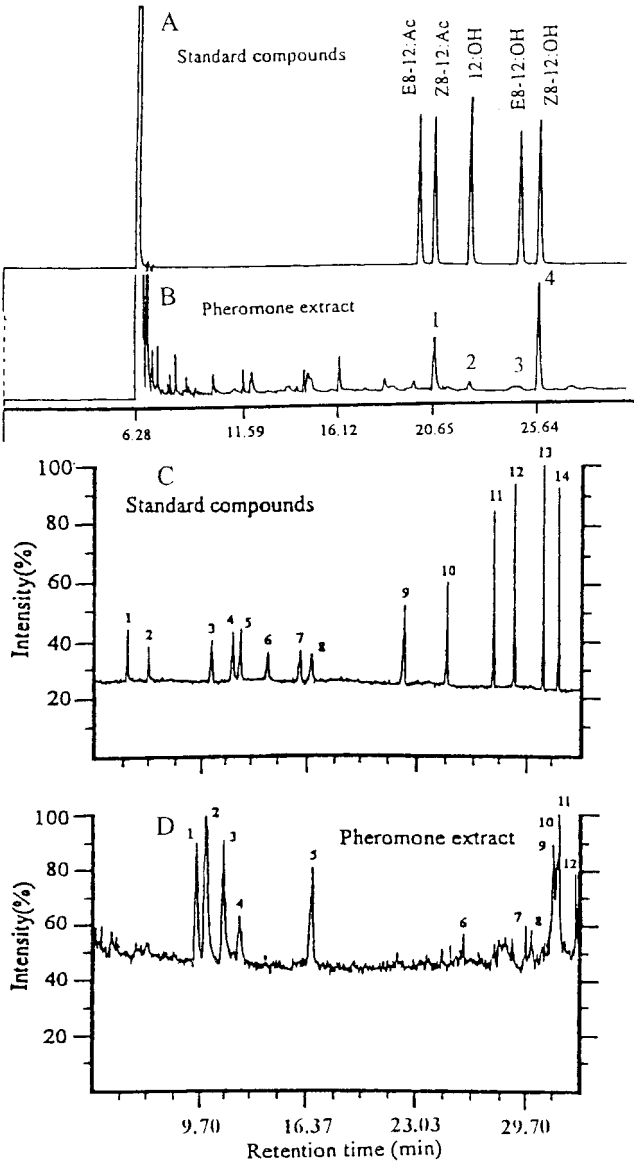


FIG. 1. Comparative GC (A, B) and GC-MS (C, D) chromatograms of pheromone extracts produced by females of *Eucosma notanthes* and standard compounds. Fourteen standard compounds are shown as peaks 1 to 14 in C :1 (10:Ac), 2 (10:OH), 3 (12:Ac), 4 (*E*8-12:Ac), 5 (*Z*8-12:Ac), 6 (12:OH), 7 (*E*8-12:OH), 8 (*Z*8-12:OH), 9 (14:Ac), 10 (14:OH), 11 (16:Ac), 12 (16:OH),13 (18:Ac) and 14(18:OH).

TABLE 1. CI MASS SPECTRAL DATA FOR THE FOURTH AND FIFTH PEAKS OF *Eucosma notanthes* PHEROMONE EXTRACTS AND STANDARD COMPOUNDS OF *Z*8-12:AC AND *Z*8-12:OH

Compound	Source	Mass spectral data, *m/z* (Intensity, %)
*Z*8-12:Ac	Synthetic	227($M+1^+$, 12.31), 165(9.23), 137(9.23), 123(21.54), 111(70.77), 97(78.46), 81(81.54), 69(100), 55(73.85), 43(23.08)
	Natural (peak 4)	227($M+1^+$, 5.88), 165(2.94), 151(2.94), 137(5.88), 123(14.71), 109(38.24), 95(61.76), 81(94.12), 67(100), 55(44.12), 43(8.82)
*Z*8-12:OH	Synthetic	185($M+1^+$, 2.60), 173(1.30), 165(1.30), 137(5.19), 123(18.18), 109(48.05), 95(67.53), 81(76.62), 69(100), 55(89.61), 41(74.03)
	Natural (peak 5)	185($M+1^+$, 1.11)), 173(1.11), 165(1.11), 149(1.11), 141(1.11), 137(5.56), 123(14.44), 109(38.89), 95(64.44), 91(4.44), 85(2.22), 81(92.22), 71(4.44), 67(100), 55(68.89), 43(8.89)

*Z*8-12:OH suggested that peak 4 and 5 were *Z*8-12:Ac and *Z*8-12:OH, respectively (Table 1). The EI mass spectra of *Z*8-12:Ac have two characteristic peaks, one with $m/z = 61$, which is a typical fragmentation peak of acetates, ($AcOH_2^+$), and the other with $m/z = 166$, which corresponds to $(M\text{-}AcOH)^+$. In contrast, the EI mass spectra of *Z*8-12:OH do not show any peak with $m/z = 61$ ($AcOH_2^+$). Therefore, it can be differentiated from acetate to alcohol. The results of our EI mass spectra of peak 4 and 5 are consistent with *Z*8-12:Ac and *Z*8-12:OH. The CI mass spectra of *Z*8-12:Ac and peak 4 both have a mother molecular ion peak with $m/z = 227$ ($M+1^+$) and all identical fragmentation patterns (Table 1). Similarly, the CI mass spectra of *Z*8-12:OH and peak 5 also have a mother molecular peak with $m/z = 185$ ($M+1^+$) and are consistent with the spectral data (Table 1). These data indicate that peak 4 corresponds to *Z*8-12:Ac and peak 5 to *Z*8-12:OH. The total amounts of *Z*8-12:Ac and *Z*8-12:OH obtained from the extraction of 52,820 females were 63.37 and 169.32 μg, respectively. This is equivalent to a ratio of *Z*8-12:OH:*Z*8-12:Ac of 2.7.

Mass spectral analysis of DMDS derivatives resolved the isomer identification of the dodecenyl acetate and dodecenol. Comparison with the data from Buser et al. (1983) and Leonhardt and DeVilbiss (1985), our derivatized standards showed the diagnostic peaks to be [M^+320 (29.2%), A^+103 (2.1%), B^+217 (64.4%)] for *Z*8-12:Ac at a retention time of 27.77 min; [M^+320 (25.2%), A^+103 (2.0%), B^+217 (61.9%)] for *E*8-12:Ac at 28.03 min; [M^+278 (44.7%), A^+103 (2.5%), B^+175 (29.6%)] for *Z*8-12:OH at 25.23 min; and [M^+278 (39.3%), A^+103 (2.6%), B^+175 (28.1%)] for *E*8-12:OH at 25.53 min. The derivatives of two components from the pheromone gland extracts also gave diagnostic peaks of [M^+320 (31.7%), A^+103

(2.2%), B^+217 (69.0%)], at a retention time of 27.78 min; and [M^+278 (39.7%), A^+103 (2.3%), B^+175 (27.5%)], at 25.30 min, confirming their identities as *Z*8-12:Ac and *Z*8-12:OH, respectively.

Field Tests. In the initial comparison of three isomers, *Z*7-12:Ac, *Z*8-12:Ac and *Z*9-12:Ac, as trap lures, the greatest numbers of *E. notanthes* males were captured in traps baited with *Z*8-12:Ac. Other isomers either failed to capture any male or the number of males captured was less than that in unbaited traps (ANOVA $F = 3.96$, $df = 79$, $0.01 < P < 0.001$) (Figure 2A). The total number of *E. notanthes* males captured was 134.

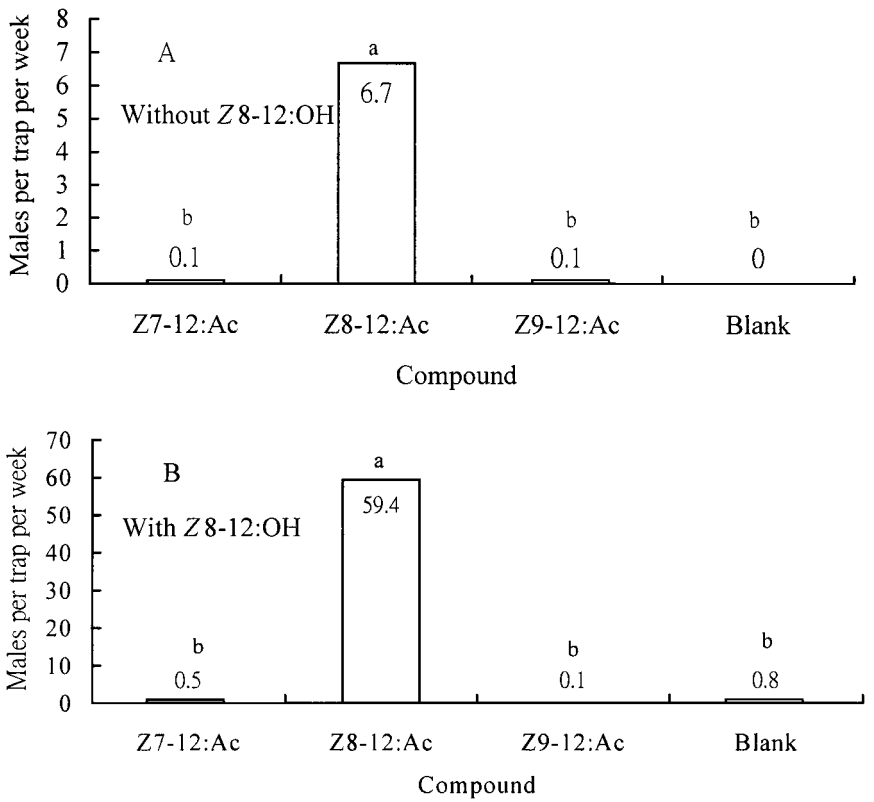


FIG. 2. Mean numbers of male *Eucosma notanthes* moths captured in carambola orchards in traps baited with three isomers, *Z*7-12:Ac, *Z*8-12:Ac, and *Z*9-12:Ac, alone (A) or with equal amount *Z*8-12:OH (B). Experiments were conducted at Changhua, Taiwan, on the following dates: experiment A, July 2–August 6, 1997; experiment B, August 6–September 10, 1997. Bars with the same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).

In the second experiment, where three isomers, *Z*7-12:Ac, *Z*8-12:Ac and *Z*9-12:Ac, were mixed individually with *Z*8-12:OH in ratio of 1 : 1. The greatest numbers of *E. notanthes* males were captured in traps baited with *Z*8-12:Ac and *Z*8-12:OH. In the other two mixtures, the numbers of males captured were nearly 0 and not different from those in unbaited traps (ANOVA $F = 31.41$, $df = 79$, $P < 0.001$) (Figure 2B). The total number of males captured in this experiment was 1,214.

In the third experiment, in which *Z*8-12:Ac and *Z*8-12:OH were compared, the greatest numbers of males were captured in traps baited with *Z*8-12:Ac, ranging from 13 to 208 males/trap/week (mean 60.1), while those captured with *Z*8-12:OH and in the blank were only 1.1 and 0 males/trap/week, respectively (ANOVA $F = 15.4$, $df = 35$, $P < 0.001$) (data not shown). The total number of males captured was 734.

In the fourth experiment, in which comparison of various ratios of *Z*8-12:Ac and *Z*8-12:OH were made, the greatest numbers of males were captured in traps baited with *Z*8-12:Ac and *Z*8-12:OH at a load ratio of 100 : 100. Significant numbers of males were also captured in traps baited with ratios of 100 : 1 and 100 : 200. Capture of males with the remaining ratios were rather small and did not differ from those in unbaited traps (ANOVA $F = 47.87$, $df = 119$, $P < 0.001$) (Figure 3A). The total number of males captured was 2,420.

In the fifth experiment, where the ratios of *Z*8-12:Ac and *Z*8-12:OH were 100 : 5, 100 : 10, 100 : 50, 100 : 100 and 100 : 150, the numbers of *E. notanthes* males captured in traps baited with a load ratio of 100 : 100 did not differ from those with 100 : 50 and 100 : 150, while they were greater than those captured in traps baited with two other load ratios of 100 : 5 and 100 : 10. (ANOVA $F = 12.69$, $df = 143$, $P < 0.001$) (Figure 3B). The total number of males captured was 2,115.

In the sixth experiment, where three ratios of *Z*8-12:Ac and *Z*18-12:OH were 100 : 100, 100 : 150, and 100:270, the numbers of *E. notanthes* males captured in traps baited with a load ratio of 100 : 100 did not differ from those with 100 : 150, while they were greater than those baited with 100 : 270. (ANOVA $F = 16.96$, $df = 99$, $P < 0.001$) (Figure 3C). The total number of males captured was 4,539.

In the last experiment, where *Z*8-12:Ac and *E*8-12:Ac were used, in ratios ranging from 100 : 0 to 0 : 100, the greatest numbers of males were captured in traps baited with a load ratio of 100 : 0. This was not greater than numbers of males captured in traps baited with these compounds at a load ratio of 99.5 : 0.5. Almost no moths was captured in traps baited with these compounds in other load ratios (ANOVA $F = 3.93$, $df = 253$, $P < 0.001$) (Figure 4). A total of 1,285 males were captured.

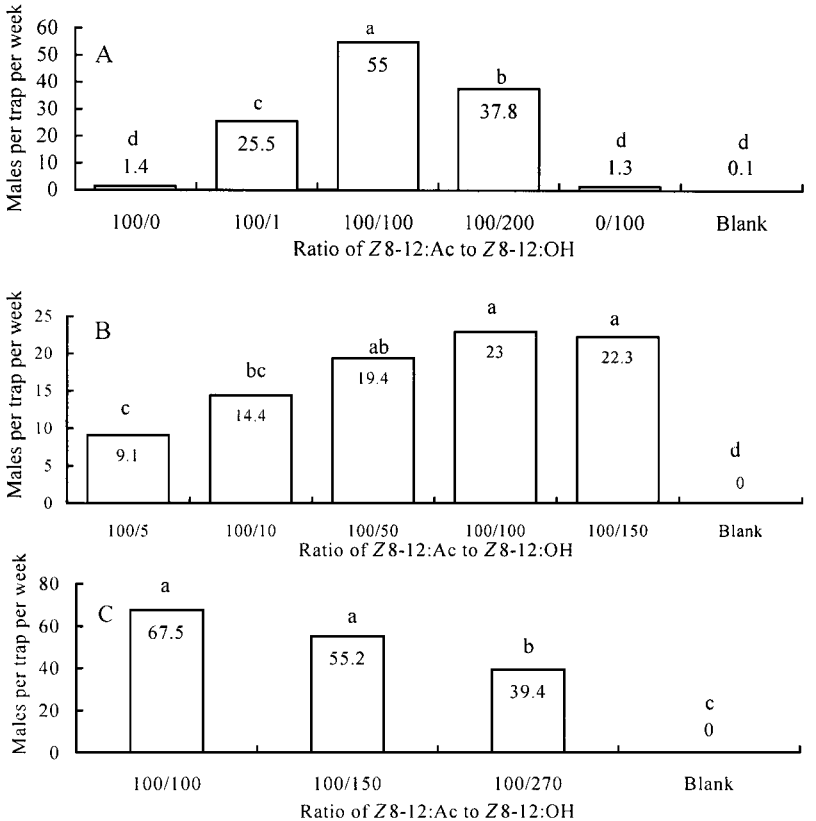


FIG. 3. Mean numbers of male *Eucosma notanthes* moths captured in traps baited with different blend ratios of *Z*8-12:Ac mixed with *Z*8-12:OH in carambola orchards. The first comparison (A) consisted of a broader range of ratios, while comparison of (B) and (C) consisted of a range of ratios closer to that found in the best efficiency lure for trapping carambola fruit borer. Experiments were conducted at Changhua, Taiwan, on the following dates: experiment A, March 19–April 23, 1997; experiment B, April 30–June 11, 1997; experiment C, December 22, 1999–February 10, 2000. Bars with the same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).

DISCUSSION

Our results indicate that the sex pheromone of *E. notanthes* comprises two compounds, i.e., *Z*8-12:Ac and *Z*8-12:OH. Both compounds in combinations or *Z*8-12:Ac alone can capture males, indicating that these compounds are sex pheromone components of this species.

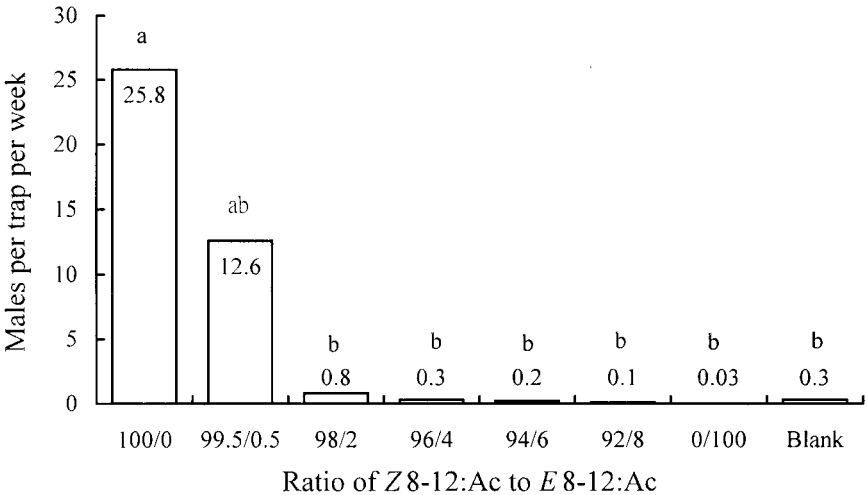


FIG. 4. Mean numbers of male *Eucosma notanthes* moths captured in traps baited with different blend ratios of *Z*8-12:Ac mixed with *E*8-12:Ac in carambola orchards at Changhua, Taiwan, from August 1 to November 11, 1996. Bars with the same letter are not significantly different by Duncan's multiple range test ($P = 0.05$).

The compound, *Z*8-12:Ac, is an attractant for more than 40 insect species, and has been found in females of three species, *Cryptophlebia leucotreta, Grapholita molesta and Hedya nubiferana*, in which it was found with other components including E8-12:Ac, 12:OH, 12:Ac, E5-12:Ac, and *Z*8-12:OH. However, only *Z*8-12:OH is reported to be an attractant of four insect species, and was identified as a sex pheromone component for the Oriental fruit moth, *G. molesta* (Kydonieus et al., 1982). The sex pheromone components of *G. molesta* have been reported to be *Z*8-12:Ac and *E*8-12:Ac, and *Z*8-12:OH and 12:OH (Roelofs et al., 1969; Cardé et al., 1979; Lacey and Sanders, 1992). In this study, two of these four components, i.e., *Z*8-12:Ac and *Z*8-12:OH, were identified as sex pheromone components of *E. notanthes.* Ratios of *Z*8-12:Ac and *Z*8-12:OH ranging from 1 : 0.5 to 1 : 1.5 were more attractive to *E. notanthes* males in orchards, whereas the ratio of these two compounds was 1 : 2.7 in hexane extracts of sex pheromone glands. A concentration of 0.5% or more of *E*8-12:Ac inhibited attraction to *E. notanthes* by the lures containing *Z*8-12:Ac, while 6–7% and 2.2% of *E*8-12:Ac was active as a pheromone component with *Z*8-12:Ac in attracting *G. molesta* and the lesser apple worm, *Grapholita prunivora*, respectively. However, when the amount of E8-12:Ac reached 23% in lures, the trap catch to males of both species decreased (Roelofs and Cardé, 1974). Therefore, isomeric purity of *Z*8-12:Ac is important for attractiveness of lures to *E. notanthes.*

In conclusion, we identified two compounds, *Z*8-12:Ac and *Z*8-12:OH, to be sex pheromone components of *E. notanthes*. Furthermore, based on field tests, *Z*8-12:Ac is regarded as the main component, while *Z*8-12:OH per se does not attract males.

Acknowledgments—This study was supported in part by a research grant from the National Science Council, R.O.C. We thank Dr. S. B. Horng of National Taiwan University, Taipei, Taiwan, for assistance in statistical analysis.

REFERENCES

BUSER, H. R., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

CARDÉ, A. M., BAKER, T. C., and CARDÉ, R. T. 1979. Identification of a four-component sex pheromone of the female oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 5:423–427.

HO, K. Y. 1985. Preliminary report on the carambola fruit borers and their control. *Plant Prot. Bull. (R.O.C.)* 27:53–62. (In Chinese)

HO, K. Y. 1988a. Studies on the methods for control of the carambola fruit borer, *Eucosma notanthes. Plant Prot. Bull. (R.O.C.)* 30:45–51. (In Chinese)

HO, K. Y. 1988b. Life history and ecology of the carambola fruit borer, *Eucosma notanthes* Meyrick, in Chia-Yi area. *Chinese J. Entomol.* 8:23–31. (In Chinese)

HUNG, C. C., and HWANG, J. S. 1991. Mass rearing method of the carambola fruit borer, *Eucosma notanthes* Meyrick. *Chinese J. Entomol. (R.O.C.)* 11:204–212. (In Chinese)

HUNG, C. C., HWANG, J. S., and HOU, R. F. 1997. Eclosion, mating and oviposition of the carambola fruit borer, *Eucosma notanthes* Meyrick. *Plant Prot. Bull. (R.O.C.)* 39:265–274. (In Chinese)

HUNG, C. C., HWANG, J. S., and HOU, R. F. 1999. Comparison of bioassay methods for attractiveness of sex pheromone components to carambola fruit borer, *Eucosma notanthes* Meyrick. *Plant Prot. Bull. (R.O.C.)* 41:165–177. (In Chinese)

HWANG, J. S., and HUNG, C. C. 1994. Formulation of sex attractant and trap designs for trapping carambola fruit borer, *Eucosma notanthes* Meyrick. *Plant Prot. Bull. (R.O.C.)* 36:31–40. (In Chinese)

HWANG, J. S., and HUNG, C. C. 1997. Mating disruption for the control of carambola fruit borer, *Eucosma notanthes* Meyrick, with synthetic sex pheromone. *Plant Prot. Bull. (R.O.C.)* 39:151–164. (In Chinese)

HWANG, J. S., HUNG, C. C., and HOU, R. F. 1996. A bioassay method of the sex pheromone of the carambola fruit borer, *Eucosma notanthes* Meyrick. *Plant Prot. Bull. (R.O.C.)* 38:119–127. (In Chinese)

HWANG, J. S., HUNG, C. C., LO, C. C., and HUNG, M. D. 1987. Sex attractant for two carambola fruit borer, *Eucosma notanthes* Meyrick and *Cryptophlebia ombrodelta* Lower. *Plant Prot. Bull. (R.O.C.)* 29:321–323. (In Chinese)

KYDONIEUS, A. F., BEROZA, M., and ZWIG, G. 1982. Insect Suppression With Controlled Release Pheromone Systems, Vol. II. CRC Press, 312 pp.

LACEY, M. J., and SANDERS, C. J. 1992. Chemical composition of sex pheromone of oriental fruit moth and rate of release by individual female moth. *J. Chem. Ecol.* 18:1421–1435.

LEONHARDT, B. A., and DEVILBISS, E. D. 1985. Separation and double-bond determination on nanogram quantities of aliphatic monounsaturated alcohols, aldehydes and carboxylic acid methyl esters. *J. Chromatogr.* 322:484–490.

ROELOFS, W. T., and CARDÉ, R. T. 1974. Oriental fruit moth and lesser apple worm attractant mixtures refined. *Environ. Entomol.* 3:586–588.

ROELOFS, W. T., COMEAU, A., and SELLE, R. 1969. Sex pheromone of the Oriental fruit moth. *Nature* 224:723.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

FEMALE SEX PHEROMONE OF BRINJAL FRUIT AND SHOOT BORER, *Leucinodes orbonalis* BLEND OPTIMIZATION

A. CORK,[1,*] S. N. ALAM,[2] A. DAS,[3] C. S. DAS,[3] G. C. GHOSH,[3] D. I. FARMAN,[1] D. R. HALL,[1] N. R. MASLEN,[1] K. VEDHAM,[4] S. J. PHYTHIAN,[1] F. M. A. ROUF,[2] and K. SRINIVASAN[5]

[1]*Natural Resources Institute*
University of Greenwich, Central Avenue
Chatham Maritime, Kent, ME4 4TB, United Kingdom

[2]*Bangladesh Agricultural Research Institute*
Gazipur-1701, Bangladesh

[3]*Rama Krishna Mission Lokasiksha Parishad*
Narendrapur, West Bengal, India

[4]*Nagarjuna Agricultural Research and Development Institute*
61, Nagarjuna Hills, Panjagutta
Hyderabad-500 082, Andhra Pradesh, India

[5]*Tractors and Farm Equipment Ltd Research Farm*
Kelambakkaam, Kancheepuram District
Tamil Nadu-603 103, India

(Received February 5, 2001; accepted April 24, 2001)

Abstract—The brinjal fruit and shoot borer, *Leucinodes orbonalis* is the major pest of eggplant in South Asia. Analysis of female pheromone gland extracts prepared from insects of Indian and Taiwanese origin confirmed (*E*)-11-hexadecenyl acetate (E11-16:Ac) as the major pheromone component with 0.8 to 2.8% of the related (*E*)-11-hexadecen-1-ol (E11-16:OH), as previously reported from Sri Lanka. The average quantity of E11-16:Ac extracted per female was estimated to be 33 ng, with a range of 18.9 to 46.4 ng when collected 2 to 3 hr into the scotophase. In field trials conducted in India, blends containing between 1 and 10% E11-16:OH caught more male *L. orbonalis* than E11-16:Ac alone. At the 1000 μg dose, on white rubber septa, addition of 1% E11-16:OH to E11-16:Ac was found to be more attractive to male *L. orbonalis* than either 0.1 or 10% E11-16:OH. Trap catch was found to be positively correlated with pheromone release rate, with the highest dose tested, 3000 μg, on white rubber septa catching more male moths than lower doses. Field and wind tunnel release rate studies confirmed that E11-16:OH released from white rubber septa and polyethylene vials at approximately twice the rate of E11-16:Ac and that the

*To whom correspondence should be addressed.

0098-0331/01/0900-1867$19.50/0

release rate of both compounds was doubled in polyethylene vials compared to white rubber septa. This difference in release rate was reflected in field trials conducted in Bangladesh where polyethylene vial dispensers caught more male moths than either black or white rubber septa, each loaded with the same 100 : 1 blend of E11-16:Ac and E11-16:OH in a 3000 μg loading.

Key Words—*Leucinodes orbonalis*, brinjal, sex pheromone, (*E*)-11-hexadecenyl acetate, (*E*)-11-hexadecen-1-ol.

INTRODUCTION

The brinjal fruit and shoot borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Pyralidae: Pyraustinae) is the major insect pest of eggplant, *Solanum melongena*, throughout Asia (Purohit and Khatri, 1973; Kuppuswamy and Balasubramanian, 1980; Allam et al., 1982). Larvae of *L. orbonalis* bore into shoots during the vegetative growth stage and later into flowers and fruits rendering fruit unfit for human consumption. Larvae block their bore holes with frass which protects them from natural enemies and most topically applied insecticides. Indigenous parasitoids of *L. orbonalis* have been recorded, notably *Trathala flavoorbitalis* Cameron (Hymenoptera: Ichneumonidae), *Eriborus argenteopilosus* Cameron (Hymenoptera: Ichneumomidae), *Phanerotoma* sp. (Hymenoptera: Braconidae) and *Campyloneura* sp. (Diptera: Cecidomyiidae) (Tewari and Krishna Moorthy, 1984; Tewari and Sardana, 1987). However, under field conditions, the level of parasitism recorded is often low (<2%) (Srinivasan, 1994), in part because of exposure to excessive amounts of insecticide used for control of *L. orbonalis*.

Eggplant is regarded as a cheap vegetable, available throughout most of the year, but production is seriously affected in many parts of the Indian sub continent because of the high cost and low efficacy of insecticide needed to ensure production of a viable crop. In West Bengal, for example, insecticide applications frequently exceed three per week. Alternative control strategies are needed but, to be effective, they require a sound understanding of the biology and ecology of the insect. As part of this process the need for an adult population monitoring system was recognized by the authors.

Adult *L. orbonalis* are not attracted to light traps but Zhu et al. (1987), working with insects from China, identified (*E*)-11-hexadecenyl acetate (E11-16:Ac) as the major component of the female sex pheromone and suggested that it was attractive to male moths in the field, although no data were presented. Subsequently, Attygalle et al. (1988) confirmed the presence of E11-16:Ac in calling virgin females using insects from Sri Lanka and, in addition, found trace amounts of (*E*)-11-hexadecen-1-ol (E11-16:OH). However, they did not report the results of any field studies to ascertain the behavioral significance of either of these two compounds. This paper reports the results of laboratory and field work

to confirm the composition of the pheromone, and to develop an effective synthetic equivalent for use in monitoring male *L. orbonalis*.

METHODS AND MATERIALS

Insect Material. Larvae were field-collected from eggplant fruit in West Bengal and Andhra Pradesh, India. Infested fruits were placed in pupation dishes filled with damp sand. Mature larvae exited the fruit and dropped into the sand where they pupated. Pupae were recovered by sieving and dispatched by air to NRI. On arrival, pupae were placed individually in Pyrex test tubes sealed with cotton wool and placed in a room maintained under a reversed light-dark cycle of 12L : 12D at 26°C and 22°C, respectively and 60% relative humidity. Laboratory-reared insects dispatched to NRI from the Asian Vegetable Research and Development Centre, Taiwan, were treated in a similar manner.

Pheromone Collection. Pheromone gland extracts were prepared 2 and 3 hr into the scotophase using 1 and 2 day old virgin female moths, by applying light pressure with forceps on the abdominal tip of female moths it was possible to evert the ovipositor and pheromone gland. The gland and exposed abdominal tip were excized with microscissors into a microvial containing hexane (50 μl). Batches of up to 10 ovipositors were prepared and stood at room temperature for 10 min before transferring the supernatant to a new microvial using a microsyringe (10 μl, Scientific Glass Engineering Ltd., Australia) and stored at -20°C.

Gas Chromatography (GC). GC analyses of pheromone gland extracts and pheromone dispensers were conducted on a Carlo Erba Mega Series 5300 instrument fitted with two Grob split/splitless injectors (240°C) and flame ionization detector (FID) (250°C). Fused silica capillary columns were used throughout the study, coated with non-polar CP-Sil 5CB (30 m $\times$ 0.32 mm; 0.25 μm; Chrompack, UK), polar CP-Wax 52CB (30 m $\times$ 0.32 mm; 0.25 μm; Carbowax 20M equivalent; Chrompack, UK) and polar DB23 (30 m $\times$ 0.25 mm; 0.25 μm; 1:1 mixture of chemically bonded methyl- and cyanopropyl-polysiloxane, J&W Scientific, Folsom CA, USA). The carrier gas was helium with an inlet pressure of 40 kPa.

All injections were made with the split value closed for 40 sec onto CP-Sil 5CB and CP-Wax 52CB columns held at 50°C for 2 min and then programmed at 20°C/min to 80°C and then at 5°C/min to 240°C. Confirmation of the isomeric composition of pheromone components was obtained by GC analysis of pheromone gland extracts on a DB23 column that was held at 70°C for two min after injection, and temperature programmed at 20°C/min to 120°C, and then at 4°C/min to 240°C. GC retention times of compounds identified in pheromone gland extracts are quoted as retention indices (RI) relative to the retention times of straight-chain hydrocarbons; thus, for example, hexadecane = 1600 RI.

Electroantennographic Detection (EAD). Linked GC-EAD analyses were carried out as described by Cork et al. (1990) by splitting half the GC effluent

to the FID and the other half to a silanized glass reservoir. Column effluent was expelled from the reservoir at 17 sec intervals for 3 sec in a stream of nitrogen (500 ml/min) over the EAD preparation. The series of EAD responses generated were then compared to identify which were significantly larger than background responses in order to determine the retention times of EAD-active compounds. GC conditions were as described above.

Mass Spectrometry (MS). Electron impact (EI) mass spectra of compounds in pheromone gland extracts were obtained from a Finnigan MAT Ion Trap Detector, model 700, interfaced with a Carlo Erba Mega Series 5160 GC under continuous ion monitoring between m/z 29–300 at 220°C. Chemical ionization (CI) mass spectra were obtained with the same equipment, but monitoring ions between m/z 60–300 with the Ion Trap set at 200°C and using *iso*-butane as the reagent gas. GC conditions were as described above except that the GC was temperature programmed at 6°C/min from 50 to 240°C with the CP-Sil 5CB column, and at 20°C/min from 50 to 100°C and then at 5°C/min to 250°C with the CP-Wax 52CB column.

Synthetic Compounds. Monounsaturated alcohols and acetates were obtained by standard Wittig and acetylenic coupling reactions (Henrick, 1977) or purchased from Shin Etsu Chemical Co. (Tokyo, Japan). Compounds used for analytical studies contained less than 1% of the opposite stereoisomer, while (*E*)-11-hexadecenyl acetate and (*E*)-11-hexadecen-1-ol used for field studies each contained 0.46% of the *Z*-isomer.

Pheromone Dispensers. Pheromone dispensers used in field and laboratory studies were prepared from white rubber septa (Sigma-Aldrich Chemical Company, Gillingham, UK, catalogue No. Z10,072-2), polyethylene vials (25 mm length, 8 mm diameter and 1.5 mm wall thickness, Just Plastics Ltd., UK) or black rubber septa (Biological Control Research Laboratories Ltd., Bangalore, India) impregnated with 0.1 ml of hexane solution containing the synthetic pheromone blend and an equivalent weight of 2,6-di-*tert*-butyl-4-methylphenol as antioxidant.

Field Trials. Field trials were conducted in India in plots of eggplant at the fruiting stage of development at the NARDI research farm, Wargal, 45 km north of Hyderabad, Andhra Pradesh and farmers' fields in the North and South 24-Paganas Districts, West Bengal. In Bangladesh, field trials were conducted in farmers' fields adjacent to the Bangladesh Agricultural Research Institute (BARI) Regional Agricultural Research Station, Jessore. No pesticides were applied for control of *L. orbonalis* at the NARDI research farm, although a foliar application of monocrotophos (0.07%) was made to seedlings before planting to protect them against sucking pests. In West Bengal and Jessore, pesticides were applied at the farmers' discretion, typically between 2 and 6 times per week.

At the NARDI research farm, insects were trapped using yellow plastic funnel traps (Ecomax, India), while white sticky delta traps (Agrisense BCS Ltd., Pontypridd, UK) were used in West Bengal and Jessore. Traps were attached at the

top and, where possible, the side of inverted L-shaped metal or wooden supports to restrict movement. Trap height was periodically adjusted to keep them at crop height, which reached between 1–1.5 m at maturity. Insects trapped in the funnel traps were killed with carbaryl impregnated wettable powder. Treatments in a replicate were randomized with at least 10 m between treatments and 50–100 m between replicates at the NARDI research farm. In West Bengal treatments were separated by 20 m and replicates placed in different villages (>2 km distant). In Jessore, replicates were placed in lines with 10 m between treatments and 15–20 m between replicates. The position of treatments within a replicate was rotated in a clockwise direction every working day at the NARDI research farm and Jessore when trap catches were recorded and insects discarded. For each rotation, trap catch data from the NARDI research farm and Jessore were combined, averaged, and statistically analyzed by analysis of variance (ANOVA) after transforming to log $(x + 1)$. Trap catch data from West Bengal were collected twice weekly but traps were not rotated. Data were converted to catch per trap per night, transformed to log $(x + 1)$ and analyzed by ANOVA. Lures were not changed throughout the duration of each trial at either location. Significant differences in treatment means at the 5% level or lower were compared by Newman-Keuls multiple range test (Statgraphics, Version 2.00).

Release Rate Measurements. White rubber septa loaded with 100, 300 and 1000 μg each of a 100 : 1 blend of E11-16:Ac and E11-16:OH were placed in a shaded position at the RKM research farm, West Bengal with averaged minimum and maximum temperatures of 22.9°C (range, 14.5 to 28.9°C) and 32.9°C (range 24 to 38.4°C), respectively. Septa were randomly collected from the field in batches of two at different time intervals and stored at 0°C before being hand-carried to NRI for analysis of residual pheromone.

Similarly, septa and polyethylene vials loaded with a 1:1 mixture of E11-16:Ac and E11-16:OH were aged in a wind tunnel (WT) at NRI at 27°C with a linear flow-rate of 5 km/hr. Dispensers aged in the WT were collected as described above for field samples and stored at -20°C. In order to extract pheromone from field and WT exposed dispensers they were immersed overnight at room temperature in a hexane solution of pentadecyl acetate (5 ml, 1 mg/ml) as an internal standard. Compounds were quantified by GC, under conditions described above, for analysis of sex pheromone gland extracts on a CP-Wax 52CB column.

RESULTS

Pheromone Identification. GC-EAD analyses of female ovipositor extracts prepared from insects of Indian and Taiwanese origin on both CP-Wax 52CB and CP-Sil 5CB GC columns indicated the presence of two EAD-active compounds. EI-MS analysis using a CP-Sil 5CB GC column confirmed the major GC peak

eluting at 1977 RI was consistent with a hexadecenyl acetate isomer showing characteristic ions at m/z 61 for an acetate functionality and m/z 222 consistent with M − 60. The anticipated hexadecen-1-ol isomer (Attygalle et al., 1988) was not observed. Since the possibility existed that trace quantities of hexadecen-1-ol were being adsorbed irreversibly onto the GC column the analyses were repeated using the CP-Wax 52CB column in CI mode. Under these conditions the hexadecenyl acetate showed characteristic ions at m/z 222 (M − 60) and m/z 283 (M + 1) and identical fragmentation pattern to synthetic (*E*)-11-hexadecenyl acetate (E11-16:Ac). Similarly, the hexadecen-1-ol isomer was observed with significant ions at m/z 241 (M + 1) and identical fragmentation pattern and GC retention time to synthetic (*E*)-11-hexadecen-1-ol (E11-16:OH). The average quantity of hexadecenyl acetate per female moth was estimated to be 33 ng, with a range of 18.9 to 46.4 ng per female moth. Trace quantities of hexadecyl acetate (16:Ac) were also observed by linked GC-MS analysis in EI mode. The mean ratio of hexadecyl acetate : hexadecenyl acetate : hexadecen-1-ol was 2.65 : 100 : 1.78 although the proportion of E11-16:OH to E11-16:Ac was higher in insects from Taiwan compared to those from India (Table 1). GC retention times of the natural compounds were compared with *E* - and *Z*-isomers of the related synthetic compounds on two polar and one non-polar column that confirmed, in each case, the natural compounds were (*E*)-11-isomers (Table 1).

Release Rate Studies. Under field conditions in West Bengal release rate studies with white rubber septa loaded with 100, 300 and 1000 μg of E11-16:Ac and 1% E11-16:OH indicated that approximately 86, 89 and 100% of E11-16:Ac, respectively remained after 40 days. After 90 days 73, 80 and 97% respectively remained, but the quantity of E11-16:OH was too low to measure accurately. In related wind tunnel studies with a 1 : 1 blend of E11-16:Ac and E11-16:OH at a dose of 100 μg each on white rubber septa and polyethylene vials, 92 and 62% E11-16:Ac and 76

TABLE 1. GC RETENTION TIMES AND RELATIVE COMPOSITION OF COMPOUNDS IDENTIFIED IN PHEROMONE GLAND WASHINGS OF *L. orbonalis* FROM INDIA AND TAIWAN

	Retention times (RI)[a]						Relative composition[b]	
	DB23		CP-Wax 52CB		CP-Sil 5CB			
Compound	Synthetic	Natural	Synthetic	Natural	Synthetic	Natural	Taiwan	India
16:Ac	2353	2351	2277	2276	1991	1991	3.3	2.0
E11-16:Ac	2382	2381	2311	2312	1978	1977	100.0	100.0
Z11-16:Ac	2400				1975		0.0	0.0
E11-16:OH	2403	2396	2390	2392	Not obs.	Not obs.	2.76	0.80
Z11-16:OH	2425		2396		Not obs.	Not obs.	0.0	0.0

[a] Retention times in equivalent chain length units relative to the retention times of straight chain hydrocarbons. Not obs. = not observed.
[b] Analyzed on a CP-Wax 52CB GC column; Normalized to E11-16:Ac (=100).

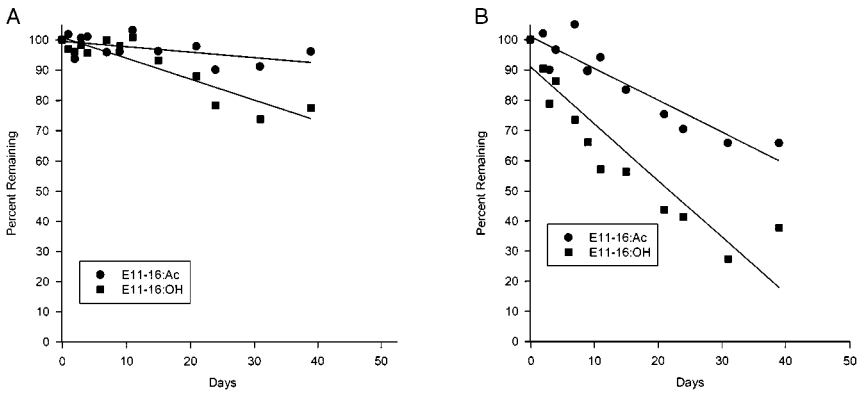


FIG. 1. Release of E11-16:Ac and E11-16:OH from white rubber septa (A) and polyethylene vials (B) in a wind tunnel (27°C, wind speed 5 km/h) with an initial loading of 100 μg per compound.

and 18% E11-16:OH, respectively, remained after 40 days exposure (Figures 1A and 1B).

Field Trials in India and Bangladesh. Trial 1 was undertaken at the NARDI research farm to determine the effect of adding E11-16:OH to E11-16:Ac had on attraction of male *L. orbonalis*. A range of blends was tested which contained between 0 and 10% E11-16:OH to E11-16:Ac at a 100 μg dose loaded on white rubber septa. The results (Table 2) showed that only blends with between 1 and 10% E11-16:OH caught more male moths than the unbaited trap (ANOVA, $F = 73.0, P < 0.001$). In order to optimize further the ratio of E11-16:Ac to E11-16:OH,

TABLE 2. TRIAL 1. CATCHES OF MALE *L. orbonalis* IN TRAPS BAITED WITH INCREASING PROPORTIONS OF E11-16:OH RELATIVE TO 100 μG DOSES OF E11-16:AC ON WHITE RUBBER SEPTA

Blends tested		Pheromone trap catches		
E11-16:Ac (μg)	E11-16:OH (μg)	Average catch per trap per night[a]	Standard error	Newman–Keuls[b]
0	0.0	0	0.0	a
100	0.0	0.11	0.06	a
100	0.01	0.0	0.0	a
100	0.1	0.17	0.0	a
100	1.0	1.05	0.22	b
100	10.0	1.72	0.05	c

[a] Andhra Pradesh, India, December 1997, 3 replicates, 6 nights.
[b] Averages followed by the same letter in a group are not significantly different $P < 0.001$ by Newman–Keuls multiple range test on log $(x + 1)$ transformed data.

TABLE 3. TRIAL 2. CATCHES OF MALE *L. orbonalis* IN TRAPS BAITED WITH INCREASING PROPORTIONS OF E11-16:OH RELATIVE TO 1000 μG DOSES OF E11-16:AC ON WHITE RUBBER SEPTA

Blends tested		Pheromone trap catches		
E11-16:Ac (μg)	E11-16:OH (μg)	Average catch per trap per night[a]	Standard error	Newman–Keuls[b]
0	0	0.38	0.14	a
1000	1	2.46	0.17	b
1000	10	3.63	0.34	c
1000	100	0.82	0.31	a
1000	1000	0.59	0.16	a

[a] Andhra Pradesh, India, May 1999, 9 replicates, 5 nights.
[b] Averages followed by the same letter in a group are not significantly different $P < 0.001$ by Newman–Keuls multiple range test on log $(x + 1)$ transformed data.

Trial 2 was conducted at the NARDI research farm. However, in this instance the dose of E11-16:Ac was increased to 1000 μg and E11-16:OH, varied from 1 to 1000 μg. The results (Table 3) showed that male moths responded maximally to blends containing between 0.1 and 1.0% E11-16:OH and that blends containing between 10 and 100% E11-16:OH were no more attractive than the unbaited trap (ANOVA, $F = 35.3$, $P < 0.001$). Trial 3, conducted both on farmers' fields in West

TABLE 4. TRIAL 3. EFFECT OF DOSE ON MALE *L. orbonalis* CATCH WITH A 100:1 MIXTURE OF E11-16:AC AND E11-16:OH ON WHITE RUBBER SEPTA

Blends tested		Pheromone trap catches		
E11-16:Ac (μg)	E11-16:OH (μg)	Average catch per trap per night[a]	Standard error	Newman–Keuls[b]
West Bengal, India, May 1999				
0	0	Not available		
100	1	0.90	0.22	ab
300	3	0.57	0.19	a
1000	10	1.58	0.45	ab
3000	30	2.27	0.70	b
Andhra Pradesh, India, May 1999				
0	0	0.34	0.18	a
100	1	5.89	1.86	bc
300	3	2.94	0.71	b
1000	10	11.09	2.53	c
3000	30	31.09	8.96	d

[a] West Bengal, 4 replicates, 30 nights; Andhra Pradesh, 7 replicates, 5 nights.
[b] Averages followed by the same letter in a group are not significantly different $P < 0.05$ by Newman–Keuls multiple range test on log $(x + 1)$ transformed data.

TABLE 5. TRIAL 4. EFFECT OF DISPENSER ON MALE *L. orbonalis* CATCH WITH A 3000 μG DOSE OF 100:1 MIXTURE OF E11-16:AC AND E11-16:OH

Dispenser	Average catch per trap per night[a]	Standard error	Newman–Keuls[b]
Polyethylene vial	3.38	1.38	c
White rubber septum	1.42	0.68	b
Black rubber septum	0.79	0.24	ab
Unbaited trap	0	0	a

[a] Jessore District, Bangladesh, November 2000, 3 replicates, 24 nights.
[b] Averages followed by the same letter in a group are not significantly different $P = 0.001$ by Newman–Keuls multiple range test on log $(x + 1)$ transformed data.

Bengal and at the NARDI research farm compared the relative attractiveness of different doses of the 100:1 blend of E11-16:Ac and E11-16:OH on white rubber septa to male *L. orbonalis*. Results from West Bengal (Table 4) suggested that the catch of male *L. orbonalis* was proportional to dose over the range tested (100 to 3000μg) despite low catches (ANOVA, $F = 3.47$, $P = 0.019$). This finding was confirmed by results from Andhra Pradesh where a clear relationship between trap catch and dose of pheromone was apparent (ANOVA, $F = 18.56$, $P < 0.001$).

The effect of dispenser type on attraction of male *L. orbonalis* was tested in farmers' fields near Jessore, Bangladesh (Trial 4). Three dispenser types were tested, polyethylene vials, white and black rubber septa. Each dispenser was loaded with the 100:1 blend of E11-16:Ac : E11-16:OH at a 3000 μg dose. The results (Table 5) confirmed that the polyethylene vials caught more male *L. orbonalis* than either white or black rubber septa at the loading tested, although both white and black septa caught more male *L. orbonalis* than the unbaited trap (ANOVA, $F = 9.03$, $P < 0.001$).

DISCUSSION

This study confirmed and extended the results of Zhu et al. (1987) and Attygalle et al. (1988). EAD-active compounds present in female sex pheromone gland extracts of *L. orbonalis* were confirmed as E11-16:Ac and E11-16:OH in the approximate ratio of 50:1 by GC-MS analysis under EI and CI ionization modes. In addition, trace quantities of 16:Ac were observed that constituted between 2 and 3.3% of E11-16:Ac. Because 16:Ac did not elicit an EAD response in linked GC-EAD analyses it was not considered to be behaviorally-active and so was not field tested.

Zhu et al. (1987) suggested that E11-16:Ac alone was attractive to male moths although they did not publish details of the field work. In this study, catches in traps baited with E11-16:Ac alone did not differ from captures in unbaited trap,

but addition of E11-16:OH increased the catches. However, the data suggested that the optimal ratio of E11-16:Ac to E11-16:OH may be dose-dependent, although further work is required to confirm this observation. The highest trap catch was obtained with the addition of 1% E11-16:OH to E11-16:Ac when presented at a 1000 μg dose in rubber septa which was consistent with the ratio of compounds found in ovipositor extracts.

Release rate data from wind tunnel studies suggested that E11-16:OH was released at approximately twice the rate of E11-16:Ac from both white rubber septa and polyethylene vials, and that the release rates of both compounds were doubled in polyethylene vials compared to rubber septa. The wind tunnel studies suggested that lures prepared from either rubber septa or polyethylene vials with a 100 μg loading of pheromone would have a field longevity in excess of one month. Related studies conducted in the field confirmed this result and suggested that loading in excess of 1000 μg of pheromone should have a field-life that substantially exceeds the four-month cropping season. However, over the winter period when night-time temperatures can drop below 10°C, polyethylene vials may prove to be more efficacious because of higher intrinsic release rates. Of concern was the observed change in ratio of pheromone components over time although its effect on attractiveness of the lures was not tested in this study.

While *L. orbonalis* is by no means the only pest of eggplant, other insect pests, such as gall midge, *Asphondylia* sp. (Diptera: Cecidomyiidae), ash weevils, *Myllcerus discolour* Boheman and *M. subfasciatus* Guerin (Coleoptera: Curculionidae), leaf feeding beetles, *Epilachna vigintioctopunctata* (Fabricius) (Coleoptera: Coccinellidae), and red spider mite, *Tetranychus curcurbitae* (Acari: Tetranychidae) rarely attain economic importance. However, even as early as 1980, Kuppuswamy and Balasubramanian (1980) noted that applying synthetic pyrethroids for control of *L. orbonalis* on eggplant significantly increased the incidence of mites. Given the heavy dependence farmers have on insecticides for control of *L. orbonalis*, alternative control strategies are urgently needed. However, poor levels of parasitism by indigenous natural enemies recorded from *L. orbonalis* and low levels of host-plant resistance so far identified (Dhankhar, 1988) have greatly hampered development of an effective IPM strategy. Nevertheless, field trials are now underway in South Asia to evaluate pheromone-baited traps together with other control options, such as removal and destruction of infested plant material, as the basis for an efficacious means of controlling *L. orbonalis* that is both sustainable and economic to the producer.

Acknowledgments—This publication is an output from research projects R7071 and R7465, funded by the UK's Department for International Development (DFID). However, DFID can accept no responsibility for any information provided or views expressed. The authors would also like to thank Dr N. S. Talekar, Asian Vegetable Research and Development Centre, for his enthusiastic encouragement and supply of insect material.

REFERENCES

ALLAM, M. A., KAMESWARA RAO, P., and KRISHNAMURTHY RAO, B. H. 1982. Chemical control of brinjal shoot and fruit borer, *Leucinodes orbonalis* Guen. with newer insecticides. *Entomon.* 7:133–135.

ATTYGALLE, A. B., SCHWARZ, J., and GUNAWARDENA, N. E. 1988. Sex pheromone of brinjal shoot and pod borer *Leucinodes orbonalis* Guenèe (Lepidoptera: Pyralidae: Pyraustinae). *Z. Naturforschung* 43:790–792.

CORK, A., BEEVOR, P. S., GOUGH, A. J. E., and HALL, D. R. 1990. Gas chromatography linked to electroantennography: A versatile technique for identifying insect semiochemicals, pp. 271–279, *in* A. R. McCaffery and I. D. Wilson (eds.). "Chromatography and Isolation of Insect Hormones and Pheromones." Plenum Press, London.

DHANKHAR, B. S. 1988. Progress in resistance studies in the eggplant (*Solanum melongena* L.) against shoot and fruit borer (*Leucinodes orbonalis Guen*) infestation. *Trop. Pest Manag.*, 34:343–345.

HENRICK, C. A. 1977. The synthesis of insect sex pheromones. Tetrahedron Report no. 34. *Tetrahedron*, 33:1–45.

KUPPUSWAMY, S., and BALASUBRAMANIAN, M. 1980. Efficacy of synthetic pyrethroids against brinjal fruit borer, *Leucinodes orbonalis* Guen. *S. Indian Hort.* 28(3):91–93.

PUROHIT, M. L., and KHATRI, A. K. 1973. Note on the chemical control of *L. orbonalis* (Guen) (Lepidoptera: Pyraustidae) on brinjal. *Indian J. Agric. Sci.* 43:214–215.

SRINIVASAN, K. 1994. Recent trends in insect pest management in vegetable crops, pp. 345–372, *in* G.S. Dhaliwal and R. Arora (eds). Trends in Agricultural Insect Pest Management. Commonwealth Publishers, New Delhi.

TEWARI, G. C., and KRISHNA MOORTHY, P. N. 1984. New records of two parasites of brinjal shoot and fruit borer, *Leucinodes orbonalis* Guen. *Entomonology* 9:63–64.

TEWARI G. C., and SARDANA, H. R. 1987. *Eriborus argenteopilosus* (Cameron)—a new parasite of *Leucinodes orbonalis* Guen. *Entomonology* 12:228–229.

ZHU, P., KONG, F., YU, S., YU, Y., JIN, S., HU, X., and XU, J. 1987. Identification of the sex pheromone of eggplant borer, *Leucinodes orbonalis* Guenèe (Lepidoptera: Pyralidae). *Z. Naturforschung* 42:1347–1348.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

PHEROMONE RESPONSE INHIBITORS OF THE CORN STALK BORER *Sesamia nonagrioides*. BIOLOGICAL EVALUATION AND TOXICOLOGY

MAGÍ RIBA,[1,*] ALBERT SANS,[1] PEP BAU,[2] GÉRARD GROLLEAU,[3] MICHEL RENOU,[3] and ANGEL GUERRERO[2,*]

[1]*Escola Tècnica Superior Enginyeria Agrària, UdL-IRTA, Rovira Roure, 177 25198 Lleida, Spain*

[2]*Department of Biological Organic Chemistry IIQAB (CSIC), Jordi Girona, 18-26 08034 Barcelona, Spain*

[3]*INRA Unité de Phytopharmacie et Médiateurs Chimiques, Route de St Cyr 78026 Versailles Cedex, France*

(Received January 26, 2001; accepted April 28, 2001)

Abstract—The behavioral activity of some trifluoromethyl ketones (TFMKs) as inhibitors of the pheromone activity of the corn stalk borer *Sesamia nonagrioides* (Lef.) (Lepidoptera: Noctuidae) is reported. The most closely-related analogue to the pheromone tested, (*Z*)-11-hexadecenyl trifluoromethyl ketone (Z11-16:TFMK), elicited a significant decrease in the number of male catches in traps baited with mixtures with the pheromone in 1:1 and 10:1 ratios in comparison to the pheromone alone. The *E* isomer of the analogue as well as two highly hydrated ketones, 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) and 1,1-difluoropentadecyl trifluoromethyl ketone, were inactive. Conversely, the saturated TFMKs *n*-dodecyl trifluoromethyl ketone and, particularly, *n*-hexadecyl trifluoromethyl ketone induced a synergistic effect when mixed with the synthetic pheromone in 10:1 ratio. However, in a wind tunnel these chemicals did not elicit any differential effect on flying moths attracted to a source containing a 10:1 blend of the analogue and the pheromone. In a dual choice bioassay with two dispensers, containing the pheromone alone, and mixtures of Z11-16:TFMK and the pheromone and separated 5 cm apart, males showed no particular preference for either dispenser. The ketone mixture, however, induced moths to execute erratic flights with frequent crosswind counter-turns and intersections with plume boundaries. The total number of contacts with the source were reduced when a control dispenser was paired with a dispenser containing Z11-16:TFMK and pheromone. Z11-16:TFMK and OTFP showed little toxicity

*To whom correspondence should be addressed. e-mail: agpqob@iiqab.csic.es. or mriba@quimica.udl.es

0098-0331/01/0900-1879$19.50/0

on mice with a LD_{50} of 1 g/kg after the 6th day of treatment. For comparitive purposes, the major component of the pheromone, Z11-16:Ac, displayed a LD_{50} of 5 g/kg 6 days after application. Our results provide additional information about the activity of the TFMKs, which might be useful for the utilization of these chemicals in future pest control studies.

Key Words—Inhibition, sex pheromone, pheromone inhibitor, *Sesamia nonagrioides*, corn stalk borer, Lepidoptera, Noctuidae, field tests, trifluoromethyl ketones, pheromone inactivation.

INTRODUCTION

Chemical communication in insects can be significantly affected by synthetic biologically-active compounds, related or not to their sex pheromones (Renou and Guerrero, 2000), consequently, many behavior-modifying chemicals have been considered as potentially useful pest control agents (Ridgway et al., 1990; Renou and Guerrero, 2000). Catabolism and inactivation of sex pheromones in insects is carried out by the action of enzymes (esterases, dehydrogenases, aldehyde oxidases) present in the male antennae (Vogt et al., 1985; Prestwich, 1987; Kasang et al., 1989) . Inhibition of these enzymes may lead to a decreased capability by the insect to detect new incoming pheromone molecules, and therefore a new approach to insect control can be envisaged (Prestwich, 1986).

The corn stalk borer *Sesamia nonagrioides* (Lef.) (Lepidoptera: Noctuidae) is a key pest of maize in the Mediterranean area (below 45°N parallel) and North Africa countries (Anonymous, 1979). Broad spectrum insecticides have been applied to control the pest but the results have been poor due to the endophitic habits of the pest, high costs and negative effects on secondary pests, such as aphids, mites and leafhoppers. The sex pheromone of the moth was identified in 1985 by Sreng et al. (Sreng et al., 1985) as a mixture of (*Z*)-11-hexadecenyl acetate (Z11-16:Ac) and (*Z*)-11-hexadecenol (Z11-16:OH). Later, Mazomenos (1989) reported two additional minor components, (*Z*)-11-hexadecenal (Z11-16:Ald) and dodecyl acetate (12:Ac), which improved the efficiency of the pheromone blend. An amended formulation based on a mixture of Z11-16:Ac, Z11-16:OH, Z11-16:Ald and 12:Ac in 77:8:10:5 was later developed by us (Sans et al., 1997). Utilization of the pheromone blend to control the pest in monitoring and mass trapping (Mazomenos et al., 1989) and mating disruption experiments (Perdiguer et al., 1992; Frérot et al., 1997) have been conducted but with relative success.

Trifluoromethyl ketones (TFMKs) are known as potent inhibitors of a number of esterases and proteases, such as acetylcholinesterase, chymotrypsin or human liver carboxylesterases (Gelb et al., 1985; Ashour and Hammock, 1987). They may also inhibit the antennal esterases present in insect olfactory tissues (Vogt et al., 1985; Prestwich and Streinz, 1988; Durán et al., 1993; Parrilla and Guerrero,

1994). In wind tunnel experiments, TFMKs were found to disrupt the orientation flight of *Spodoptera littoralis* and *S. nonagrioides* males to pheromone sources (virgin females or synthetic pheromone) (Bau et al., 1999). However, (*Z*)-1,1,1-trifluoro-14-heptadecen-2-one, a pheromone analogue of *Ostrinia nubilalis*, did not inhibit male attraction when it was coevaporated with pheromone in a wind tunnel assay (Klun et al., 1991). In the field, some TFMKs displayed inhibitory activity of the pheromone action on processionary males when mixed with the pheromone (Parrilla and Guerrero, 1994), but this type of effect was not confirmed on *S. nonagrioides* when (*Z*)-1,1,1-trifluoro-14-nonadecen-2-one, a structurally related analogue of the pheromone, was added to the natural attractant. In this case and unexpectedly, a synergistic effect on the number of catches was observed (Riba et al., 1994). These contradictory data indicate that the relationship between the antiesterase activity of TFMKs and their physiological and behavioral effects on pheromone communication is far from being completely understood, and therefore more work in this regard is needed. We report herein the behavioral activity in the laboratory and in the field of some TFMKs on the corn stalk borer as well as preliminary toxicity studies of these chemicals in mice.

METHODS AND MATERIALS

Insects. The corn stalk borer was reared in the laboratory on a slightly-modified artificial diet (Poitout and Bues, 1974). Pupae were sexed, placed in groups of 20–25 into 20 × 20 cm plastic boxes and maintained in a climatic chamber on a 16 : 8 L:D regime at 25 ± 1°C and 65 ± 10% relative humidity until emergence. Adults were provided with 10% sucrose solution, separated daily by age and kept on filter paper in plastic containers.

Chemicals. The fluoromethyl ketones tested include 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP), *n*-dodecyl trifluoromethyl ketone (12:TFMK), *n*-hexadecyl trifluoromethyl ketone (16:TFMK), (*Z*)-11-hexadecenyl trifluoromethyl ketone (Z11-16:TFMK), (*E*)-11-hexadecenyl trifluoromethyl ketone (E11-16:TFMK) and 1,1-difluoropentadecyl trifluoromethyl ketone (15:PFMK) (Figure 1). They were prepared in our laboratory according to previously published procedures (Parrilla and Guerrero, 1994; Villuendas et al., 1994) except 15:PFMK (unpublished). The major component of the pheromone (*Z*)-11-hexadecenyl acetate, (Z11-16:Ac), was kindly provided by SEDQ, S.A.

Wind Tunnel Experiments. Assays were conducted in a glass tunnel of 180 × 55 × 50 cm, as previously described (Quero et al., 1995). The active space was visualized with the aid of a SO_3 smoke dispenser (Drägerwerk, Germany) and the illumination (2–5 lux) was obtained through a dimmed fluorescent red light. A video camera (Pulnix B/W TM50), linked to a JVC-SR306E video recorder and a Panasonic TC-14S1RC monitor, was placed at 135 cm above the tunnel

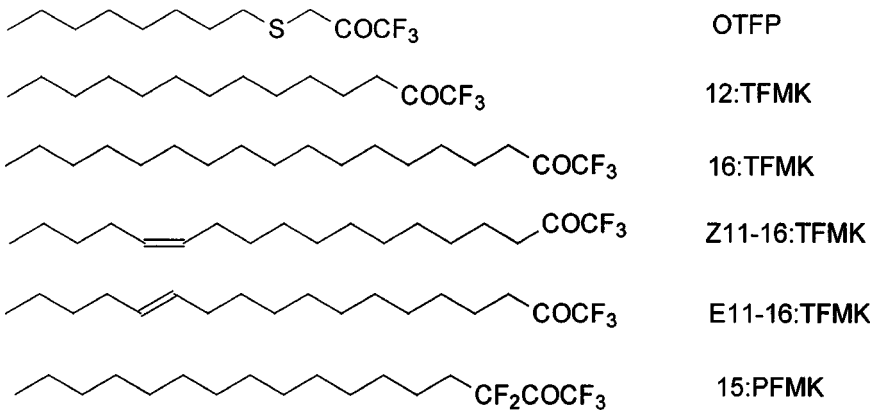


FIG. 1. TFMKs tested in wind tunnel assays and field tests against *S. nonagrioides*.

and in perpendicular position to minimize optical distortion of the flight. The camera allowed the recording of a flight path 130 cm long and 45 cm wide section of the tunnel and the video recordings were analyzed as already reported (Bau et al., 1999). The estimated cross wind dimension of the pheromone plume was 12 and 31 cm wide at 50 and 100 cm, respectively, from the source. Experiments were performed on individuals between the 3rd and the 7th h of the 2nd and 3rd scotophase. Moths were acclimatized to the wind tunnel conditions (temperature of the room 22 $\pm$ 1°C, 60 $\pm$ 10% relative humidity) for 30 min. Males were placed on a Petri dish, cut on the edge to facilitate take off of the moths, over a stainless steel jack 130 cm from the source and 18 cm from the top of the tunnel. Insects were allowed to respond for 5 min. Four types of behavior (wing fanning and taking flight TF; arrival to the middle of the tunnel HW; close approach to the lure CA; and contact with the source SC) were scored and compared with control insects. For out of plume parameters, only males which spent at least 20% of their total flight time outside the plume boundaries were considered. Flight parameters were determined on uninterrupted flights and only those males arresting at the source for a minimum period of 5 s were recorded as SC. Experiments were conducted in blocks including exposed and control insects, and statistical analysis (χ^2 homogeneity test, $P < 0.05$) was performed within every block. On each day of experimentation, three groups of 8–10 males flying towards a mixture of pheromone and trifluoromethyl ketone were compared with 10–14 control males. The attraction source was 0.1 μg of the pheromone blend (mixture of Z11-16:Ac, Z11-16:OH, Z11-16:Ald and 12:Ac in 77:8:10:5 ratio) (Sans et al., 1997). Just prior to the experiments, the required amount of attractant, dissolved in 10 μl of nanograde hexane, was applied to a brown female-shaped piece of cardboard used as dispenser. The solvent was allowed to evaporate and the dispensers were suspended 18 cm from the top and 40 cm from the upwind end of the tunnel. In the

dual choice tests, two dispensers, separated 5 cm apart from each other, were placed in parallel at the same distance from the release platform of the insects. For each flight track the following parameters were recorded: flight distance, flight duration, ground speed, turning frequency, track width, track angle, course angle and drift angle (Marsh et al., 1978), and analyzed for significance (*LSD* test, $P < 0.05$). The activity of the saturated 12:TFMK and 16:TFMK was tested by recording all types of behavior flying to a source consisting of mixtures of the analogue with the pheromone in 10:1 ratio compared to those elicited by the pheromone alone.

Field Tests. The experiments were conducted in infested maize fields of the Lleida province (northeastern Spain) during the 2nd and the 3rd generations of the insect. In the trials from September 2 to October 22 1998, the following compounds were tested: 12:TFMK, OTFP, Z11-16:TFMK and E11-16:TFMK. The field was divided into 4 independent blocks (replications), separated at least 50 m from each other. Funnel traps were hung at a height of 1.5 m and separated 25 m from each other within the block. Every block comprised 5 randomized treatments, those of the TFMKs and the synthetic pheromone, which had the same composition as that used in the wind tunnel experiments (see above). As dispensers, rubber septa were impregnated with mixtures of 0.1 mg of the synthetic pheromone and 1 mg of the inhibitor. Traps were rotated every week and the number of catches recorded. In 1999, the experiments were conducted from July 20 to October 15 and three TFMKs were tested: 12:TFMK, Z11-16:TFMK and 16:TFMK. The dispensers contained blends of the pheromone and the inhibitor in 1:1, 1:10 and 1:0.1 ratios, although 16:TFMK was not tested in mixtures with the synthetic attractant in the last ratio. The amount of pheromone was 0.1 mg in all cases. Three different blocks, separated 100 m apart from each other, were considered and each block comprised 9 randomized treatments, 8 mixtures of TFMK + pheromone and the pheromone alone. In the 2000 experiments, three different blocks with 8 treatments each were set up and the tests were conducted from July 26 to October 11. Baits contained mixtures of the fluoromethyl ketones Z11-16:TFMK, 16:TFMK and 15:PFMK with the pheromone in 1:1 and 10:1 ratios and the pheromone alone. The amount of pheromone was again 0.1 mg. All data were transformed ($\sqrt{x} + 0.5$) and analyzed for significance (Student's t-test, $P < 0.05$). In all experiments dispensers were replaced in the middle of the season flight.

Toxicology. Acute toxicity to vertebrates was determined orally on male and female Swiss mice (22–30 g). Test compounds (OTFP and Z11-16:TFMK) were diluted in corn oil and the appropriate volume of the solution to achieve a 500–1000 mg/kg dose (maximum 0.5 ml of solution per mouse) was introduced into the mouth of the animal, via a syringe equipped with a cannula for small animals. Mice were fed regular rodent diet and water, and weighed the day before treatment and for 7 consecutive days after injection or up to the day of death. Mortality was monitored daily during one week. Toxicity of the major pheromone

component Z11-16:Ac at doses of 500, 1000 and 5000 mg/kg was also determined for comparison purposes.

RESULTS

Wind Tunnel. S. nonagrioides males are delicate and sensitive to several environmental stimuli, such as type and shape of the dispenser, temperature and humidity. We have found that even under careful control and handling conditions the responses can be highly variable from day–to–day of experimentation. This variability induced us to establish a 50% threshold of control moths completing the behavioral sequence to validate the flight results of the day. When 10:1 mixtures of the saturated TFMKs (12:TFMK and 16:TFMK) with the synthetic pheromone blend were used in the source, the number of males displaying the different types of behavior were not significantly different regardless of the presence or absence of the analogue at the source (Figure 2). In this case, the track of the moths resembled that of the insects attracted to pheromone alone. A representative flight track of males flying towards a source containing a mixture of 100 ng of the pheromone blend and 1 μg of 12:TFMK relative to control (100 ng of the pheromone blend) is shown in Figure 3. The flight was quite straight with few counter-turns and intersections with the plume until the insect reached the proximity of the lure. The flight parameters of exposed and control insects were similar, the differences being

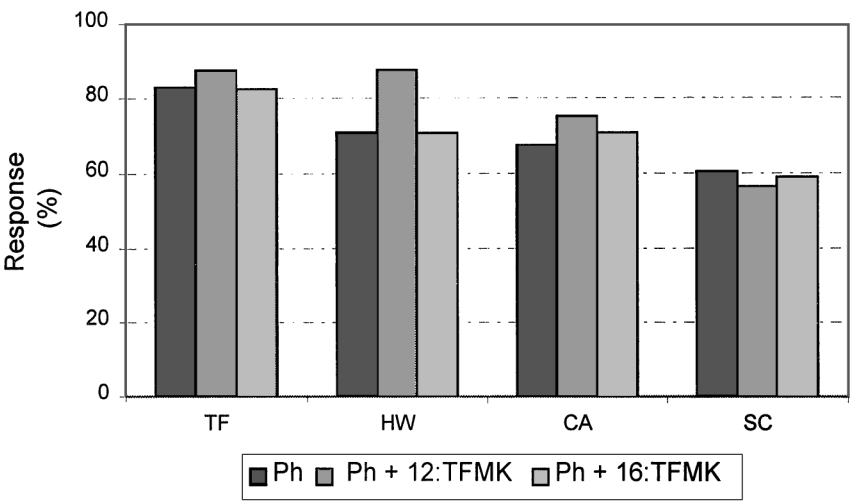


FIG. 2. Response of *S. nonagrioides* males in a wind tunnel to pheromone (Ph, $N = 58$) or to sources containing mixtures of 10 ng of the synthetic pheromone blend and 100 ng of 12:TFMK (Ph + 12:TFMK, $N = 32$) or 16:TFMK (Ph + 16:TFMK, $N = 34$).

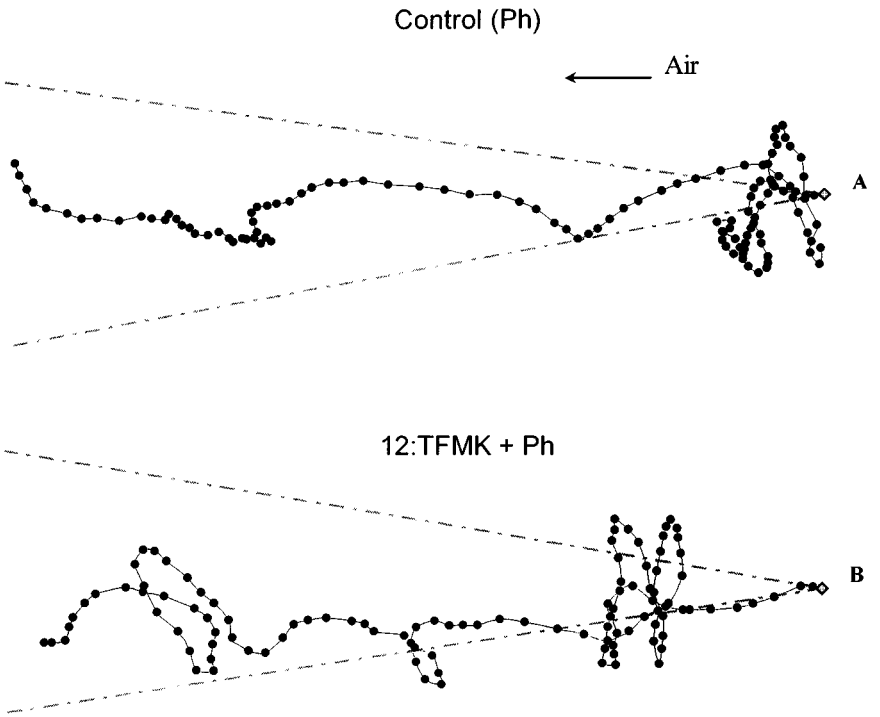


FIG. 3. Representative flight tracks of *S. nonagrioides* males flying upwind towards a dispenser containing a mixture of 100 ng of pheromone blend and 1 μg of 12:TFMK (bait B) relative to control (100 ng of pheromone, bait A). Black dots represent the insect positions at 0.1 s intervals.

statistically insignificant both when the insect was flying inside or outside the plume (Table 1). As expected, moths flying out of the plume displayed higher track width, track angle and course angle than insects flying inside the plume. The drift angle appeared higher but the difference was not significant (Table 1).

In the dual choice assay and in order to test whether the insects were able to discriminate between two overlapping plumes separated 5 cm at their origin, preliminary results showed that males were differently attracted to two lures baited with 100 ng and 1 μg of synthetic pheromone, respectively. Thus, while 17% ($N = 6$ out of 36) of insects were attracted to the lower-dose dispenser, 69% ($N = 25$ out of 36) contacted the source containing the higher dose. Baits with identical pheromone doses (100 ng) elicited no preference on the number of males contacting the source (36% and 43% of contacts in each bait, $N = 20$ and 24 out of 56, respectively) (control test, Figure 4). When serial doses of 1, 5 and 10 μg of Z11-16:TFMK were added to 100 ng of synthetic pheromone (treatment lure)

TABLE 1. SELECTED PARAMETERS FROM TRACKS OF *S. nonagrioides* MALES FLYING UPWIND TOWARDS A SOURCE CONTAINING A MIXTURE OF 100 NG OF PHEROMONE AND 1 μG OF 12:TFMK RELATIVE TO CONTROL (100 NG OF PHEROMONE)

	Inside the plume		Outside the plume	
Parameter	Control ($N=11$)	12:TFMK + pheromone ($N=15$)	Control ($N=10$)	12:TFMK + pheromone ($N=11$)
Total flight distance (cm)	200^a (16.0)	225^a (22.6)	—	—
Total flight duration (s)	4.5^a (0.5)	5.5^a (0.5)	—	—
Ground speed (cm/s)	20.4^a (2.4)	16.9^a (1.2)	19.4^a (1.5)	17.4^a (1.2)
Turning frequency (turns/s)	1.5^a (0.8)	1.7^a (0.5)	3.6^a (0.2)	4.2^a (0.25)
Track width (cm)	7.1^b (1.3)	5.1^b (0.5)	15.5^a (2.0)	11.8^a (1.0)
Track angle (degrees)	50.4^b (2.6)	57.0^b (2.3)	79.5^a (3.6)	73.8^a (3.5)
Course angle (degrees)	34.6^b (2.6)	37.3^b (2.1)	55.8^a (3.2)	51.3^a (3.0)
Drift angle (degrees)	15.8^b (1.5)	19.7^{ab} (1.4)	23.7^a (1.5)	22.6^a (1.3)

Note. Means (±SEM) within a row followed by the same letter are not significantly different ($P < 0.05$), LSD text).

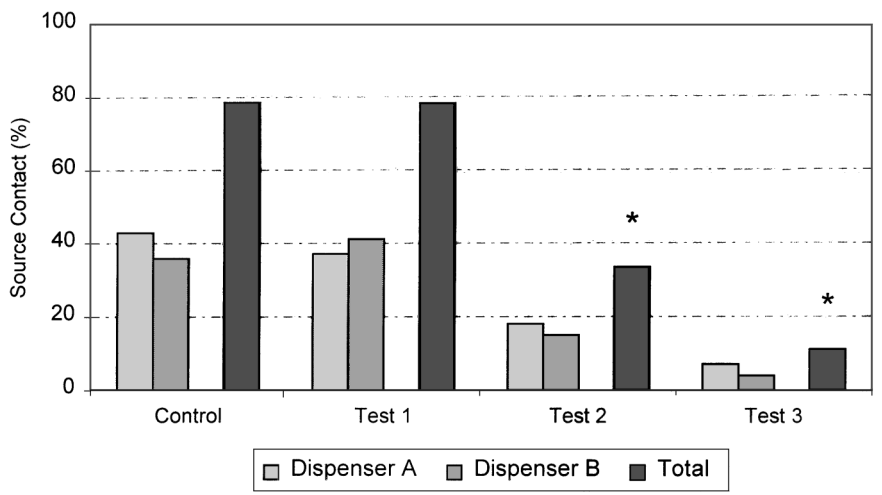


FIG. 4. Source contact behavior of *S. nonagrioides* males in dual choice tests of Z11-16: TFMK with the pheromone. In control test both dispensers contain 100 ng of the pheromone blend. Dispenser A contained 100 ng of pheromone in all cases. In tests 1–3, dispenser B contained a mixture of 100 ng of pheromone and 1, 5 and 10 μg of Z11-16:TFMK, respectively. Bar in black represents the sum of contacts of both dispensers in each test. The asterisk denotes significantly different values from control (χ^2 homogeneity test, $P < 0.05$).

relative to 100 ng of pheromone (reference lure), insects showed no particular preference for either source, irrespective of the amount of inhibitor present in the treatment lure. However, while no inhibition was noted when 1 μg of the inhibitor was present in the bait (78% of moths contact one source or the other vs 79% in the control experiment) (test 1, Figure 4), addition of 5 or 10 μg of the inhibitor elicited lower attraction of males to the source (33% of total number of contacts for 5 μg and 11% for 10 μg, $P < 0.05$) (tests 2–3, Figure 4). At the highest dose, only 1 and 2 insects out of 28 successfully contacted the treatment and the reference lure, respectively, while the percentage of insects arriving at the vicinity of the bait was 57% out of 93% that had taken flight. A representative flight track of a moth flying in this type of test vs a control insect is shown in Figure 5. The moths exhibited erratic progress toward the source, flying across the wind with a

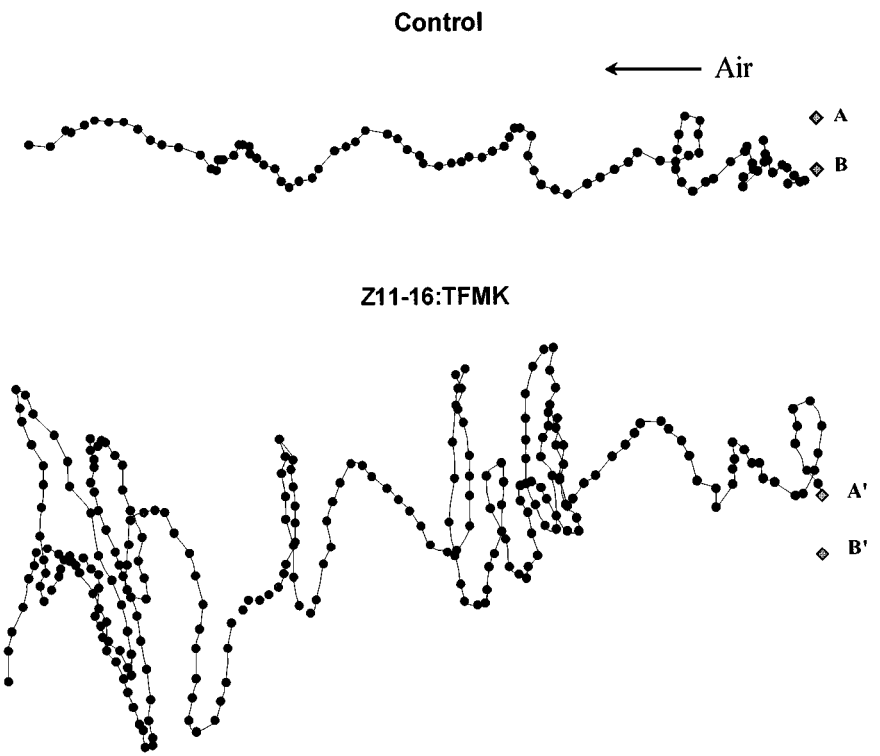


FIG. 5. Representative flight tracks of *S. nonagrioides* males flying upwind towards two dispensers. In the control test both dispensers (A and B) contained 100 ng of pheromone, whereas in the test with Z11-16:TFMK dispenser A′ contained a mixture of 100 ng of pheromone and 1 μg of the inhibitor and dispenser B′ only 100 ng of pheromone. Black dots represent the insect position at 0.1 s intervals.

TABLE 2. SELECTED PARAMETERS OF A FLIGHT TRACK OF *S. nonagrioides* MALES FLYING UPWIND TOWARDS TWO DISPENSERS. IN THE CONTROL TEST, DISPENSERS CONTAINED 100 NG OF PHEROMONE; IN THE TEST WITH Z11-16:TFMK, ONE DISPENSER CONTAINED A MIXTURE OF 100 NG OF PHEROMONE AND 1 μG OF THE INHIBITOR AND THE OTHER DISPENSER ONLY 100 NG OF PHEROMONE

Parameter	Control ($N = 12$)	Z11-16:TFMK ($N = 21$)
Total flight distance (cm)	161.6^b (12.7)	303^a (28.2)
Total flight duration (s)	11.9^a (2.3)	16.7^a (1.6)
Ground speed (cm/s)	14.6^a (1.0)	18.6^a (0.7)
Turning frequency (turns/s)	2.2^a (0.2)	1.7^b (0.1)
Track width (cm)	4.6^b (0.6)	7.9^a (0.7)
Track angle (degrees)	52.2^b (3.1)	67.3^a (1.6)
Course angle (degrees)	32.6^b (1.6)	48.0^a (1.7)
Drift angle (degrees)	19.6^a (1.6)	19.4^a (1.7)

Note. Means ($\pm$ SEM within a row followed by the same letter are not singificantly different ($P < 0.05$, LSD test).

frequent zigzag movement. The track width was higher in insects flying in plumes containing the TFMK (7.9 vs 4.6 cm), as were the track (67.3° vs 52.2°) and the course angles (48.0° vs 32.6°), while the drift angle (the difference between the course angle and the track angle) remained constant (Table 2). Total flight distance was also higher in TFMK-exposed moths (303 vs 161.6 cm). Flight duration to reach the source (16.7 vs 11.9 s) and the ground speed (18.6 vs 14.6 cm/s) did not differ (Table 2).

Field Tests. The effect of the fluorinated ketones was determined by comparing the number of males caught by mixtures of the chemicals with the pheromone relative to the pheromone alone. In preliminary tests carried out in 1998, a 10:1 mixture of Z11-16:TFMK with the pheromone decreased ($P < 0.05$) in the number of catches with regard to the pheromone (total catches/trap 8.3 ± 3.5 vs 87.8 ± 15.1) (Figure 6). Conversely, OTFP and E11-16:TFMK had no effect when mixed with the pheromone in the same ratio. Surprisingly, the saturated ketone, 12:TFMK, induced a clear synergistic effect (over 2 $\times$ fold) relative to the pheromone alone (Figure 6). In 1999, the activity of the chemicals was evaluated in several mixtures (0.1:1, 1:1, and 10:1) with the pheromone. The effect of 12:TFMK was not significant this time, although baits containing the ketone with the pheromone in 1:1 and 10:1 ratio caught more males than those with the natural attractant. However, 16:TFMK, increased (4 $\times$ fold) the level of captures when mixed with the pheromone at the highest ratio ($P < 0.05$). The inhibitory activity of the unsaturated analogue Z11-16:TFMK was again recorded, particularly in 10:1 mixtures with the pheromone ($P < 0.05$) in which the total number of catches per trap was 11.3 ± 5.7 compared to 56.3 ± 19.1 with the pheromone (Figure 7). In the 2000

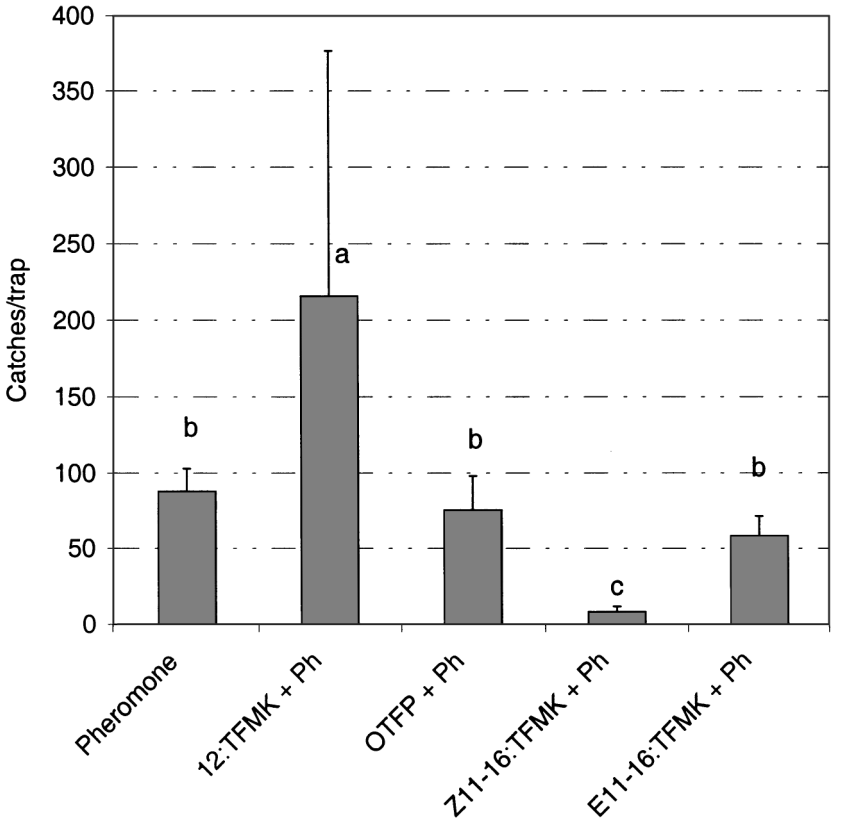


FIG. 6. Number of *S. nonagrioides* males caught in traps baited with mixtures of different trifluoromethyl ketones (12:TFMK, OTFP, Z11-16:TFMK and E11-16:TFMK) and pheromone in 10:1 ratio, compared with the number of males caught by the pheromone blend. Rubber septa were used as dispensers. The amount of pheromone present in all baits was 0.1 mg. Bars (±SEM) containing the same letter are not significantly different according to the Student t-test ($P < 0.05$). Tests were performed in an infested maize field in the province of Lleida (Spain) from September 2 to October 22, 1998.

tests, 16:TFMK again increased the number of catches (over 4 × fold) relative to the pheromone when mixed with the natural attractant in 10:1 ratio. No effect was observed in 1:1 blends (Figure 8). Conversely, Z11-16:TFMK decreased the number of catches even in 1:1 mixtures with the pheromone (total catches/trap 18.6 ± 9.0 vs 53.3 ± 11.3). The inhibitory effect of Z11-16:TFMK increased with dose, and, in the total number of males caught per trap was only 5.0 ± 2.6, 10:1 analogue:pheromone blends. Intrinsically, the analogue was unattractive. In a preliminary experiment, the pentafluoromethyl ketone 15:PFMK was

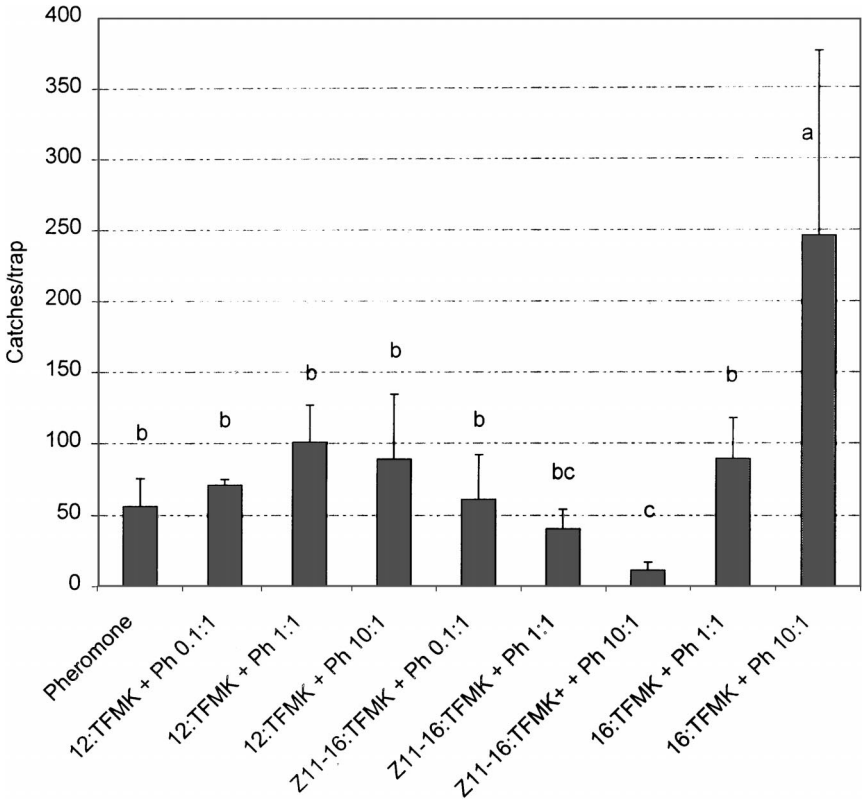


FIG. 7. Number of *S. nonagrioides* males caught in traps baited with mixtures of trifluoromethyl ketones (12:TFMK, Z11-16:TFMK and 16:TFMK) and the pheromone blend at 0.1 : 1, 1 : 1 and 10 : 1 ratios compared with the pheromone alone. Tests were conducted in an infested maize field in the province of Lleida (Spain) from July 20th to October 15th 1999. The amount of the pheromone present in all baits was 0.1 mg. Bars (±SEM) containing the same letter are not significantly different according to the Student t-test ($P < 0.05$).

practically inactive when blended with the pheromone at the highest proportion (Figure 8).

Toxicology. Mortality was not observed when Z11-16: TFMK and OTFP were administered at 500 mg/kg after 7 days. At 1000 mg/kg, all mice survived for 5 days when treated with OTFP and 4 days with Z11-16:TFMK. Administration of up to 2000 mg/kg of the major component of the pheromone induced no death after the 7-day treatment, while a higher dose of the chemical (5000 mg/kg) resulted in 25% mortality by the 5th day of treatment (Table 3). The mean weight gain of the animals was 31% (±4) when 500 mg/kg of Z11-16:Ac was administered.

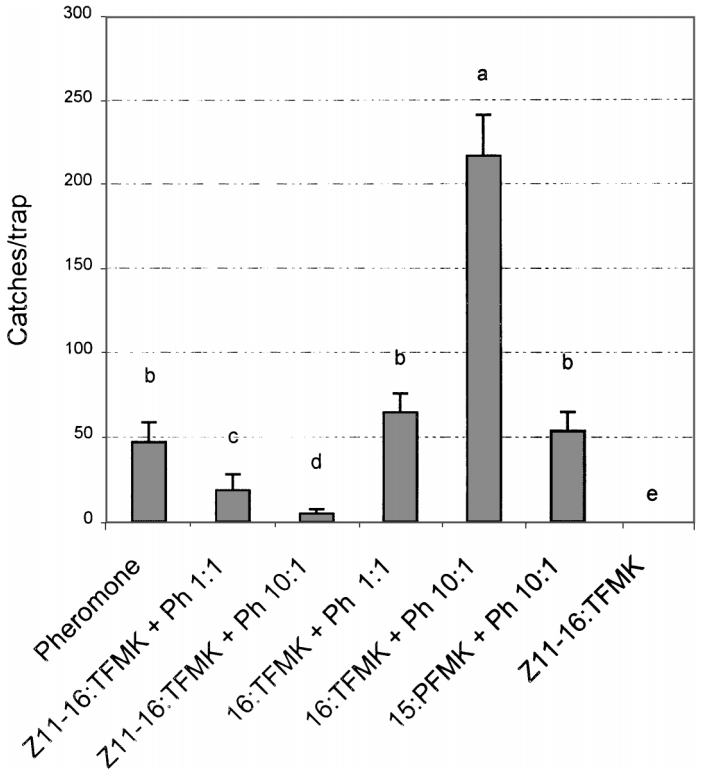


FIG. 8. Number of *S. nonagrioides* males caught in traps baited with mixtures of trifluoromethyl ketones (Z11-16:TFMK, 16:TFMK and 15:PFMK) and the pheromone in 1:1 and 10:1 ratios compared with pheromone alone. The assay was conducted in an infested maize field in the province of Lleida (Spain) from July 26 to October 11, 2000 and the dispensers replaced on September 6. The amount of the pheromone present in all baits was 0.1 mg. Bars (±SEM) containing the same letter are not significantly different according to the Student t-test ($P < 0.05$).

When mice were fed with the same amount of Z11-16:TFMK and OTFP the mean weight gains were 33% (±10) and 14% (±7), respectively. By contrast, doses of 1000 mg/kg of the TFMKs and 2000 mg/kg of the natural attractant caused mice to gain no weight.

DISCUSSION

Inhibition of the catabolic enzymes of the sex pheromone in insects has been considered a potential approach for pest control (Prestwich, 1986; Renou and

TABLE 3. MORTALITY OF SWISS MICE FED WITH DIFFERENT DOSES OF THE TRIFLUOROMETHYL KETONES Z11-16:TFMK AND OTFP AND THE MAJOR COMPONENT OF THE PHEROMONE OF *S. nonagrioides*, Z11-16:Ac[a]

Compound (dose per kg)	*n*	Days 1–4	Day 5	Day 6	Day 7
Z11-16:Ac					
500 mg	6	0	0	0	0
1000 mg	10	0	0	0	0
2000 mg	5	0	0	0	0
5000 mg	8	0	25	50	75
Z11-16:TFMK					
500 mg	6	0	0	0	0
1000 mg	7	0	14.3	42.9	42.9
OTFP					
500 mg	6	0	0	0	0
1000 mg	4	0	0	50	75

[a] Values are expressed in cumulative percentages of mortality.

Guerrero, 2000). In this regard, TFMKs reversibly inhibit the antennal esterases responsible for catabolism of pheromone molecules (Vogt et al., 1985; Prestwich and Streinz, 1988; Durán et al., 1993; Rosell et al., 1996), possibly through formation of a stable hemiacetal of tetrahedral geometry with a serine residue at the active site of the enzyme (Linderman et al., 1988; Rosell et al., 1996). These fluorinated chemicals elicited low intrinsic electroantennographic activity (Prestwich and Streinz, 1988; Riba et al., 1994), but reduced EAG pheromone responses in several insect species, such as *S. littoralis*, *Mamestra brassicae* and *Heliothis zea* (Renou et al., 1997), and inhibited the single sensillum responses (SSR) of the pheromone receptor cells of *S. littoralis* (Renou et al., 1997) and *Antheraea polyphemus* (Pophof, 1998; Pophof et al., 2000).

In field tests, the pheromone analogue Z11-16:TFMK consistently inhibited the attractant activity of the synthetic pheromone of the corn borer. In contrast, OTFP was ineffective as an inhibitor, possibly due to interaction/adsorption with the rubber septa since solvent extraction of freshly prepared dispensers containing 1 mg of the chemical failed to appreciably recover the compound. Likewise, E11-16:TFMK was inactive as inhibitor when mixed with the pheromone in 10:1 ratio. In the wind tunnel OTFP induced erratic flights by males flying to the source containing the chemical (Bau et al., 1999). Similarly, topical application of OTFP decreased the number of treated males contacting the pheromone source (Bau et al., 1999). The saturated analogue 12:TFMK, which showed an interesting antiesterase activity in *S. littoralis* (Durán et al., 1993), induced a synergistic effect in 10 : 1 mixtures with the pheromone. The effect was not significant, however, in 1999. 16:TFMK, a more closely related analogue of the pheromone, consistently

elicited a remarkable synergistic effect (4× fold) on the number of catches in a similar formulation both in 1999 and 2000 tests. It should be noted that (*Z*)-1,1,1-trifluoro-14-nonadecen-2-one (Z12-17:TFMK), the one-carbon homologue of Z11-16:TFMK, when added to the natural attractant in 10:1 ratio provoked a 68% increase in the number of catches relative to the pheromone (Riba et al., 1994) and increased the selectivity of the pheromone formulation with regard to other habitat-sharing species. Z12-17:TFMK was a poor inhibitor of the antennal esterase of *Plutella xylostella*, which shares Z11-16:Ac as one main pheromone component with *S. nonagrioides* (Prestwich and Streinz, 1988). These results suggest that small modifications of the analogue structure may imply, in an analogous way to the pheromone itself, a strong behavioral significance, as already described (Renou and Guerrero, 2000 and references cited therein).

We tried to confirm in wind tunnel bioassays the apparent synergistic effect of the two saturated TFMKs found in the field. However, no differential effect was found in any type of behavior when males were attracted to mixtures of the TFMK and the pheromone. Several factors may account for the discrepancy between the field and laboratory results. First, the global release rate of the inhibitor-pheromone blend in the field is clearly different from that in the wind tunnel, mainly due to the different types of dispensers used (the release rate from the cardboard being higher than the rubber septa by some orders of magnitude). For this reason, a 500× higher dosage of the pheromone blend on a rubber septum was required to elicit equal responses *Grapholita molesta* than the natural attractant on filter paper (Valeur and Löfstedt, 1996) and several olefinic compounds were released from rubber caps at approx. $10^3\times$ fold lower rate than from filter paper or cotton pads (Preiss and Priesner, 1988). In addition, and in preliminary tests, showed that a female-shape stimulus was an important factor for attractancy (Sans, unpublished data), although visual cues may be secondary in comparison to an accurate sex pheromone blend (Shorey and Gaston, 1970; Carpenter and Sparks, 1982). Second, the different diffusion rate of the antagonist and the natural pheromone, provided that they do not interact with the support, should imply a different proportion of the compounds in the air. This effect would be independent of the intrinsically different vapor pressure of the chemicals, as noticed by the retention times of 12:TFMK, 16:TFMK, Z11-16:TFMK, Z11-16:Ac, Z11-16:OH, Z11-16:Ald and 12:Ac which have been determined as 13.925, 23.463, 23.351, 28.17, 25.015, 23.539 and 18.835 min, respectively, on a DB-1 capillary column. Third, the effects in the wind tunnel are usually established within a relatively short distance (130 cm from the release platform of the insects to the bait), whereas in the field the flying attraction over much greater distances should be more determinant in assessing the activity of any specific inhibitor/synergist considered. This has been duly addressed in the literature, i.e., Hathaway et al. (1974) found that addition of undecanol to codlemone in 20:1 ratio reduced male catches of *Laspeyresia pomonella* by ca. 50%, while in a wind tunnel, higher than 1000×

fold overdose of the analogue was required to reduce orientation flight responses significantly.

In dual choice tests, males showed no preference for either dispenser when these were baited with identical pheromone doses. However, the moths clearly discriminated between the two overlapping plumes when the lures contained different amounts of the pheromone blend. In the presence of Z11-16:TFMK, the insects displayed no particular choice for either lure, but the ketone significantly decreased arrestment of the upwind flight, particularly at high doses. This indicates that the inhibitor not only exerts its antagonistic effect when evaporated with the pheromone from the same dispenser, but also on insects flying in an overlapping plume originated by a close pheromone source. This fact may have remarkable implications in future field studies since the inhibitor could eventually display its antagonistic action on natural pheromone released by females. Moths executed frequent crosswind counter-turns with a high number of intersections with plume boundaries, very much resembling the track of males topically treated with the chemical onto the antenna (Bau et al., 1999). The successful orientation of the males indicates that the odor filaments emanating from the two sources were sufficiently separated to be distinguished by the flying insects, which agrees with the fine-scale structure of odor plumes reported (Murlis and Jones, 1981; Mafra-Neto and Cardé, 1994; Baker et al., 1998).

Several TFMKs tested have shown a remarkable antiesterase activity *in vitro*. Thus, 12:TFMK displayed an IC_{50} of 4.4 μM in *S. littoralis* (Durán et al., 1993); 16:TFMK IC_{50} of 63 μM (Durán et al., 1993), OTFP of 0.08 μM (Rosell et al., 1996) and Z11-16:TFMK IC_{50} of 1.59 μM (Rosell et al., 1996); while in *S. nonagrioides* Z11-16:TFMK showed an IC_{50} of 123.7 μM (Quero et al., unpublished). However, their distinct activity *in vivo* point to a different mechanism(s) of action and not only to an antiesterase effect (Renou et al., 1997). Among the possible operating mechanisms, it has been suggested the one involving transport of the TFMK through binding with the pheromone binding proteins (PBP) of *A. polyphemus*, and subsequent competition with the PBP-pheromone complex at the pheromone receptor sites (Pophof et al., 2000). In the same trend, we found that some aliphatic TFMKs bind to the PBP of the processionary moth *Thaumetopoea pityocampa* in competition with pheromone molecules (Feixas et al., 1995). However, the structural deficiency of the analogue, which occurs with the saturated TFMKs, makes it unable to interact properly with the receptor site and to trigger adequate receptor cell responses, as shown by the lack of activity in SSR (Bau, 2000), to provoke a successful behavioral output. In the case of the unsaturated Z11-16:TFMK, the presence of the double bond with the right location and stereochemistry as in the pheromone skeleton may be a determinant positive feature for the recognition and the transduction processes (Camps et al., 1988; Renou and Guerrero, 2000). The variety of effects observed by the TFMKs tested in the field, cannot be fully explained since many different parameters may enter into play and affect the overall

behavior of the moths, as discussed above. In toxicity tests, Z11-16:TFMK and OTFP showed little toxicity to mice with an LD_{50} of 1 g/kg after the 6th day of experimentation. The major component of the pheromone, Z11-16:Ac, presented an LD_{50} of 5 g/kg after the same time of treatment, a value that is in the average of mammalian toxicity data available for Lepidoptera pheromones (Weatherston and Minks, 1995). Our data agree with the low acute toxicity to mice of several substituted TFMKs reported earlier (Hammock et al., 1982; Abdel-Aal et al., 1984) and support the lack of mammalian toxicity shown by other pheromones (Weatherston and Minks, 1995).

In summary, new data about the inhibition of pheromone action by a structurally related TFMK have been gathered. Our promising results, such as inhibition of the pheromone action in the field, inhibition of number of contacts to the pheromone source when it is close to another containing the analogue, and low toxicity, should stimulate the study of using TFMKs in future pest control strategies, as it has been proposed for juvenile hormone esterase (Hammock and Philpott, 1992; Bonning et al., 1995).

Acknowledgments—We gratefully acknowledge CICYT (AGF 97-1217-C02-01, AGF 97-1217-C02-02), EC (FAIR CT96-1302), and Comissionat per a Universitats i Recerca (1997SGR 00021) for financial support. We also thank SEDQ, S.A. for free samples of starting materials and Kenogard S.A. for previous support.

REFERENCES

ABDEL-AAL, Y. A. I., ROE, R. M., and HAMMOCK, B. D. 1984. Kinetic properties of the inhibition of juvenile hormone esterase by two trifluoromethyl ketones and O-ethyl-S-phenylphosphoramidothiolate. *Pest. Biochem. Physiol.* 21:232–241.

ANONYMOUS. 1979. Distribution maps of pests. Series A (Agricultural), Map No. 399 Pest: *Sesamia nonagrioides* (Lef.). Commonwealth Agricultural Bureau, London.

ASHOUR, M.-B. A., and HAMMOCK, B. D. 1987. Substituted trifluoroketones as potent selective inhibitors of mammalian carboxylesterases. *Biochem. Pharm.* 36:1869–1879.

BAKER, T. C., FADAMIRO, H. Y., and COSSÉ, A. A. 1998. Moth uses fine tuning for odour resolution. *Nature* 393:530.

BAU, J. (2000). Activitat electrofisiològica i comportamental de feromones sexuals de Lepidòpters i dels seus inhibidors. PhD Thesis. University of Barcelona, 230 pp.

BAU, J., MARTÍNEZ, D., RENOU, M., and GUERRERO, A. 1999. Pheromone-triggered orientation flight of male moths can be disrupted by trifluoromethyl ketones. *Chem. Senses* 24:473–480.

BONNING, B. C., HOOVER, K., BOOTH, T. F., DUFFEY, S., and HAMMOCK, B. D. 1995. Development of a recombinant baculovirus expressing a modified juvenile hormone esterase with potential for insect control. *Arch. Insect Biochem. Physiol.* 30:177–194.

CAMPS, F., FABRIÀS, G., GASOL, V., GUERRERO, A., HERNÁNDEZ, R., and MONTOYA, R. 1988. Analogs of the sex pheromone of processionary moth *Thaumetopoea pityocampa:* Synthesis and biological activity. *J. Chem. Ecol.* 14:1331–1346.

CARPENTER, J. E., and SPARKS, A. N. 1982. Effects of vision on mating behavior of the male corn earworm. *J. Econ. Entomol.* 75:248–250.

DURÁN, I., PARRILLA, A., FEIXAS, J., and GUERRERO, A. 1993. Inhibition of antennal esterases of the Egyptian armyworm *Spodoptera littoralis* by trifluoromethyl ketones. *Bioorg. Med. Chem. Lett.* 3:2593–2598.

FEIXAS, J., PRESTWICH, G. D., and GUERRERO, A. 1995. Ligand specificty of pheromone-binding proteins of the processionary moth. *Eur. J. Biochem.* 234:521–526.

FRÉROT, B., GUILLON, M., BERNARD, P., MADRENNES, L., SCHEPPER, B., MATHIEU, F., and COEUR, A. 1997. Mating disruption of corn stalk borer, *Sesamia nonagrioides* Lef. (Lep. Noctuidae), pp. 119–128, *in* P. Witzgall and H. Arn (eds.). Technology Transfer in Mating Disruption. IOBC, Montpellier.

GELB, M. H., SVAREN, J. P., and ABELES, R. H. 1985. Fluoroketone inhibitors of hydrolytic enzymes. *Biochemistry* 24:1813–1817.

HAMMOCK, B. D., and PHILPOTT, M. L. 1992. Juvenile hormone esterase for insect control. US Patent US 5,098,706.

HAMMOCK, B. D., WING, K. D., MCLAUGHLIN, J., LOVELL, V. M., and SPARKS, T. C. 1982. Trifluoromethyl ketones as possible transition state analog inhibitors of juvenile hormone esterase. *Pest. Biochem. Physiol.* 17:76–88.

HATHAWAY, D. O., MCGOVERN, T. P., BEROZA, M., MOFFITT, H. R., MCDONOUGH, L. M., and BUTT, B. A. 1974. An inhibitor of sexual attraction of male codling moths to a synthetic sex pheromone and virgin females in traps. *Environ. Entomol.* 3:522–524.

KASANG, G., NICHOLLS, M., and VON PROFF, L. 1989. Sex pheromone conversion and degradation in antennae of the silkworm moth *Bombyx mori* L. *Experientia* 45:81–87.

KLUN, J. A., SCHWARZ, M., and UEBEL, E. C. 1991. European corn borer: Pheromonal catabolism and behavioral response to sex pheromone. *J. Chem. Ecol.* 17:317–334.

LINDERMAN, R. J., LEAZER, J., ROE, R. M., VENKATESH, K., SELINSKY, B. S., and LONDON, R. E. 1988. ^{19}F NMR Spectral evidence that 3-octylthio-1,1,1-trifluoropropan-2-one, a potent inhibitor of insect juvenile hormone esterase, functions as a transition state analog inhibitor of acetylcholinesterase. *Pest. Biochem. Physiol.* 31:187–194.

MAFRA-NETO, A., and CARDÉ, R. T. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144.

MARSH, D., KENNEDY, J. S., and LUDLOW, A. R. 1978. An analysis of anemotactic zigzagging flight in male moths stimulated by pheromone. *Physiol. Ent.* 3:221–240.

MAZOMENOS, B. E. 1989. Sex pheromone components of corn stalk borer *Sesamia nonagrioides* (Lef.). *J. Chem. Ecol.* 15:1241–1247.

MAZOMENOS, B. E., BARDAS, D., and PANTAZIMAZOMENOU, A. 1989. *Sesamia nonagrioides* sex pheromone: Field attactiveness use of pheromone traps for monitoring and control. *Acta Phytopatol. Entomol. Hungar.* 24:153–156.

MURLIS, J., and JONES, C. D. 1981. Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

PARRILLA, A., and GUERRERO, A. 1994. Trifluoromethyl ketones as inhibitors of the processionary moth sex pheromone. *Chem. Senses* 19:1–10.

PERDIGUER, A., GIMENO, F., AGUILAR, L., EIZAGUIRRE, M., RIBA, M., and SANS, A. 1992. Ensayos de confusión sexual en *Sesamia nonagrioides. Inv. Agr. Prot. Veg.* 7:253–260.

POITOUT, S., and BUES, R. 1974. Élevage des chenilles de vingt-huit espèces de lepidoptères Noctuidae et deux espèces d'Arctiidae sur milieu artificiel simple. Particularités de l'élevage selon les espèces. *Ann. Zool. Ecol. Anim.* 6:431–441.

POPHOF, B. 1998. Inhibitors of sensillar esterase reversibly block the responses of moth pheromone receptor cells. *J. Comp. Physiol. A* 183:153–164.

POPHOF, B., GEBAUER, T., and ZIEGELBERGER. 2000. Decyl-thio-trifluoropropanone, a competitive inhibitor of moth pheromone receptors. *J. Comp. Physiol.* 186:315–323.

PREISS, R., and PRIESNER, E. 1988. Responses of male codling moths (*Laspeyresia pomonella*) to codlemone and other alcohols in a wind tunnel. *J. Chem. Ecol.* 14:797–813.

PRESTWICH, G. D. 1986. Fluorinated sterols, hormones and pheromones: Enzyme-targeted disruptants in insects. *Pest. Sci.* 37:430–440.

PRESTWICH, G. D. 1987. Chemical studies of pheromone reception and catabolism, pp. 473–527, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

PRESTWICH, G. D., and STREINZ, L. 1988. Haloacetate analogs of pheromones: Effects on catabolism and electrophysiology in *Plutella xylostella. J. Chem. Ecol.* 14:1003–1021.

QUERO, C., CAMPS, F., and GUERRERO, A. 1995. Behavior of processionary males (*Thaumetopoea pityocampa*) induced by sex pheromone and analogs in a wind tunnel. *J. Chem. Ecol.* 21:1957–1969.

RENOU, M., and GUERRERO, A. 2000. Insect parapheromones in olfaction research and semiochemical-based pest control strategies. *Ann. Rev. Entomol.* 48:605–630.

RENOU, M., LUCAS, P., MALO, E., QUERO, C., and GUERRERO, A. 1997. Effects of trifluoromethyl ketones and related compounds on the EAG and behavioural responses to pheromones in male moths. *Chem. Senses* 22:407–416.

RIBA, M., EIZAGUIRRE, M., SANS, A., QUERO, C., and GUERRERO, A. 1994. Inhibition of pheromone action in *Sesamia nonagrioides* by haloacetate analogues. *Pest. Sci.* 41:97–103.

RIDGWAY, R. L., SILVERSTEIN, R. M., and INSCOE, M. N., (eds.) (1990). Behavior-Modifying Chemicals for Insect Management. New York, Marcel Dekker.

ROSELL, G., HERRERO, S., and GUERRERO, A. 1996. New trifluoromethyl ketones as potent inhibitors of esterases: ^{19}F NMR spectroscopy of transition state analog complexes and structure-activity relationships. *Biochem. Biophys. Res. Comm.* 226:287–292.

SANS, A., RIBA, M., EIZAGUIRRE, M., and LOPEZ, C. 1997. Electroantennogram, wind tunnel and field responses of male Mediterranean corn borer, *Sesamia nonagrioides*, to several blends of its sex pheromone components. *Ent. Exp. Appl.* 82:121–127.

SHOREY, H. H., and GASTON, L. K. 1970. Sex pheromones of noctuid moths. XX. Short-range visual orientation by pheromone-stimulated males of *Trichoplusia ni. Ann. Entomol. Soc. Am.* 63:829–832.

SRENG, I., MAUME, B., and FRÉROT, B. 1985. Analyse de la sécretion phéromonale produite par les femelles vierges de *Sesamia nonagrioides* (Lef.) (Lepidoptère, Noctuidae). *C.R. Acad. Sci. Paris III* 301:439–442.

VALEUR, P. G., and LÖFSTEDT, C. 1996. Behaviour of male oriental fruit moth, *Grapholita molesta*, in overlapping sex pheromone plumes in a wind tunnel. *Ent. Exp. Appl.* 79:51–59.

VILLUENDAS, I., PARRILLA, A., and GUERRERO, A. 1994. An efficient and expeditious synthesis of functionalized trifluoromethyl ketones through lithium-iodine exchange reaction. *Tetrahedron* 50:12673–12684.

VOGT, R. G., RIDDIFORD, L. M., and PRESTWICH, G. D. 1985. Kinetic properties of a pheromone-degrading enzyme: The sensillar esterase of *Antheraea polyphemus. Proc. Natl. Acad. Sci. USA* 82:8827–8831.

WEATHERSTON, I., and MINKS, A. K. 1995. Regulation of semiochemicals—global aspects. *Pest Manage. Rev.* 1:1–13.

Journal of Chemical Ecology, Vol. 27, No. 9, September 2001 (© 2001)

REMOVAL OF DISSOLVED BROWN ALGAL PHLOROTANNINS USING INSOLUBLE POLYVINYLPOLYPYRROLIDONE (PVPP)

GUNILLA B. TOTH* and HENRIK PAVIA

Tjärnö Marine Biological Laboratory
Göteborg University
SE-452 96 Strömstad, Sweden

(Received January 23, 2001; accepted May 6, 2001)

Abstract—Tannins, a large and diverse group of phenolic secondary metabolites, are common in terrestrial plants and marine brown algae. It is sometimes desirable to remove the tannins from plant or algal extracts, e.g., when isolating enzymes and nucleic acids, when using certain colorimetric methods to quantify the tannin content, or to create reliable controls when using tannins in experimental studies. Insoluble polyvinylpolypyrrolidone (PVPP) can be used to specifically remove tannins from solution. In the present study, we evaluated the effect of different factors (amount of PVPP, number of PVPP treatments, type of solvent, pH, and incubation time) on the PVPP removal of dissolved brown algal phlorotannins. Our results imply that there is a limited amount of phlorotannins that can bind to a given amount of PVPP, and that it is preferable to use low quantities of PVPP repeatedly, compared to using fewer treatments with a high amount of PVPP. Furthermore, we found no consistent effect on the removal of phlorotannins due to solvent type (acetone, methanol, distilled water or filtered seawater). There was a slight decrease in the amount of phlorotannins removed from extracts with increasing pH when repeatedly treated with PVPP. All phlorotannins were removed from extracts with pH $\leq$ 6.2, and 89% of the initial phlorotannin content was removed at pH 9.7. These results are compared with previous methodological studies on tannin removal with PVPP. Furthermore, the implications of phlorotannin removal in analytical and ecological investigations are discussed.

Key Words—Bioassay, enzyme, Folin-Ciocalteus, pH, phlorotannins, polyphenolic compounds, polyvinylpolypyrrolidone, PVPP, solvent, tannins.

*To whom correspondence should be addressed. e-mail: gunilla.toth@marbot.gu.se

0098-0331/01/0900-1899$19.50/0

INTRODUCTION

Polyphenolic secondary metabolites are a large and diverse group of chemical compounds, which are common both in terrestrial plants and in aquatic macrophytes (Waterman and Mole, 1994). One class of polyphenolic compounds that has received much attention from ecologists during the last decades is the tannins (see Harborne, 1983; Karban and Baldwin, 1997), which are water-soluble compounds with the ability to precipitate proteins from solution. They are thought to act as chemical defenses in plants either by having direct toxic effects (e.g., Berenbaum, 1984; Martin and Martin, 1984; Steinly and Berenbaum, 1985), or by decreasing the digestibility of food (Tugwell and Branch, 1992) or the assimilation efficiency of herbivores (Boettcher and Targett, 1993). Tannins can be divided into three chemically distinct groups: condensed tannins, hydrolyzable tannins, and phlorotannins. Condensed tannins are associated with the production of lignin and are common in lignified higher plants, but also occur in some herbs. Hydrolyzable tannins occur in some green alga and in angiosperms, while phlorotannins are restricted to brown algae (Waterman and Mole, 1994). Phlorotannins are the least complex group of tannins and consist of phloroglucinol (1,3,5, trihydroxybenzene) units that are linked by carbon—carbon or carbon—oxygen bonds. They have a wide range of molecular sizes (400 to 400,000 Da) and can occur in variable concentrations (0.5–20% of the dry weight) in the epidermal cortex in brown algae (Ragan and Glombitza, 1986). Phlorotannins are commonly believed to have defensive or protective functions, e.g., against herbivores, bacteria, fungi, fouling organisms and UV-B radiation (see Ragan and Glombitza, 1986; Targett and Arnold, 1998, for references), to function in wound healing processes (Fagerberg and Dawes, 1976), or in cell-wall construction (Schoenwaelder and Clayton, 1998).

Tannins form strong complexes with proteins either reversibly by hydrogen bonding through peptide or amide linkages, or irreversibly by covalent condensations (Loomis and Battaile, 1966; Appel, 1993; Waterman and Mole, 1994; Stern et al., 1996a). This may interfere with, or even prevent, the isolation of enzymes or nucleic acids from plant extracts (e.g., Loomis and Battaile, 1966; Porebski and Bailey, 1997; Koonjul et al., 1999). Furthermore, removal of tannins may be desirable during analytical quantification of total plant phenolic compounds (e.g., Yates and Peckol, 1993; Van Alstyne, 1995; Peckol et al., 1996; Stern et al., 1996b), or when using tannin extracts in experimental studies (e.g., Pavia et al., 1997; Pavia and Toth, 2000; Toth and Pavia, 2000). The strong hydrogen bonding capacity can be used to remove tannins by adding substances that contain groups similar to peptide bonds e.g., nylon or polyvinylpolypyrrolidone (PVPP). Optimal conditions for binding of tannins to PVPP has been evaluated previously (Andersen and Sowers, 1968), but the experimental treatments were not replicated and the data were not statistically analyzed. However, the results suggested that successive

treatments of plant extracts with insoluble PVPP in 1–10 vol% methanol under acidic conditions (pH 3.5) were favorable for specifically removing tannins from solution (Andersen and Sowers, 1968). The aim of the present study was to evaluate the optimal conditions for PVPP removal of brown algal phlorotannins in an experimentally and statistically rigorous way. We performed a series of laboratory experiments where we evaluated the influence of different solvents, extract pH, quantities of PVPP, number of PVPP treatments, and incubation times on the removal of dissolved phlorotannins.

METHODS AND MATERIALS

Collection and Preparation of Algal Phlorotannin Samples. Phlorotannins were extracted from individuals of the brown seaweed species *Ascophyllum nodosum* (L.) Le Jolis, collected randomly from different sites outside Tjärnö Marine Biological Laboratory at the Swedish West Coast (58°54′N, 11°07′E). *A. nodosum* is common at sheltered rocky shores in the north Atlantic, where it can form large stands in the intertidal zone. In the area of investigation, it has a phlorotannin content of 4–15% of the plant's dry weight (Pavia and Åberg, 1996; Pavia et al., 1999). Seaweed individuals were brought back to the laboratory, cleaned of all visible epiphytes and debris, and immediately frozen (−70°C) and freeze-dried. The freeze-dried algal material was homogenized to a fine powder and phlorotannins were extracted in aqueous acetone (60 vol%) on a shaking rack in darkness at 4°C for 24 hr. The extract was centrifuged and filtered (0.2 μm) to remove algal particles. Acetone was evaporated *in vacuo* and the extract was filtered again to remove precipitated lipophilic substances. Fresh extracts were prepared daily and the Folin-Ciocalteus (F-C) analysis for quantification of total phenolic compounds (Van Alstyne, 1995) was used to determine the concentration of F-C reactive compounds. This analysis measures all substances with aromatic hydroxyl groups and does not discriminate between phlorotannins and non-phlorotannin compounds. The effects of different extract solvents, extract pH, quantities of PVPP, number of PVPP treatments, and incubation times on the PVPP removal of the dissolved F-C reactive compounds were determined under different conditions in three separate experiments.

Effects of Solvent and Incubation Time on PVPP-Phlorotannin Binding. In the first experiment, we studied the influence of different solvents (10 vol% acetone and methanol) and incubation times (10 and 30 min) on the ability of PVPP (CAS no. 25249-54-1) to remove F-C reactive compounds from solution. The initial concentration of F-C reactive compounds was 470 mg/l and the pH of the extracts was 5.8. Extract aliquots (5 ml) and PVPP (25 mg/ml) were placed in small brown glass flasks (20 ml) and placed on a shaking rack for the desired incubation time. Two replicates of each treatment were prepared. After incubation, the samples were

centrifuged and the supernatant was filtered (0.2 μm) to remove the PVPP. The concentration of F-C reactive compounds was measured and the amount removed (% of initial concentration) was calculated. Data were analyzed using a two-way analysis of variance (ANOVA) with solvent (2 levels) and incubation time (2 levels) as fixed orthogonal factors.

Effect of Solvent, pH and PVPP Treatment on PVPP-Phlorotannin Binding. In the second experiment, the effects of different solvents, pH and PVPP treatments on the removal of dissolved F-C reactive compounds were studied. Since this experiment was conducted over four days, four different extracts with initial concentrations of F-C reactive compounds between 450–600 mg/l were used. The initial pH of the extracts was 5.8, and was lowered to 3.5 using concentrated hydrochloric acid (0.3 μl HCl/ml extract) in the low pH treatments. Extract aliquots (5 ml), based on distilled water, acetone, or methanol and with different pH, were placed together with low and high (10 or 25 mg/ml) amounts of PVPP in brown glass flasks as in the first experiment. Six replicates of each treatment were prepared and incubated for 10 min. After incubation, PVPP was removed by centrifugation and the supernatant was filtered (0.2 μm) as in the first experiment. The concentration of F-C reactive compounds was measured in two replicates. This procedure was repeated twice with 10 mg fresh PVPP/ml extract, and after each PVPP treatment the concentration of F-C reactive compounds was measured in two replicates. The amount of F-C reactive compounds removed (% of initial concentration) was calculated, and data were analyzed using a three-way ANOVA with solvent (3 levels), pH (2 levels) and PVPP treatment (6 levels) as fixed orthogonal factors.

Effect of pH on PVPP-Phlorotannin Binding. The phlorotannin extract used in the third experiment was purified according to the method described in Steinberg and van Altena (1992), and the phlorotannins were dissolved in filtered seawater (15%). The initial phlorotannin concentration of the extract was 500 mg/l and the initial pH was 6.2. In this experiment, we tested the effect of different pH's on the removal of the dissolved phlorotannins. The extract was divided into three equal volumes and a few μl of concentrated HCl or NaOH was added to adjust the pH to 2.9 and 9.7. Extract aliquots (5 ml) were placed together with PVPP (10 mg/ml) in brown glass flasks as in the previous experiments. Two replicates were prepared and treated three times with PVPP, after which the phlorotannin content was measured as in the second experiment. The amount of phlorotannins removed (% of initial phlorotannin concentration) was calculated, and data were analyzed using a one-way ANOVA with pH as a fixed 3-level factor.

Prior to all analysis, data were tested for homogeneity of variances with Cochran's test (Underwood, 1997). Multiple mean comparisons were made with the Student Newman Keuls (SNK) test (Underwood, 1997).

RESULTS

There was no difference in the amount of F-C reactive compounds removed from extracts containing 10 vol.% acetone or methanol (ANOVA, $F_{1,4} = 0.10$, $P = 0.77$) in the first experiment (Figure 1). Furthermore, incubating the extracts with PVPP for 10 or 30 min removed similar amounts of F-C reactive compounds, since there was no difference between different incubation times (ANOVA, $F_{1,4} = 0.01$, $P = 0.93$, Figure 1).

There was a difference in the amount of F-C reactive compounds removed from extracts with different solvents and pH when subjected to different PVPP treatments, as indicated by the three-way interaction between the factors solvent, pH and PVPP treatment (Table 1) in the second experiment. The SNK test revealed that there was an overall increase in the amount of F-C reactive compounds removed when the extracts were treated three times with PVPP, irrespective of the amount PVPP used in the first treatment (10 or 25 mg PVPP/ml, Figure 2). Furthermore, in accordance with the first experiment, there was no consistent difference in the amount of F-C reactive compounds removed due to the extract solvent

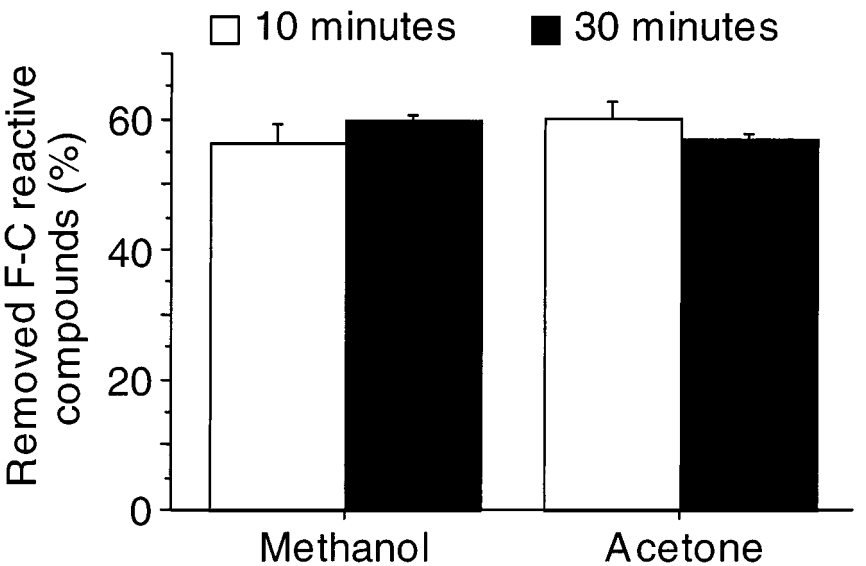


FIG. 1. Removal of Folin-Ciocalteus (F-C) reactive compounds (% of initial concentration) from *Ascophyllum nodosum* extracts based on different solvents (10 vol% methanol or acetone) treated once with 25 mg PVPP/ml extract for 10 or 30 min incubation. The pH of the extracts was 5.8. There were no significant differences among treatments. Error bars show + SE ($N = 2$).

TABLE 1. THREE WAY ANALYSIS OF VARIANCE (ANOVA) OF THE AMOUNT OF F-C REACTIVE COMPOUNDS REMOVED FROM *Ascophyllum nodosum* EXTRACTS WITH DIFFERENT SOLVENTS AND pH, SUBJECTED TO DIFFERENT PVPP TREATMENTS IN THE SECOND EXPERIMENT[a]

Source	*df*	MS	*F*	*P*
Solvent (S)	2	6.78	3.57	0.04
pH	1	178.54	93.92	<0.001
PVPP treatment (PVPP)	6	8353.55	4394.56	<0.001
S × pH	2	16.66	8.76	<0.001
S × PVPP	12	5.56	2.93	0.005
pH × PVPP	6	335.94	176.73	<0.001
S × pH × PVPP	12	4.79	2.52	0.014
Residual	42	1.90		

[a] Data on mean values and standard errors are presented in Figure 2.

(Figure 2). The only difference ($P < 0.05$) in the amount of F-C reactive compounds removed due to extract solvent was found when extracts with pH 5.8 were treated twice, first with high and then with low amount of PVPP. Significantly, fewer F-C reactive compounds were removed from the distilled water extracts compared to the methanol and acetone extracts (Figure 2), although the difference was relatively small. The pH of extracts affected the removal of F-C reactive compounds either positively or negatively depending on the PVPP treatment. The largest differences between pH treatments were found when extracts were treated once with a high amount of PVPP. There was a higher removal of F-C reactive compounds from extracts at pH 3.5 compared to extracts at pH 5.8, while the opposite was found in extracts that were treated once with a lower amount of PVPP (Figure 2).

There was a difference in the amount of phlorotannins removed from extracts with different pH (ANOVA, $F_{2,3} = 809.47$, $P < 0.0001$) by three successive PVPP treatments. The SNK test revealed that the phlorotannin removal was most effective from extracts with pH 2.9 and 6.2 and less effective from extract with pH 9.7 (Figure 3).

DISCUSSION

The results in the present study show that the effectiveness of phlorotannin removal with PVPP is dependent on the number of PVPP treatments and the amount of PVPP used in each treatment, i.e., it appears that a limited amount of phlorotannin can bind to a given amount of PVPP. Increasing the incubation time from 10 to 30 min did not increase the amount of F-C reactive material removed from a

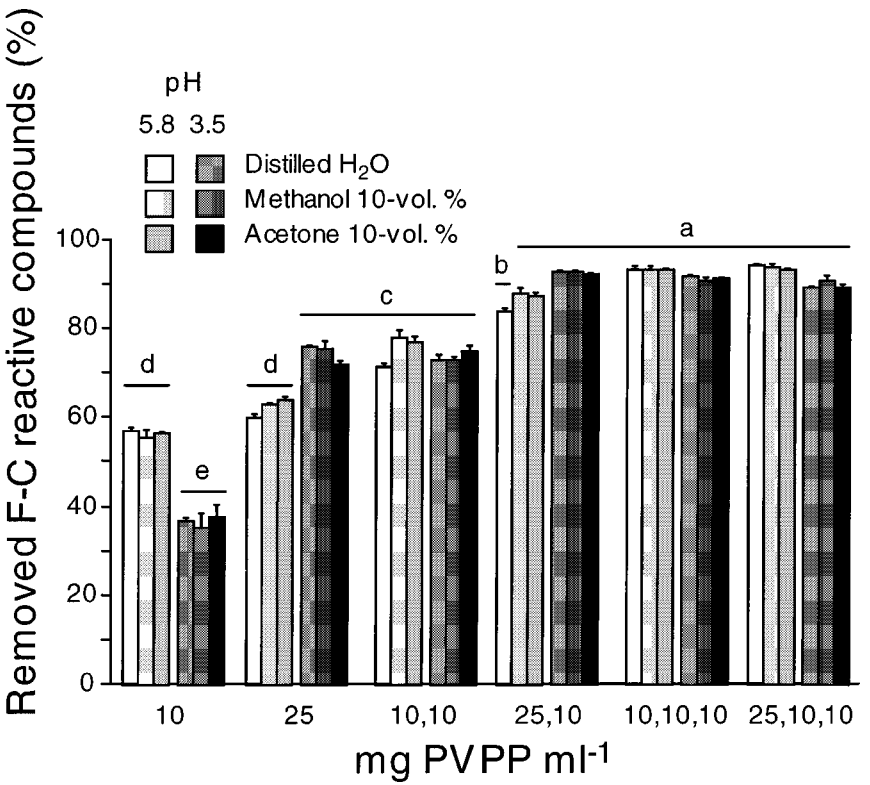


FIG. 2. Removal of Folin-Ciocalteus (F-C reactive) compounds (% of initial concentration) from *Ascophyllum nodosum* extracts based on different solvents (distilled water, 10 vol% methanol or acetone) with different pH (5.8 or 3.5) subjected to different PVPP treatments (10 or 25 mg PVPP/ml in the first treatment and subsequent treatments with 10 mg PVPP/ml). Letters above horizontal bars indicate significant differences among treatments based on Student-Newman-Keuls multiple-comparisons tests ($P < 0.05$). Error bars show + SE ($N = 2$).

single PVPP treatment in the first experiment. In accordance with the findings in previous studies (Andersen and Sowers, 1968; Targett et al., 1995), we found that three successive 10 min PVPP incubations were effective in removing the F-C reactive material from solution. These treatments removed approximately 90% of the F-C reactive compounds from extracts in the second experiment. The remaining F-C reactive substances were probably non-phlorotannin compounds with aromatic hydroxyl-groups, which did not bind to PVPP (Yates and Peckol, 1993; Van Alstyne, 1995; Peckol et al., 1996; Stern et al., 1996b). In the third experiment, when more purified phlorotannin extracts with different pH were treated repeatedly

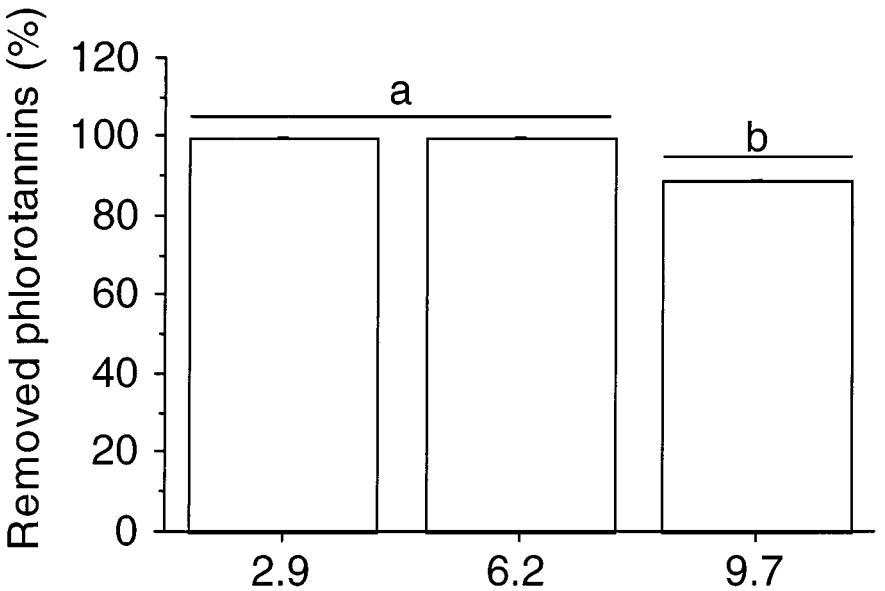


FIG. 3. Removal of phlorotannins (% of initial concentration) from purified *Ascophyllum nodosum* extracts with different pH (2.9, 6.2, and 9.7) after three successive PVPP treatments (10 mg PVPP/ml extract). The extracts were based on artificial seawater (15%). Letters above horizontal bars indicate significant differences among treatments based on Student-Newman-Keuls multiple-comparisons tests ($P < 0.05$). SE was 0.101 at pH 2.9, 0.013 at pH 6.2, and 0.364 at pH 9.7 ($N = 2$).

with PVPP, all (>99%) of the phlorotannins were removed after three successive PVPP treatments both in extracts with pH 2.9 and 6.2. The PVPP removal of dissolved phlorotannins was independent of the solvent used, i.e., there was no consistent difference in the phlorotannin removal between extracts based on distilled water compared to methanol or acetone. Furthermore, 10 mg PVPP/ml extract was preferable to 25 mg/ml, since a larger proportion of the extract was lost due to absorption to the dry PVPP powder when using the high amount of PVPP. Given these results, the initial concentration of the phlorotannin extract probably affects the number of PVPP treatments that are required to remove all phlorotannins. An extract with a higher initial phlorotannin concentration than used in the present study (i.e., >600 mg/l) may require more than three successive PVPP treatments. However, the results in the present study imply that it is preferable to increase the number of PVPP treatments, rather than the amount of PVPP in a given treatment, when the phlorotannin content of the extract increases.

It has been reported that PVPP is most effective in removing tannins from solutions with a low pH (Andersen and Sowers, 1968), and this statement has been

referred to by several authors (Yates and Peckol, 1993; Targett et al., 1995; Van Alstyne, 1995; Stern et al., 1996b). A low pH has been regarded as favorable since tannins are thought to bind to the PVPP molecules with hydrogen bonds (Loomis and Battaile, 1966; Waterman and Mole, 1994; Stern et al., 1996a). The experiment performed by Andersen and Sowers (1968) was not replicated, and, therefore, it is not possible to state that there actually was a significant difference in the tannin removal between extracts with different pH (3.5–8.5). In the present study, we demonstrated that, when treating the extracts repeatedly with PVPP, there is no difference in the amount of phlorotannins removed if pH is increased from 3.5, which was regarded as the optimal pH by Andersen and Sowers (1968), to 6.2. The effectiveness of phlorotannin removal decreases if the pH is increased even further, although three successive PVPP treatments still removed 89% of the phlorotannins in the extract with pH 9.2. The fact that there is no need to adjust the pH of the extract is advantageous if PVPP treated phlorotannin extracts are prepared for the purpose of being used as controls in experimental studies (see discussion of ecological implications below).

Use of PVPP to remove phlorotannins from brown algal extracts has been criticized due to the fact that a single PVPP treatment failed to remove all (i.e., 100%) of the phloroglucinol or phlorotannin content from solution (Van Alstyne, 1995; Stern et al., 1996b). However, calculating the loss of F-C absorbance from solutions treated with PVPP (100 mg/ml) in the study by Van Alstyne (1995) shows that phloroglucinol removal from the 80 vol% methanol solution without BSA was approximately 94% of the initial concentration. Furthermore, PVPP removed 95–97% of the F-C reactive compounds from *Fucus gardneri* extracts. Van Alstyne (1995) pointed out that PVPP might have a greater affinity for complex phlorotannins than for phloroglucinol. This may be correct, but the difference between the removal of dissolved phloroglucinol and phlorotannins in the study by Van Alstyne (1995) is not large, and would perhaps not be statistically significant if tested. Stern et al. (1996b) found that a single PVPP treatment (20 mg/ml) only removed 20% of the Folin-Denis absorbance in purified phlorotannin extracts from *Fucus vesiculosus*, a species that is closely related to *A. nodosum*. We found that when purified, *A. nodosum* phlorotannin extracts (according to the method described in Steinberg and van Altena, 1992) were treated with PVPP (3×10 mg/ml) in the third experiment, no color formation was detected with the F-C method, i.e. all phlorotannins were removed. These results are also in accordance with the results of Targett et al. (1995) who found that 90% of the phenolic compounds were removed from crude methanol extracts of *A. nodosum* after three successive PVPP treatments (15 mg/ml). Furthermore, PVPP removed 98% of dissolved phloroglucinol, between 87–95% of the phlorotannins from crude methanol extracts of *Lobophora variegata*, and between 90–99% of the phlorotannins from purified extracts of *F. vesiculosus* (Targett et al., 1995). Therefore, we suggest that PVPP can be effective in removing both phloroglucinol and phlorotannins

from solution, especially when the PVPP treatments are repeated several times.

The principal implications of the results found in the present study concern several fields of application including both basic and applied research areas. For example, the protein binding properties of tannins make it difficult to isolate enzymes (Loomis and Battaile, 1966; Kranner and Grill, 1996) and nucleic acids (Porebski and Bailey, 1997; Koonjul et al., 1999; Chen et al., 2000) from plant or algal material, and therefore, removal of tannins is often essential. Furthermore, some common methods that are used to quantify total phenolic compounds are based on reactions with free hydroxyl groups and do not discriminate between phenolic and non-phenolic compounds (Waterman and Mole, 1994). Therefore, measurements before and after removal of tannins may be required for proper quantification of tannins in plants (Makkar et al., 1993) and algae (Yates and Peckol, 1993; Peckol et al., 1996). Removal or isolation of tannins may also be important when using bioassays involving tannins in medical (Wall et al., 1969; Béress et al., 1993) or ecological (Pavia and Toth, 2000; Toth and Pavia, 2000) studies. Hypotheses concerning the activity of tannins against different types of threats are usually tested in bioassays using more or less purified plant extracts (Geiselman and McConnell, 1981; Steinberg, 1988; Pavia and Toth, 2000). Problems commonly arise when interpreting the results from such bioassays, since other plant extract properties may correlate and/or interact with the tannins and affect the outcome of the bioassay. To be able to rigorously address questions concerning the protective and defensive functions of tannins, they must either be purified or removed from the extract. By removing tannins with PVPP, a control containing all other chemical compounds in the extract can be created (c.f. Pavia and Toth, 2000).

In conclusion, we suggest that when repeatedly using a small amount of PVPP at pH ≤ 6.2, PVPP can be effective in removing all dissolved brown algal phlorotannins. Furthermore, there is no need to use organic solvents or to adjust the pH of the extracts, which is advantageous when using this method to create controls without phlorotannins in ecological experiments.

Acknowledgements—We are grateful to all staff and students at the Tjärnö Marine Biological Laboratory for their helpfulness and hospitality, and to two anonymous reviewers who provided valuable comments on an earlier draft of the manuscript. Financial support was provided by the research foundation of Adlerbertska. H. P. was supported by the Foundation for Strategic Research through the Marine Science and Technology (MASTEC) program.

REFERENCES

ANDERSEN, R. A., and SOWERS, J. A. 1968. Optimum conditions for binding of plant phenols to insoluble polyvinylpyrrolidone. *Phytochemistry* 7:293–301.

APPEL, H. 1993. Phenolics in ecological interactions: the importance of oxidation. *J. Chem. Ecol.* 19:1521–1552.

BERENBAUM, M. 1984. Effects of tannin ingestion on two species of papilionid caterpillars. *Entomol. Exp. Appl.* 34:245–250.

BÉRESS, A., WASSERMANN, O., BRUHN, T., and BÉRESS, L. 1993. A new procedure for the isolation of anti-HIV compounds (polysaccharides and polyphenols) from the marine alga *Fucus vesiculosus. J. Nat. Prod.* 56:478–488.

BOETTCHER, A. A., and TARGETT, N. M. 1993. Role of polyphenolic molecular size in reduction of assimilation efficiency in *Xiphister mucosus. Ecology* 74:891–903.

CHEN, G. Y. J., JIN, S., and GOODWIN, P. H. 2000. An improved method for isolation of total RNA from *Malva pusilla* tissues infected with *Colletotrichum gloeosporioides. J. Phytopath.* 148: 57–60.

FAGERBERG, W. R., and DAWES, C. J. 1976. Studies on *Sargassum*. I. A light microscope examination of the wound, regeneration process in mature stipes of *S. filipendula. Am. J. Bot.* 63:110–119.

GEISELMAN, J. A., and MCCONNELL, O. J. 1981. Polyphenols in brown algae *Fucus vesiculosus* and *Ascophyllum nodosum*: Chemical defeses against the marine herbivorous snail, *Littorina littorea. J. Chem. Ecol.* 7:1115–1133.

HARBORNE, J. B. 1983. Introduction to Ecological Biochemistry. London Academic Press, London.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. The University of Chicago Press. Chicago.

KOONJUL, P. K., BRANDT, W. F., FARRANT, J. M., and LINDSEY, G. G. 1999. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenol contamination of RNA. *Nucleic Acids Res.* 27:915–916.

KRANNER, I., and GRILL, D. 1996. Determination of glutathione and glutathione disulphide in lichens: A comparison of frequently used methods. *Phytochem. Anal.* 7:24–28.

LOOMIS, W. D., and BATTAILE, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5:423–438.

MAKKAR, H. P. S., BLUMMEL, M., BOROWY, N. K., and BECKER, K. 1993. Gravimetric-determination of tannins and their correlations with chemical and protein precipitation methods. *J. Sci. Food Agr.* 61:161–165.

MARTIN, M. M., and MARTIN, J. S. 1984. Surfactants: Their role in preventing the precipitation of proteins by tannins in insect guts. *Oecologia* 61:342–345.

PAVIA, H., and ÅBERG, P. 1996. Spatial variation in polyphenolic content of *Ascophyllum nodosum. Hydrobiologia* 326/327:199–203.

PAVIA, H., and TOTH, G. B. 2000. Induced chemical resistance to herbivory in the brown seaweed *Ascophyllum nodosum. Ecology* 81:3212–3225.

PAVIA, H., CERVIN, G., LINDGREN, A., and ÅBERG, P. 1997. Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum. Mar. Ecol. Prog. Ser.* 157:139–146.

PAVIA, H., TOTH, G. B., and ÅBERG, P. 1999. Trade-offs between phlorotannin production and annual growth in the brown seaweed *Ascophyllum nodosum. J. Ecol.* 87:761–771.

PECKOL, P., KRANE, J. M., and YATES, J. L. 1996. Interactive effects of inducible defense and resource availability on phlorotannins in the North Atlantic brown alga *Fucus vesiculosus. Mar. Ecol. Prog. Ser.* 138:209–217.

POREBSKI, S., and BAILEY, L. G. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Reporter* 15: 8–15.

RAGAN, M. A., and GLOMBITZA, K.-W. 1986. Phlorotannins, brown algal polyphenols. *Prog. Phyc. Res.* 4:129–241.

SCHOENWAELDER, M. E. A., and CLAYTON, M. N. 1998. Secretion of phenolic substances into the zygote wall and cell plate in embryos of *Hormosira* and *Acrocarpia* (Fucales, Phaeophyceae) *J. Phycol.* 34:969–980.

STEINBERG, P. D. 1988. Effects of quantitative and qualitative variation in phenolic compounds on feeding in three species of marine invertebrate herbivores. *J. Exp. Mar. Biol. Ecol.* 120:221–237.

STEINBERG, P. D., and VAN ALTENA, I. 1992. Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. *Ecol. Monogr.* 62:189–222.

STEINLY, B. A., and BERENBAUM, M. 1985. Histopathological effects of tannins on the midgut epithelium of *Papilio polyxenes* and *Papilio glaucus. Entomol. Exp. Appl.* 39:3–10.

STERN, J. L., HAGERMAN, A. E., STEINBERG, P. D., and MASON, P. K. 1996a. Phlorotannin–protein interactions. *J. Chem. Ecol.* 22:1877–1899.

STERN, J. L., HAGERMAN, A. E., STEINBERG, P. D., WINTER, F. C., and ESTES, J. A. 1996b. A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J. Chem. Ecol.* 22:1273–1293.

TARGETT, N. M., and ARNOLD, T. M. 1998. Predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. *J. Phycol.* 36:195–205.

TARGETT, N. M., BOETTCHER, A. A., TARGETT, T. E., and VROLIJK, N. H. 1995. Tropical marine herbivore assimilation of phenolic-rich plants. *Oecologia* 103:170–179.

TOTH, G., and PAVIA, H. 2000. Lack of phlorotannin induction in the brown seaweed *Ascophyllum nodosum* in response to increased copper concentrations. *Mar. Ecol. Prog. Ser.* 192:119–126.

TUGWELL, S., and BRANCH, G. M. 1992. Effects of gut surfactants on kelp polyphenol defenses. *Ecology* 73:205–215.

UNDERWOOD, A. J. 1997. Experiments in Ecology: Their Logical Design and Interpretation Using Analysis of Variance. Cambridge University Press, Cambridge.

VAN ALSTYNE, K. L. 1995. Comparison of three methods for quantifying brown algal polyphenolic compounds. *J. Chem. Ecol.* 21:45–58.

WALL, M. E., TAYLOR, H., AMBROSIO, L., and DAVIS, K. 1969. Plant antitumor agents III: A convenient separation of tannins from other plant constituents. *J. Pharm. Sci.* 58:839–841.

WATERMAN, P. G., and MOLE, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publication, Oxford.

YATES, J. L., and PECKOL, P. 1993. Effects of nutrient availability and herbivory on polyphenolics in the seaweed *Fucus vesiculosus. Ecology* 74:1757–1766.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

HERBIVORE-INDUCED VOLATILE PRODUCTION BY *Arabidopsis thaliana* LEADS TO ATTRACTION OF THE PARASITOID *Cotesia rubecula*: CHEMICAL, BEHAVIORAL, AND GENE-EXPRESSION ANALYSIS

REMCO M. P. VAN POECKE,[1] MAARTEN A. POSTHUMUS,[2] and MARCEL DICKE[1,*]

[1]*Laboratory of Entomology*
Wageningen University
P.O. Box 8031 6700 EH Wageningen, The Netherlands

[2]*Laboratory of Organic Chemistry*
Wageningen University
Dreijenplein 8 6703 HB Wageningen, The Netherlands

(Received November 30, 2000; accepted June 9, 2001)

Abstract—Many plant species defend themselves against herbivorous insects indirectly by producing volatiles in response to herbivory. These volatiles attract carnivorous enemies of the herbivores. Research on the model plant *Arabidopsis thaliana* (L.) Heynh. has contributed considerably to the unraveling of signal transduction pathways involved in direct plant defense mechanisms against pathogens. Here, we demonstrate that *Arabidopsis* is also a good candidate for studying signal transduction pathways involved in indirect defense mechanisms by showing that: (1) Adult females of *Cotesia rubecula*, a specialist parasitic wasp of *Pieris rapae* caterpillars, are attracted to *P. rapae*-infested *Arabidopsis* plants. (2) *Arabidopsis* infested by *P. rapae* emits volatiles from several major biosynthetic pathways, including terpenoids and green leaf volatiles. The blends from herbivore-infested and artificially damaged plants are similar. However, differences can be found with respect to a few components of the blend, such as two nitriles and the monoterpene myrcene, that were produced exclusively by caterpillar-infested plants, and methyl salicylate, that was produced in larger amounts by caterpillar-infested plants. (3) Genes from major biosynthetic pathways involved in volatile production are induced by caterpillar feeding. These include *AtTPS10*, encoding a terpene synthase involved in myrcene production, *AtPAL1*, encoding phenylalanine ammonia-lyase involved in methyl salicylate production, and *AtLOX2* and *AtHPL*, encoding lipoxygenase and hydroperoxide lyase, respectively, both involved in the production of green leaf volatiles.

*To whom correspondence should be addressed; e-mail: marcel.dicke@users.ento.wag-ur.nl

0098-0331/01/1000-1911$19.50/0

AtAOS, encoding allene oxide synthase, involved in the production of jasmonic acid, also was induced by herbivory.

Key Words—*Pieris rapae*, Lepidoptera, Hymenoptera, tritrophic interactions, foraging behavior, headspace analysis, terpenoids, green leaf volatiles, methyl salicylate, nitriles.

INTRODUCTION

To defend themselves against herbivorous insects, plants use both direct and indirect mechanisms (Karban and Baldwin, 1997; Dicke, 1999). Direct defense mechanisms have a direct effect on the herbivore, e.g., by negatively affecting the physiology of the herbivore with toxic or antinutritional compounds, or by interfering with the behavior of the herbivore with repelling or deterring compounds (Karban and Baldwin, 1997). Indirect defense mechanisms promote the effectiveness of natural enemies of the herbivore, i.e., predators or parasitoids. One way of promoting the effectiveness of natural enemies is by producing volatiles in response to herbivory that can attract predators or parasitoids. This kind of tritrophic interaction has been shown for many, mainly agriculturally important, plant species. Plants capable of this type of interaction belong to at least 12 plant families, in combination with a diverse range of herbivore and natural enemy species (Dicke, 1999).

Among the volatiles induced by herbivory, terpenoids, lipoxygenase-derived volatiles, and methyl salicylate are commonly found. These compounds are produced via different biosynthetic pathways.

Terpenoids are a large and diverse group of compounds, present in most plant species (Gershenzon and Croteau, 1991). Although the early steps in terpenoid production can be divided into a cytosolic and a plastidic pathway (Lichtenthaler, 1999), products from both pathways are used by terpene synthases (TPS) (Bohlmann et al., 1998, 2000), to produce mono-, sesqui-, and diterpenes.

Lipoxygenase-derived or "green leaf" volatiles are also common in plants. Important enzymes involved in their biosynthesis are lipoxygenase (LOX) (Bell and Mullet, 1993) and hydroperoxide lyase (HPL) (Bate et al., 1998). Another branch of the lipoxygenase pathway produces the plant hormone jasmonic acid, which is involved in the induction of direct (Karban and Baldwin, 1997; McConn et al., 1997) and indirect (Hopke et al., 1994; Dicke et al.; 1999; Gols et al., 1999; Thaler, 1999) defense mechanisms. For the production of jasmonic acid, the products from LOX are used by the enzyme allene oxide synthase (AOS) instead of HPL (Laudert et al., 1996).

Methyl salicylate is a volatile derivative of the plant hormone salicylic acid (Lee et al., 1995). The role of salicylic acid in direct defense mechanisms of plants against pathogens has been extensively reviewed (e.g., Dempsey et al., 1999). Methyl salicylate is produced via the phenyl propanoid pathway. A key enzyme in

the phenyl propanoid pathway is phenylalanine ammonia-lyase (PAL) (Lee et al., 1995; Mauch-Mani and Slusarenko, 1996). Besides (methyl) salicylate, many other products are produced from this pathway, such as lignin and flavonoids (Dixon and Paiva, 1995).

Signal-transduction pathways involved in herbivore-induced plant volatiles have only recently been studied (e.g., Hopke et al., 1994; Paré and Tumlinson, 1997; Boland et al., 1999; Dicke et al., 1999). However, there is a wealth of knowledge on signal transduction pathways in direct defenses against both insects and pathogens (e.g., Karban and Baldwin, 1997; Pieterse et al., 1998). Much of the knowledge has been obtained by taking a molecular genetic approach (e.g., Delaney et al., 1994). The incorporation of the model plant of molecular genetics, *Arabidopsis thaliana*, has contributed enormously to progress in this field (e.g., Dietrich et al., 1994; Pieterse et al., 1998; Thomma et al., 1998).

To date, only a few studies on insect–plant interactions have used *Arabidopsis* as a host plant (Grant-Petterson and Renwick, 1996; Mauricio and Rausher, 1997; McConn et al., 1997; Mauricio, 1998; Reymond et al., 2000; van Loon et al., 2000; Stotz et al., 2000), and only one of those focussed on indirect defenses (van Loon et al., 2000). Yet, *Arabidopsis* is known to be consumed by insects, such as caterpillars of *Pieris rapae* (Yano and Ohsaki, 1993; van Loon et al., 2000), and these caterpillars are hosts to parasitoids such as *Cotesia rubecula* (Agelopoulos and Keller, 1994; Geervliet et al., 1994). Several crucifers are known to produce herbivore-induced volatiles in response to *P. rapae* damage, and *C. rubecula* is attracted to these (Blaakmeer et al., 1994; Geervliet et al., 1994, 1997).

This indicated to us that *Arabidopsis* might be a good candidate for studying signal transduction in indirect defenses in plants. In the present study, we investigate (1) whether *Arabidopsis* produces volatiles induced by *P. rapae* feeding that attract *C. rubecula* parasitoids, (2) the composition of the herbivore-induced volatile blend, and (3) whether genes from major biosynthetic pathways involved in the production of the identified volatiles are induced by caterpillar feeding.

METHODS AND MATERIALS

Plants. Arabidopsis thaliana (L.) Heynh., ecotype Columbia, was grown from seed in a greenhouse (20–30°C, 50–70% relative humidity, 8L : 16D). A few days prior to plant treatment, 9- to 10-week-old plants were transferred to a climate room (23 ± 1°C, 50–70% relative humidity, 10 klux, 8L : 16D). All plants used were in the vegetative state.

Insects. Pieris rapae was reared on Brussels sprout plants (*Brassica oleracea gemmifera* cv. Icarus) in a climate room (21 ± 1°C, 50–70% relative humidity, 16L : 8D). The parasitoid *Cotesia rubecula* was reared on *P. rapae* larvae feeding on Brussels sprout plants, under greenhouse conditions (25 ± 5°C, 50–70% relative

humidity, 16L : 8D). Every year a fresh colony was initiated with individuals collected from fields near Wageningen, The Netherlands. For bioassay experiments, *C. rubecula* pupae were collected and transferred to a gauze cage in a climate room (23 ± 1°C, 50–70% relative humidity, 16L : 8D). The emerging wasps were provided with water and honey. Adult wasps that did not have contact with plant material or caterpillars (no ovipostion experience) are referred to as naive wasps.

Plant Treatments. For headspace collection and analysis, caterpillar-infested plants were obtained by placing 12 first-instar *P. rapae* on each plant. For the two-choice bioassay and gene expression analysis, caterpillar-infested plants were obtained by placing two first-instar *P. rapae* on each leaf with a main vein longer than 3 cm, resulting on average in 24 larvae per plant. In both cases, larvae had fed on the plants for 24 hr before they were used in experiments. Caterpillar-infested plants are referred to as a plant–herbivore complex (PHC).

To obtain artificially damaged (AD) plants, all leaves with a main vein longer than 3 cm were rubbed with carborundum powder no. 180 (Cats Import, Hoogvliet, The Netherlands) on a moist cotton pad, just before an experiment (Mattiacci et al., 1995).

Control (C) plants did not receive any treatment, but were of the same age and size as treated plants, and had been transferred to the climate room at the same time.

Bioassay. Parasitoid flight experiments were conducted in a wind-tunnel set-up (25 ± 5°C, 50–70% relative humidity, 0.7 klux) described by Geervliet et al. (1994), with a wind speed of 0.2 m/sec.

For two-choice flight experiments, two odor sources were placed at the upwind end of the wind tunnel. Each odor source consisted of eight *Arabidopsis* plants that received the same treatment. These eight plants were placed in a 2 × 4 arrangement, with the three plants closest to the center and upwind end of the wind-tunnel on a 4.5 cm elevation (Figure 1). An 11 × 19 cm clear Plexiglas rectangle was placed, at the upwind end of each odor source, on the elevation, to create some air turbulence. The distance between the odor sources was 14 cm.

One day before a bioassay, 4- to 6-day-old, naive *C. rubecula* wasps were sexed and the males removed. Just prior to the bioassay, an individual female wasp was placed onto a microscope slide with one leaf from a caterpillar-damaged *Arabidopsis* from which the caterpillars had been removed. The wasp plus leaf plus slide were transported to the middle of the release cylinder in the wind tunnel, which was 60 cm downwind from the odor sources (Figure 1).

The flight behavior of the wasps was observed. Only flights that resulted in the first landing on one of the odor sources were recorded as a choice. Landings on other parts of the wind tunnel besides the release cylinder or odor sources were recorded as no choice. If the wasp remained in/on the release cylinder for longer than 15 min, this was also recorded as no choice. After a choice or no choice, the wasp was removed.

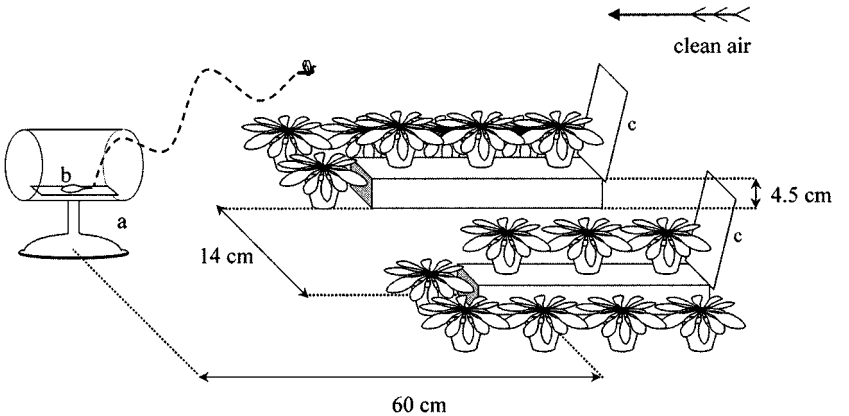


FIG. 1. Wind-tunnel set-up. Two odor sources, each consisting of eight *Arabidopsis* plants were placed 60 cm upwind of the release cylinder (a). Three plants were placed on a 4.5-cm elevation for each odor source. The distance between the two odor sources was 14 cm. Wasps were transported to the release cylinder on a microscope slide with one leaf from a caterpillar-damaged *Arabidopsis* from which caterpillars had been removed (b). Clean laminar air was blown over the odor sources towards the release cylinder at a speed of 0.2 m/sec. Plexiglas rectangles (c) were placed at the upwind end of the odor sources to create some turbulence.

All combinations of the three odor sources (PHC-C, AD-C, PHC-AD) were tested on the same day. For each combination of odor sources, plant positions were changed from left to right and vice versa, to compensate for unforeseen asymmetric effects. In total, 50 wasps in six days were tested per odor combination.

Choices between two odor sources in the bioassay were statistically analyzed by using a chi-square test. Differences in the percentage of wasps making a choice (responsiveness) among the three combinations of odor sources were tested using a contingency table on the absolute numbers.

For the no-choice flight experiment, one odor source was placed at the upwind end of the wind tunnel. The odor source consisted of one *Arabidopsis* plant placed on a 4.5-cm elevation. To create some air turbulence, an 11- $\times$ 19-cm clear Plexiglas rectangle was placed at the upwind end of the odor source on the elevation. Selection and handling of the wasps was the same as for the two-choice experiments. On one day, the behavior of 10 female wasps was subsequently recorded. Only flights that resulted in the first landing on the odor source were recorded as a successful landing. Landings on parts of the wind tunnel other than the release cylinder or odor sources were recorded as nonsuccessful landings. If wasps remained in/on the release cylinder for longer than 20 min, this was also recorded as a nonsuccessful landing. After a successful or nonsuccessful landing, the wasp was removed. On day 0, a single, undamaged *Arabidopsis* plant was used as an odor source, and the

behavior of the 10 female wasps was recorded. After this test, a single first-instar *P. rapae* caterpillar that had just hatched was placed on the *Arabidopsis* plant. After 1, 2, 3, 4, and 6 days, this plant was used as odor source for the bioassay. Every day, the behavior of a new set of 10 females was recorded. This experiment was repeated three times. After pooling the data for every infestation period, differences between the number of wasps making a successful landing among the subsequent days was tested using a contingency table. A Spearman rank correlation test was used to analyze whether more days of feeding resulted in greater attraction.

Headspace Collection and Analysis. For dynamic headspace collection, 40 plants per treatment were used and experiments were performed in a climate room ($23 \pm 1^{\circ}$C, 50–70% relative humidity, 10 klux). Soil was removed from the roots by rinsing with tap water, and the plants were placed into a 5-liter glass jar on wet filter paper. The jar was closed with a glass lid having an air inlet and air outlet. A viton O-ring was placed between the lid, and jar and the lid was closed tightly with a metal clamp. Pressurized air was filtered over silica gel, a molecular sieve, and activated charcoal. The resulting cleaned air was led through the jar with plants for 6 hr, at a flow rate of 240 ml/min. To collect the headspace, the airstream was filtered at the outlet with 90 mg Tenax-TA in a glass tube. Subsequently, the Tenax tube was closed with a 1/4-in Swagelok cap and transferred to the GC-MS for analysis.

Collected volatiles were released from the Tenax by using a Thermodesorption Cold Trap set-up (Chrompack, Middelburg, The Netherlands) as described by Mattiacci et al. (1995) and transferred to the analytical gas chromatography column (Supelcowax 10 or Rtx-200, 60 m $\times$ 0.25 mm ID, 0.25-μm film thickness). The initial linear velocity of the helium carrier gas was 22 cm/sec. The temperature of the column was raised from 40°C to 270°C at 4°C/min. Volatiles were analyzed by a mass spectrometer (Finnigan MAT 95) and identified by comparison of the mass spectra with those in the NIST98 library, and the Wageningen Mass Spectral Database of Natural Products, and by checking the retention index, unless stated otherwise.

Headspace collections of control (C) plants were carried out simultaneously, in parallel, with each of the two treatments (AD and PHC) to minimize variation among plant batches and day-to-day variation. Collections were made in triplicate.

When comparing damaged (either AD or PHC) with undamaged control (C) plants, a compound was considered to be emitted in larger amounts, if that compound showed a larger peak area in all three replicates of damaged plants, compared with the three replicates of undamaged control plants, or if that compound showed a larger peak area in two of the three replicates of damaged plants, and the third (and smallest) peak area was equal to the largest peak area of the three replicates of undamaged control plants. The latter occurred mostly when the compound was not detected in any of the three replicates of undamaged control plants and detected in two of the three replicates of damaged plants.

Gene Expression Analysis. The protocol for RNA isolation, described by Chang et al. (1993) was followed. Leaf material from 5 or 6 plants was pooled to obtain a total of 5 g, and the protocol was adjusted to these 5 g. Isolated RNA was DNase treated (DNase I, GibcoBRL) using an RNase inhibitor (RNaseOUT, GibcoBRL) to preserve RNA quality. The concentration of total RNA was spectrophotometrically determined.

From the total RNA, 10 μg was separated on a formaldehyde–agarose gel, blotted on a nylon membrane (Hybond-N+, Amersham) (Sambrook et al., 1989), and UV cross-linked. These northern blots were prehybridized and hybridized at 65°C in hybridization buffer (Church and Gilbert, 1984). The blots were washed for 15 min under medium stringency conditions (one wash with 2 × SSC, 0.1% SDS at room temperature, one wash with 2 × SSC, 0.1% SDS at 55°C).

All probes were made by using the RadPrime DNA Labeling System (GibcoBRL). Templates for the probes were obtained by PCR from cDNA clones (*AtTPS03*, *AtTPS10*, *AtLOX2*, *AtAOS*, *AtHPL*) or by RT-PCR from *Arabidopsis* total RNA (*AtPAL1*, *AtPAL2*, *AtPAL3*). Plasmids containing full-length cDNAs of *AtTPS03* and *AtTPS10* were provided by Jörg Bohlmann (see Bohlmann et al., 2000). The plasmids containing cDNAs of *AtLOX2*, *AtAOS*, and *AtHPL* were described by Bell and Mullet (1993), Laudert et al. (1996), and Bate et al. (1998), respectively. Primers used for PCR were 5′-TAT-ACG-CGA-CCG-CCC-TTG-AG-3′ and 5′-GGC-GGA-GCT-TCG-AAC-TAC-AGA-G corresponding to bases 172827–172846 and 174843–174864 of *AtTPS03* (GenBank Accession No. Z97341); 5′-TTC-CGA-CTT-TTC-AGG-CAA-CAT-3′ and 5′-GAG-AGC-TGG-TCG-GAG-AAT-ACG-3′ corresponding to bases 327–347 and 1247–1267 of *AtTPS10* (GenBank Accession No. AF178535); 5′-GGA-TCG-ATA-AGA-CCG-CAG-AAC-ATG-GAG-3′ and 5′-GCT-GTC-GAC-TCA-TAG-AAC-TCT-TTT-AAG-G-3′ corresponding to bases 1969–1994 and 2364–2391 of *AtLOX2* (Bell and Mullet, 1993) (underlined parts do not correspond to the plasmid template); 5′-CTT-TTC-ACC-GGT-ACT-TAC-ATG-CCG-3′ and 5′-GAT-AAC-CGT-AAA-GCA-TCT-CAC-CG-3′ corresponding to bases 447–470 and 1274–1291 of *AtAOS* (Laudert et al., 1996); 5′-TCA-CAG-CTT-CCC-CTC-CGT-ACA-ATG-3′ and 5′-CCG-GAG- TCA-CCA-GTA-ATC-GTA-TCA-3′ corresponding to bases 147–170 and 1487–1510 of *AtHPL* (Bate et al., 1998); 5′-AAG-AGC-AAC-GGA-GGA-GGA-GTG-G-3′ and 5′-GTG-GCG-GAG-TGT-GGC-AAT-GT-3′ corresponding to bases 150–171 and 603–622 of *AtPAL1* (Ohl et al., 1990); 5′-AAC-GGC-ACC-GCA-TTA-CAA-ACA-GAA-3′ and 5′-AGA-TCC-AAC-CGC-CGT-GCC-ATT-A- 3′ corresponding to bases 1838–1861 and 2455–2476 of *AtPAL2* (GenBank Accession no. L33678); and 5′-AGC-GAG-TGG-AGG-AGG-ACC-GAC-AGT-3′ and 5′-GGG-CGC-CCG-ATG-AGG- AAT-3′ corresponding to bases 2117–2140 and 3242–3259 of *AtPAL3* (GenBank Accession no. L33679). Ubiquitin expression served as a constitutive control.

For first-strand cDNA synthesis, 10 μg of total RNA was used together with M-MLV Reverse Transcriptase, Random Primers and RNaseOUT (GibcoBRL). One twentieth of the cDNA mixture was used for subsequent PCR reactions with Taq DNA polymerase (GibcoBRL).

All mRNA analyses were done in triplicate, using new sets of plants.

RESULTS

Bioassay. Cotesia rubecula females clearly preferred volatiles from *Pieris rapae*-infested (plant–herbivore complex, PHC) or artificially damaged (AD) *Arabidopsis* plants over those from undamaged plants (C). Wasps preferred odors from PHC over AD (Figure 2). The percentage of wasps making a choice did not differ among the PHC-C (66%), AD-C (70%), or PHC-AD (82%) combinations (contingency table, $P = 0.17$).

The two-choice test was performed using eight *Arabidopsis* plants per odor source with, in the case of PHC, on average 24 first-instar *P. rapae*. To test whether a single *Arabidopsis* plant damaged by a single *P. rapae* also attracted *C. rubecula* females, we performed a no-choice test (Figure 3). The results show that a single plant infested by a single caterpillar attracts the parasitoid. The number of attracted wasps differed among the different days (contingency table, $P < 0.001$). More days of feeding resulted in more attractive plants (Spearman's rank correlation test, $P < 0.0025$).

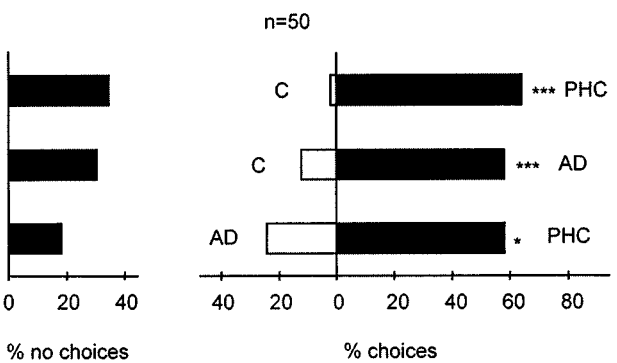


FIG. 2. Responses of *C. rubecula* to herbivore-infested (PHC), artificially damaged (AD), and undamaged (C) *Arabidopsis* in a two-choice set-up. Asterisks indicate a significant difference within a choice test: $^{***}P < 0.001$, $^{*}P < 0.05$ (χ^2). The left part of the figure shows the percentage of wasps that did not land on one of the two odor sources, for each odor combination. The right part of the figure shows the percentages of wasps landing on either odor source, also for each odor combination. In total, 50 wasps were tested per combination.

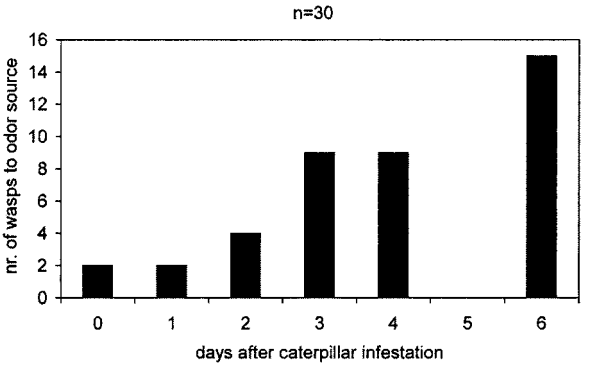


FIG. 3. Response of *C. rubecula* to a single herbivore-infested *Arabidopsis* plant in a no-choice set-up. At day 0, an undamaged *Arabidopsis* plant was tested for attractiveness to *C. rubecula* females. Subsequently, a single *P. rapae* larvae that had just hatched was placed on the plant. After 1, 2, 3, 4, and 6 days of infestation, the plant was tested again for attractiveness. In total, 30 wasps were tested in three replicate experiments per infestation period.

Headspace Analysis. A total of 38 compounds derived from different biosynthetic pathways were identified in the headspace of *Arabidopsis* plants (Figure 4), including methyl salicylate, terpenoids, green leaf volatiles, sulfides, nitriles, alcohols, aldehydes, ketones, and two cycloheptadienes.

Artificially damaged plants emitted larger amounts of volatiles than undamaged plants (Figure 4A), including more green leaf volatiles [(*Z*)-3-hexen-1-ol (peak 14), (*Z*)-3-hexen-1-yl acetate (15), and 1-hexanol (16)]. Emission of terpenoids [β-ionone (4) and β-cyclocitral (5)], sulfides [dimethyl disulfide (8) and dimethyl trisulfide (9)], alcohols [3-pentanol (20), 1-penten-3-ol (21), and 2-ethyl-1-hexanol (23)], and ketones [3-pentanone (29) and 1-penten-3-one (30)] was also induced by artificial damage (Figure 4A).

Caterpillar-infested plants emitted slightly larger amounts of volatiles than undamaged plants (Figure 4B). Caterpillar-infested plants emitted more methyl salicylate (1), terpenoids [myrcene (2) and β-ionone (4)], sulfides [dimethyl disulfide (8) and dimethyl trisulfide (9)], nitriles [5-(methylthio)-pentanenitrile (10) and 6,7-dithiaoctanenitrile (11)], green leaf volatiles [(*Z*)-3-hexen-1-ol (14) and 1-hexanol (16)], some alcohols [1-pentanol (19), 1-penten-3-ol (21), and 2-penten-1-ol (22)], and ketones (1-penten-3-one and 2-pentanone) than undamaged plants (Figure 4B). In fact, the monoterpene myrcene (2), the two nitriles (10 and 11), and the alcohol 2-penten-1-ol (22) were exclusively found in the volatile blend of caterpillar-infested plants. Methyl salicylate (1), the two nitriles (10, and 11), the alcohol 1-pentanol (19), and the ketone 2-pentanone (28) were induced by caterpillar feeding (Figure 4B), but not by artificial damage (Figure 4A). The green leaf

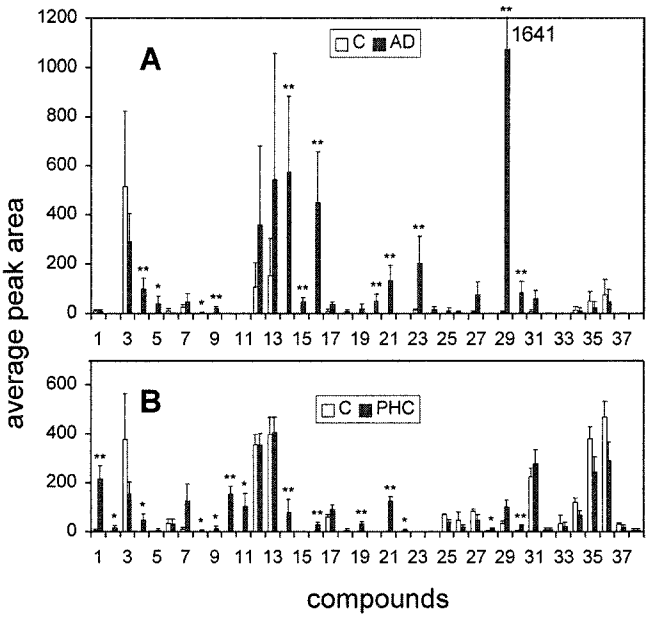


FIG. 4. Headspace analysis of herbivore-infested (PHC), artificially damaged (AD), and undamaged (C) *Arabidopsis*. Average peak areas of three replicates are shown. Error bars indicate the standard error. Compounds marked with ** showed larger peak areas in all three replicates of treated plants (either AD or PHC), compared with the three replicates of undamaged control plants (C). Compounds marked with * showed larger peak areas in two of the three replicates of treated plants, the peak area of the third (and smallest) peak area being equal to the largest peak area of the three replicates of undamaged control plants. Compound numders are: (1) methyl salicylate, (2) myrcene, (3) limonene, (4) β-ionone, (5) β-cyclocitral, (6) longifolene, (7) (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (8) dimethyldisulfide, (9) dimethyltrisulfide, (10) 5-(methylthio) pentanenitrile, (11) 6, 7-dithiooctanenitrile, (12) 6-[(*Z*)-1-butenyl]-1,4-cycloheptadiene, (13) 6-butyl-1,4-cycloheptadiene, (14) (*Z*)-3-hexen-1-ol, (15) (*Z*)-3-hexen-1-yl acetate, (16) 1-hexanol, (17) hexanal, (18) 1-butanol, (19) 1-pentanol, (20) 3-pentanol, (21) 1-penten-3-ol, (22) 2-penten-1-ol, (23) 2-ethyl-1-hexanol, (24) 1-octen-3-ol, (25) 1-nonanol, (26) 1-dodecanol, (27) 1-tetradecanol, (28) 2-pentanone, (29) 3-pentanone, (30) 1-penten-3-one, (31) 4-methyl-3-heptanone, (32) 4-methyl-4-hepten-3-one, (33) heptanal, (34) octanal, (35) nonanal, (36) decanal, (37) undecanal, and (38) 1-nonene.

volatile (*Z*)-3-hexen-1-yl acetate (15) and the alcohol 3-pentanol (20) were found only in the blend of artificially damaged plants. In contrast to herbivore-infested plants, artificially damaged plants showed induced emission of the terpenoid β-cyclocitral (5), the alcohol 2-ethyl-1-hexanol (23), and the ketone 3-pentanone (29). Two remarkable compounds that made up a large part of the blend emitted by

undamaged, artificially damaged, and caterpillar-infested plants were tentatively identified as 6-[(*Z*)-1-butenyl]-1,4-cycloheptadiene (12; ectocarpene) and 6-butyl-1, 4-cycloheptadiene (13; dictyotene), based on mass spectra.

Gene Expression Analysis. The expression of some genes (*AtLOX2*, *AtAOS*, and *AtPAL2*) could be detected using northern blot analysis. For other genes (*AtPAL1*, *AtPAL3*, *AtTPS03*, *AtTPS10*, and *AtHPL*), northern blot analysis resulted in weak or undetectable radioactivity signals (data not shown). In those cases, RT-PCR was used. Gene expression was considered to be induced by *P. rapae* feeding if all three replicates from caterpillar-damaged plants showed higher expression than the three replicates from undamaged plants.

Expression of three genes from the lipoxygenase pathway, *AtLOX2* (Bell and Mullet, 1993), *AtHPL* (Bate et al., 1998), and *AtAOS* (Laudert et al., 1996), was induced by caterpillar feeding (Figure 5A, B). The expression of two genes involved in terpenoid biosynthesis was examined: *AtTPS03* and *AtTPS10*. Expression of *AtTPS03*, a novel monoterpene synthase gene (Bohlmann, unpublished results), and *AtTPS10*, myrcene/ocimene synthase gene (Bohlmann et al., 2000), was also induced by 24 hr of caterpillar feeding (Figure 5B).

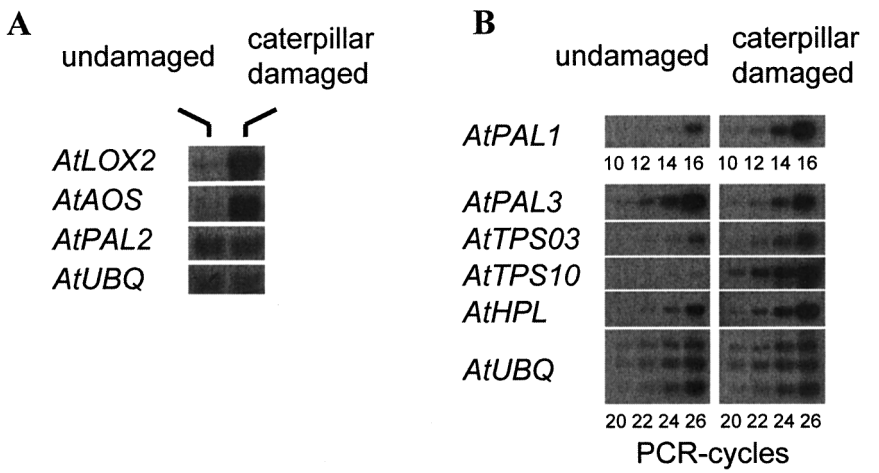


FIG. 5. Induction of gene expression by herbivore damage. RNA was extracted from undamaged *Arabidopsis* plants or *Arabidopsis* plants that had been subjected to 24 hr of *Pieris rapae* caterpillar feeding. For northern analysis (A), 5 μg of RNA was loaded in each lane. Northern blot analysis was performed for *AtLOX2*, *AtAOS*, and *AtPAL2*. RT-PCR analysis and subsequent blotting and radioactive labeling were performed for *AtPAL1* (10, 12, 14, and 16 PCR cycles), *AtPAL3*, *AtTPS03*, *AtTPS10*, and *AtHPL* (20, 22, 24, and 26 PCR cycles) (B). Ubiquitin expression served as a constitutive control, both for northern and RT-PCR (20, 22, 24, and 26 PCR cycles) analyses. Note that autoradiography films were exposed differently for different genes; therefore, comparison of expression levels among genes is not possible.

Three genes encoding for phenylalanine ammonia-lyase, an enzyme involved in the production of methyl salicylate, have been isolated in *Arabidopsis*,: *AtPAL1* (Ohl et al., 1990), *AtPAL2*, and *AtPAL3* (Wanner et al., 1995). *AtPAL1* was clearly induced by caterpillar feeding, whereas *AtPAL2* and *AtPAL3* were not (Figure 5A, B)

DISCUSSION

The attraction of carnivorous arthropods by plants in response to herbivory has been intensively studied over the past 15 years (Turlings et al., 1995; Dicke, 1999). This phenomenon has been shown for a large number of plants in various families, including the Brassicaceae (Dicke, 1999). Our data show that *Arabidopsis thaliana* plants are able to do so as well. Damage results in the production of volatiles that attract *C. rubecula*, a specialist parasitoid of *Pieris rapae*. The parasitoids prefer volatiles from caterpillar-infested or mechanically damaged plants over those from undamaged plants. They also distinguish between artificially damaged and caterpillar-infested plants, preferring the latter (Figure 2). This resembles the findings for agricultural plants in the Brassicaceae family with the same herbivore and parasitoid species (Agelopoulos and Keller, 1994; Geervliet et al., 1994). The difference in attractiveness between artificially damaged and caterpillar-infested plants cannot be explained by a response to odors emitted by the caterpillars themselves or their by-products. *Pieris rapae* larvae or their by-products are only slightly attractive to *C. rubecula* females (Geervliet et al., 1994). Moreover, the wasps do not distinguish between caterpillar-infested plants and previously infested plants from which the caterpillars and their by-products were removed 24 hr after infestation (Geervliet et al., 1994). Additionally, feeding damage of only a single *P. rapae* caterpillar on a single *Arabidopsis* plant results in increased attractiveness of the plant to *C. rubecula* females (see Figure 3). This is before the larva reaches the third instar, so the biomass of the caterpillar is small, and feces production is at a relatively low level, compared to later instars.

Recently, it was demonstrated that seed production of *Arabidopsis* plants, on which caterpillars of *P. rapae* are feeding that had been parasitized by *C. rubecula*, is similar to the seed production of undamaged plants and greater than the seed production of plants fed upon by unparasitized caterpillars (van Loon et al., 2000). This finding shows that *Arabidopsis'* fitness may benefit greatly from the attraction of parasitoid wasps by means of herbivore-induced volatiles.

Both artificially damaged and caterpillar-infested plants emit larger amounts of volatiles than undamaged plants (Figure 4). The volatile blend of damaged plants, either artificially or by caterpillar feeding, contains compounds that could not be detected in the blend of undamaged plants. These blends are quite similar, although some differences can be found. Artificially damaged plants showed

induced emission of especially green leaf volatiles and ketones, but also of alcohols, terpenoids, and sulfides. Caterpillar-pinfested plants showed induced emissions of methyl salicylate and nitriles, but also of alcohols, ketones, sulfides, and terpenoids. The nitriles and the terpenoid myrcene were not detected in blends of artificially damaged plants and undamaged plants. Methyl salicylate (Dicke et al., 1990, 1998; C.E.M. Van den Boom et al., unpublished data) and terpenoids (Dicke et al., 1990, 1998; Turlings et al., 1990; Loughrin et al., 1994) are known to be induced by herbivory in many plant species. Both methyl salicylate and several terpenoids are attractants for predatory mites (Dicke et al., 1990). Moreover, terpenoids are likely to be involved in the attraction of parasitoids (Turlings et al., 1991). Nitriles are induced by spider mite feeding on cucumber (Takabayashi et al., 1994) and on apple (Takabayashi et al., 1991). However, in cruciferous plants such as Brussels sprouts (Blaakmeer et al., 1994) and red and white cabbage (Geervliet et al., 1997), methyl salicylate, terpenoids, and nitriles are either not induced by herbivory or only very slightly. Therefore, induction of methyl salicylate, the terpenoids myrcene and (3*E*,7*E*)-4, 8,12-trimethyl-1,3,7,11-tridecatetraene, and the nitriles 5-(methylthio) pentanenitrile and 6,7-dithiooctanenitrile by *P. rapae* infestation is noteworthy.

In all plant species studied so far, artificial damage leads to the emission of green leaf volatiles (e.g., Dicke et al., 1990; Turlings et al., 1990; Whitman and Eller, 1990; Takabayashi et al., 1991). It is not surprising that green leaf volatiles are also present in the volatile blends of all caterpillar-infested plant species tested so far (e.g., Whitman and Eller, 1990; Turlings et al., 1990) and now including *Arabidopsis*. In Brussels sprouts and red and white cabbage, they are highly induced by caterpillar feeding, forming the largest part of the volatile blends (Blaakmeer et al., 1994; Mattiacci et al., 1995; Geervliet et al., 1997). Females of the parasitoid wasps *Microplitis croceipes* and *Netelia heroica* are attracted to individual green leaf volatiles (Whitman and Eller, 1990).

The presence of two compounds in the volatile blend of *Arabidopsis*, tentatively identified as the cycloheptadienes ectocarpene and dictyotene is remarkable, as these compounds are not common among higher plants. They are mostly known as sex pheromones in algae (Boland, 1995). Yet, they have been found in some higher plants, e.g., in *Senecio isatideus* (Bohlmann et al., 1979). These compounds are not induced by caterpillar feeding in *Arabidopsis*.

Emission of volatiles after herbivory can be a passive release of stored compounds but also can result from an increased production of volatiles. Using isotope labeling techniques, Paré and Tumlinson (1997) showed that in response to herbivory, cotton produces some volatiles *de novo* and others not. In Lima bean and cucumber, the activity of a terpenoid-producing enzyme is induced by herbivore damage (Bouwmeester et al., 1999). Here, we studied the expression of genes involved in early (*AtLOX*, *AtPAL1*, *AtPAL2*, and *AtPAL3*) or late (*AtTPS03*, *AtTPS10*, and *AtHPL*) steps of several biosynthetic pathways of volatiles emitted

by *Arabidopsis* (Figure 5). Gene expression levels correlated well with the emission of induced volatiles.

Bohlmann and coworkers recently reported the isolation of a terpene synthase from *Arabidopsis*, *AtTPS10*. Functional characterization showed that the AtTPS10 protein is a myrcene/ocimene synthase. Myrcene is the major product of the enzyme, and ocimene is produced in lower amounts (Bohlmann et al., 2000). Both myrcene emission and *AtTPS10* gene expression are induced by 24 hr of caterpillar feeding, indicating that herbivory leads to induced myrcene production. Expression of a novel monoterpene synthase gene, *AtTPS03* (Bohlmann, unpublished results), also was induced by caterpillar feeding (Figure 5B).

Three PAL genes have been identified in *Arabidopsis* (Wanner et al., 1995). Mauch-Mani and Slusarenko (1996) have shown that a major function of PAL in *Arabidopsis* is the production of salicylic acid. As methyl salicylate is a derivative of salicylic acid (Lee et al., 1995), it is likely that induced PAL gene expression can lead to increased production of methyl salicylate. This is supported by our data, which show an induced *AtPAL1* expression after caterpillar feeding (Figure 5B) and an increased emission of methyl salicylate. Expression of the other two known genes encoding for PAL in *Arabidopsis*, *AtPAL2* and *AtPAL3*, was not induced. Possibly, these two genes are involved in other processes within the phenyl propanoid pathway than production of methyl salicylate. Similar expression patterns were reported by Reymond et al. (2000) after 3 hr of *P. rapae* feeding on *Arabidopsis*, although they found a weak induction of *AtPAL3*.

Genes important in the production of green leaf volatiles, such as *AtLOX2* (Bell and Mullet, 1993) and *AtHPL* (Bate et al., 1998), are induced in caterpillar-infested *Arabidopsis* (Figure 5A, B). However, the products formed by lipoxygenase are also used by allene oxide synthase (AOS), involved in jasmonic acid production. Expression of the *AtAOS* gene in *Arabidopsis* is also induced by caterpillar feeding (Figure 5A). Therefore, we cannot predict whether increased *AtLOX* and *AtHPL* expression will lead to increased green leaf volatile production, increased jasmonic acid production, or both. Jasmonic acid is an important elicitor involved in induced indirect defenses of plants. In *Vicia faba* (Blechert et al., 1995) and tobacco (McCloud and Baldwin, 1997), caterpillar feeding results in increased endogenous jasmonic acid levels. Jasmonic acid treatment induces volatile emissions in several plant species (Boland et al., 1995) including Lima bean (Hopke et al., 1994; Dicke et al., 1999), maize (Hopke et al., 1994), and gerbera (Gols et al., 1999), resulting in the attraction of carnivores (Dicke et al., 1999; Gols et al., 1999), and leads to higher parasitism rates of caterpillars feeding on tomato (Thaler, 1999). Expression of *AtLOX2*, *AtAOS*, and *AtHPL* is already induced at 3 hr of *P. rapae* feeding on *Arabidopsis* (Reymond et al., 2000).

To our knowledge, no genes involved in the production of nitriles have been identified in *Arabidopsis* so far.

Herbivory-induced expression of genes involved in volatile production indicates that *Arabidopsis* is actively producing volatiles following herbivory. Moreover, differences in attraction of *C. rubecula* and composition of volatile blends of artificially damaged and caterpillar-infested plants suggest that herbivory leads to production of *C. rubecula*–attracting volatiles that are distinct from the volatiles emitted after artificial damage. However, mimicking mechanical aspects of caterpillar damage correctly is difficult if not impossible. Expression of *AtLOX2* (Bell and Mullet, 1993), *AtHPL* (Bate et al., 1998), *AtAOS* (Laudert et al., 1996), and *AtPAL1* (Mizutani et al., 1997) is also induced by artificial damage. Further experiments are needed and currently in progress to investigate whether *Arabidopsis* indeed produces volatiles specific for herbivory compared to mechanical damage.

CONCLUSIONS

This study on indirect plant defense combines behavioral and chemical analyses with gene expression analysis. Our data show that *Pieris rapae*-infested *Arabidopsis thaliana* attract *Cotesia rubecula* parasitoids. Caterpillar-infested plants release volatiles from several major biosynthetic pathways, and genes involved in the production of these volatiles are induced by herbivory. Many mutants and transgenic lines of *Arabidopsis* are available that are altered in signal transduction pathways of direct defense mechanisms or in the biosynthesis of secondary plant metabolites. In addition, the entire genome sequence of *Arabidopsis* has been completed, and knowledge of direct defense mechanisms is steadily increasing (e.g., Pieterse and van Loon, 1999). Therefore, *Arabidopsis* constitutes an interesting tool to study signal transduction of induced indirect defense in plants.

Acknowledgments—We thank Maarten Koornneef, Kees van Loon, Corné Pieterse, and Joop van Loon for their advice on this research and valuable comments on the manuscript; Herman Dijkman, Leo Koopman, Frans van Aggelen, and Andre Gidding for the insect rearing; Casper Pillen for rearing of *Arabidopsis* plants; Jörg Bohlmann and Jonathan Gershenzon for the *AtTPS03* and *AtTPS10* plasmids; Steven Rothstein, Jocelyne Le Tarte, and Richard Stahl for providing the *AtLOX2*, *AtAOS*, and *AtHPL* plasmids; and the PhD discussion group of the Laboratory of Entomology for comments on this manuscript. M.D. was partly funded by the Uyttenboogaart-Eliasen Foundation, Amsterdam.

REFERENCES

AGELOPOULOS, N. G. and KELLER, M. A. 1994. Plant-natural enemy association in the tritrophic system, *Cotesia rubecula–Pieris rapae*–Brassicaceae (Crucifera): II. Preference of *C. rubecula* for landing and searching. *J. Chem. Ecol.* 20:1735–1748.

BATE, N. J., SIVASANKAR, S., MOXON, C., RILEY, J. M. C., THOMPSON, J. E., and ROTHSTEIN, S. J. 1998. Molecular characterization of an *Arabidopsis* gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. *Plant Physiol.* 117:1393–1400.

BELL, E. and MULLET, J. E. 1993. Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. *Plant Physiol*. 103:1133–1137.

BLAAKMEER, A., GEERVLIET, J. B. F., LOON, J. J. A., POSTHUMUS, M. A., VAN BEEK, T. A., and DE GROOT, A. E. 1994. Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: Consequences for in-flight host location by *Cotesia* parasitoids. *Entomol. Exp. Appl*. 73:175-182.

BLECHERT, S., BRODSCHELM, W., HOLDER, S., KAMMERER, L., KUTCHAN, T. M., MUELLER, M. J., XIA, Z. Q., and ZENK, M. H. 1995. The octadecanoic pathway: Signal molecules for the regulation of secondary pathways. *Proc. Natl. Acad. Sci. USA* 92:4099–4105.

BOHLMANN, F., ZDERO, C., BERGER, D., SUWITA, A., MAHANTA, P., and JEFFREY, C. 1979. Neue furanoeremophilane und weitere inhaltsstoffe aus Südafrikanischen *Senecio*-arten. *Phytochemistry* 18:79–93.

BOHLMANN, J., MEYER-GAUEN, G., and CROTEAU, R. 1998. Plant terpenoid synthases: Molecular biology and phylogenetic analysis. *Proc. Natl. Acad. Sci. USA* 95:4126-4133.

BOHLMANN, J., MARTIN, D., OLDHAM, N. J., and GERSHENZON, J. 2000. Terpenoid secondary metabolism in *Arabidopsis thaliana*: cDNA cloning, characterization and functional expression of a myrcene/ocimene synthase. *Arch. Biochem. Biophys.* 375:261–269.

BOLAND, W. 1995. The chemistry of gamete attraction: Chemical structures, biosynthesis, and (a)biotic degradation of algal pheromones. *Proc. Natl. Acad. Sci. USA* 92:37–43.

BOLAND, W., HOPKE, J., DONATH, J., NÜSKE, J., and BUBLITZ, F. 1995. Jasmonic acid and coronatin induce odor production in plants. *Angew. Chem. Int. Ed. Engl*. 34:1600–1602.

BOLAND W., KOCH, T., KRUMM, T., PIEL, J., and JUX, A. 1999. Induced biosynthesis of insect semiochemicals in plants, pp. 110–131, *in* D. J. Chadwick and J. A. Goode (eds.). Insect-Plant Interactions and Induced Plant Defense. John Wiley & Sons, New York.

BOUWMEESTER, H. J., VERSTAPPEN, F. W. A., POSTHUMUS, M. A., and DICKE, M. 1999. Spider mite-induced (3*S*)-(*E*)-neridol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. *Plant Physiol*. 121:173–180.

CHANG, S., PURYEAR, J., and CAIRNEY, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11:113–116.

CHURCH, G. and GILBERT, W. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 92:4114–4119.

DELANEY, T. P., UKNES, S., VERNOOIJ, B., FRIEDRICH, L.,WEYMANN, K., NEGROTTO, D., GAFFNEY, T., GUTRELLA, M., KESSMANN, H., WARD, E., and RYALS, J. 1994. A central role of salicylic acid in plant disease resistance. *Science* 266:1247–1250.

DEMPSEY, D. A., SHAH, J., and KLESSIG, D. F. 1999. Salicylic acid and disease resistance in plants. *Crit. Rev. Plant Sci.* 18:547–575.

DICKE, M. 1999. Evolution of induced indirect defense of plants, pp. 62–88, *in* R. Tollrian and C. J. Harvell (eds.). The Ecology and Evolution of Inducible Defenses. Princeton University Press, Princeton, New Jersey.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A. E. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DICKE, M., TAKABAYASHI, J., POSTHUMUS, M. A., SCHUTTE, C., and KRIPS, O. E. 1998. Plant–phytoseiid interactions mediated by herbivore-induced plant volatiles: Variation in production of cues and in response of predatory mites. *Exp. Appl. Acar*. 22:311–333.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in Lima bean plants. *J. Chem. Ecol*. 25:1907–1922.

DIETRICH, R. A., DELANEY, T. P., UKNES, S. J., WARD, E. R., RYALS, J. A., and DANGL, J. L. 1994. *Arabidopsis* mutants simulating disease resistance response. *Cell* 77:565–577.

DIXON, R. A. and PAIVA, N. L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7:1085–1097.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73:289–297.

GEERVLIET, J. B. F., POSTHUMUS, M. A., VET, L. E. M., and DICKE, M. 1997. Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *J. Chem. Ecol*. 23:2935–2954.

GERSHENZON, J. and CROTEAU, R. 1991. Terpenoids, pp. 165–219, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites, Vol. 1. Academic Press, New York.

GOLS, R., POSTHUMUS, M. A., and DICKE, M. 1999. Jasmonic acid induces the production of gerbera volatiles that attract the biological control agent *Phytoseiulus persimilis*. *Entomol. Exp. Appl.* 93:77–86.

GRANT-PETERSSON, J. and RENWICK, J. A. A. 1996. Effects of ultraviolet-B exposure of *Arabidopsis thaliana* on herbivory by two crucifer-feeding insects (Lepidoptera). *Environ. Entomol*. 25:135–142.

HOPKE, J., DONATH, J., BLECHERT, S., and BOLAND, W. 1994. Herbivore-induced volatiles: The emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a β-glucosidase and jasmonic acid. *FEBS Lett.* 352:146–150.

KARBAN, R. and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, Illinois.

LAUDERT, D., PFANNSCHMIDT, U., LOTTSPEICH, F., HOLLÄNDER-CZYTKO, H., and WEILER, E. W. 1996. Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. *Plant Mol. Biol*. 31:323–335.

LEE, H. L., LÉON, J., and RASKIN, I. 1995. Biosynthesis and metabolism of salicylic acid. *Proc. Natl. Acad. Sci. USA* 92:4076–4079.

LICHTENTHALER, H. K. 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu. Rev. Plant. Physiol. Plant Mol. Biol*. 50:47–65.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., TURLINGS, T. C. J., and TUMLINSON, J. H. 1994. Diurnal cycle of emission of induced volatile terpenoids herbivore-injured cotton plants. *Proc. Natl. Acad. Sci. USA* 91:11836–11840.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1995. β-Glucosidase: An elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92:2036–2040.

MAUCH-MANI, B. and SLUSARENKO, A. J. 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell* 8:203–212.

MAURICIO, R. 1998. Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *Am. Nat.* 151:20–28.

MAURICIO, R. and RAUSHER, M. D. 1997. Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution* 5:1435–1444.

MIZUTANI, M., OHTA, D., and SATO, R. 1997. Isolation of a cDNA and a genomic clone encoding cinnamate-4-hydroxylase from *Arabidopsis* and its expression manner in planta. *Plant Physiol.* 113:755–763.

MCCLOUD, E. S. and BALDWIN, I. T. 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris*. *Planta* 203:430–435.

MCCONN, M., CREELMAN, R. A., BELL, E., MULLET, J. E., and BROWSE, J. 1997. Jamonate is essential for insect defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 94:5473–5477.

OHL, S., HEDRICK, S. A., CHORY, J., and LAMB, J. C. 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. *Plant Cell* 2:837–848.

PARÉ, P. W. and TUMLINSON, J. H. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol*. 114:1161–1167.

PIETERSE, C. M. J. and VAN LOON, L. C. 1999. Salicylic acid-independent plant defence pathways. *Trends Plant Sci.* 4:52–58.

PIETERSE, C. M. J., VAN WEES, S. C. M., VAN PELT, J. A., KNOESTER, M., LAAN, R., GERRITS, H., WEISBEEK, P. J., and VAN LOON, L. C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10:1571–1580.

REYMOND, P., WEBER, H., DAMOND, M., and FARMER, E. E. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* 12:707–719.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

STOTZ, H. U., PITTENDRIGH, B. R., KROYMANN, J., WENIGER, K., FRITSCHE, J., BAUIKE, A., and MITCHELL-OLDS, T. 2000. Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not diamondback moth. *Plant Physiol*. 124:1007–1017.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: Relative influence of plant and herbivore. *Chemoecology* 2:1–6.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1994. Volatile herbivore-induced terpenoids in plant-mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol*. 20:1329–1354.

THALER, J. S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399:686–687.

THOMMA, B. P. H. J., EGGERMONT, K., PENNICKX, I. A. M. A., MAUCH-MANI, B.M., VOGLSANG, R., CAMMUE, B. P. A., and BROEKAERT, W. F. 1998. Separate jasmonate-dependent and salicylate-dependent defense- response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA* 95:15107–15111.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., TUMLINSON, J. H., HEATH, R. R., PROVEAUX, A. T., and DOOLITTLE, R. E. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol*. 17:2235–2251.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL P. J., RÖSE U. S. R., LEWIS, W.J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92:4169–4174.

VAN LOON, J. J. A., DE BOER, J. G., and DICKE, M. 2000. Parasitoid–plant mutualism: Parasitoid attack of herbivore increases plant reproduction. *Entomol. Exp. Appl*. 97:219–227.

WANNER, L. A., LI, G., WARE, D., SOMSSICH, I. E., and DAVIS, K. R. 1995. The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. *Plant Mol. Biol.* 27:327–338.

WHITMAN, D. W. and ELLER, F. J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69–75.

YANO, S. and OHSAKI, N. 1993. The phenology and intrinsic quality of wild crucifers that determine the community structure of their herbivorous insects. *Res. Pop. Ecol*. 35:151–170.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

VOLATILES FROM *Ficus hispida* AND THEIR ATTRACTIVENESS TO FIG WASPS

QISHI SONG,[1,2,*] DARONG YANG,[1] GUANGMING ZHANG,[1] and CHONGREN YANG[2]

[1]*Xishuangbanna Tropical Botanical Garden*
Chinese Academy of Sciences
Kunming, Yunnan 650223, China

[2]*Laboratory of Phytochemistry*
Kunming Institute of Botany
Chinese Academy of Sciences
Kunming, Yunnan 650204, China

(Received November 30, 2000; accepted June 13, 2001)

Abstract— Volatile compositions of receptive (ready to be pollinated), postpollinated, and postparasitized figs, and leaves of *Ficus hispida* were analyzed. Differences among them were examined, and the specificity of fig wasp attractiveness was investigated. Linalool was the major constituent of steam-distilled oil of either male or female receptive figs, while dibutyl phthalate was the major compound of the oils of postparasitized and postpollinated figs. In petroleum ether extracts, palmitic oil, and 9,12-octadecadienoic acid were the main constituents of male and female receptive figs, while hexadecanoic acid ethyl ester was the major compound of postparasitized and postpollinated figs. In dichloromethane extracts, linalool was the major constituent of male and female receptive figs, 1-hydroxylinalool was the major component of male postparasitized figs, and 1-hydroxylinalool and benzyl alcohol were the major constituents of female postpollinated figs. Bioassays with sticky traps showed that *Ceratosolen solmsi-marchal* was attracted to dichloromethane extracts of male and female receptive figs and to petroleum ether extracts of female receptive figs, but was not attracted to dichloromethane and petroleum ether extracts of male postparasitized and female postpollinated figs. Figs were attractive to pollinating wasps only at the receptive stage. The volatile constituents of receptive figs were different from those of postpollinated or postparasitized figs. From a receptive to a postpollinated state, figs changed in their volatile composition. Some compounds disappeared or decreased in amount. These include linalool, linalool oxide, α-terpeneol, and 2,6-dimethyl-1,7-octadiene-3,6-diol, which may act as the attractants of the wasps. Others increased in amount, or several additional chemicals appeared. These include dibutyl phthalate, 1-hydroxylinalool, and

*To whom correspondence should be addressed.

0098-0331/01/1000-1929$19.50/0

benzyl alcohol, which may be repellents of the wasps. That dichloromethane extracts of male and female receptive figs showed similar activities in attracting fig wasps indicates that receptive figs of both sexes are similarly attractive to fig wasps, which is further supported by their similar volatile composition. Leaf extract was not attractive to the wasps.

Key Words—*Ficus hispida*, *Ceratosolen solmsimarchali*, fig volatile, chemical attraction.

INTRODUCTION

Specialist insects often choose their host plants carefully. They may feed on one or only a few closely related plant species. Although visual factors may be important (Rausher, 1978; Owens and Prokopy, 1986), the search for and identification of preferred host plants are usually olfactory (Miller and Strickler, 1984). The nondirected dispersal flight of the specialist insect is converted into active search behavior once specific host volatile information is perceived (Cardé, 1984).

Mutualism between fig (*Ficus*) and fig wasp (*Agaonidae*) is species-specific. Each species of *Ficus* is generally pollinated by one specific species of fig wasp (Ramirez, 1974; Wiebes, 1979). Fig trees are totally dependent on wasps for pollination, and as a reward, figs provide food for wasp larval development inside the fruit (Janzen, 1979). The high degree of specificity that pollinating wasps show to their particular fig species led to the assumption that the trees attract their specific pollinators through the release of volatile chemicals when the figs are ready to be pollinated (Ramirez, 1970; Hills et al., 1972; Galil, 1977; Janzen, 1979). Olfactory attraction is considered a reasonable way by which fig trees attract huge number of these tiny specific pollinators from far away. Although chemical attraction was suggested more than 50 years ago (Condit, 1947), studies on it are still preliminary (Barker, 1985; Baijnath et al., 1986; Bronstein, 1987, 1992; van Noort et al., 1989; Ware et al., 1993; Ware and Compton, 1994; Gibernau et al., 1997; Grison et al., 1999). An investigation using fig-bearing trees and arrays of sticky traps baited with figs suggested that the wasps are attracted to the trees by volatiles emanating from the figs and that wasps are specifically attracted to figs of their host species only at the time when figs are ready to be pollinated (Ware and Compton, 1994).

In order to elucidate the chemical mechanisms underlying the specific attractiveness of fig trees to their pollinating wasps, we analyzed the chemical constituents of volatiles from receptive (ready to be pollinated), postpollinated, and postparasitized figs of *Ficus hispida* L. and the difference in composition of volatiles among these states of figs. Using arrays of sticky traps baited with fig volatiles, we investigated the specificity of fig wasp attraction to these volatiles.

METHODS AND MATERIALS

Materials. *Ficus hispida* L. is a pioneer tree growing in abundance in the wastelands of the tropics. It is gynodioecious and bears fruit year round. Our previous investigation showed that two or three pollinating wasps were found inside each receptive fig and that all of them were *Ceratosolen solmsimarchali* Mayr (Yang et al., 1997).

Fresh male and female receptive figs, female postpollinated, and male post-parasitized figs and leaves were collected from fruit-bearing healthy *F. hispida* trees inside Xishuangbanna Tropical Botanical Garden, Yunnan, China. Receptive figs are flowering inflorescences that receive pollinating wasps. Figs at different developing stages were determined by fig dissection. Fresh leaves were taken from female and male trees and mixed at a 1 : 1 ratio.

Collection of Volatiles. For steam distillations, immediately following collection, all materials were ground and subjected to vacuum steam distillation for 6 hr at 100°C. Distillates were extracted 3 times with diether ether and dried over sodium sulfate. The oils obtained were then frozen. For solvent extraction, fresh ground plant materials were soaked twice in petroleum ether (30–60°C) or dichloromethane for 24 hr at room temperature. Extracts from each soaking were combined and reduced in volume at 30°C by rotary evaporator. Extracts were dried over sodium sulfate and frozen.

Analysis of Volatiles. Frozen samples were subjected to gas chromatography–mass spectrometry by using a Finnigan 4510 GC/MS/DC. An HP-5 column (30 m long, 0.25 mm ID) was used under the following conditions: 10 psi head pressure, split flow ratio 30 : 1, and oven temperature program of 80–250°C at 5°C/ min . The carrier gas was helium, the injector was maintained at 230°C, and injection volume was 0.2 μl. Mass spectra conditions: ion source, EI; temperature, 175°C; electron energy, 70 eV; signal-enhancing voltage, 1300 V; bulb current, 0.25 mA; IS scan. Data analyses were carried out by using the EPA/NIH/MASS database (NBS Library database). Retention times and mass spectra of compounds detected in the samples were compared with those of authentic compounds.

Bioassay of Volatiles. A 20 cm^{-2} colorless plastic plate sprayed with odorless peach gum was used as a sticky trap. A cotton ball soaked with 0.2 ml dichloromethane solution of the sample (concentration 10% v/v) was placed at the center of the sticky plate to attract fig wasps. Sticky traps were placed on poles. Two arrays of three sticky traps for each treatment were placed 3 m from the fig tree canopy. The plates were positioned 1 m away from each other and 1.5 m above ground. Cotton balls with 0.2 ml dichloromethane were used as solvent controls. The sticky traps were place at 6:30 AM in May 2000. Insects trapped were recorded and collected at 1200 hr and 1800 hr for two days. The number of the wasps used

is the total of wasps trapped in two days. ANOVA of data were done with Winks software.

RESULTS

Volatiles of figs from steam distillation were present in small quantities, about 20 μl/kg fresh wt of fig material. Compounds may have been altered by heat. Headspace extraction is nondestructive, but yield of volatiles is even less. Therefore, lower polarity solvents (petroleum ether and dichloromethane) were used to extract enough volatiles from the figs for bioassay. Solvent extraction may also yield some nonvolatile compounds. The following are the comparative analyses of volatiles from figs and leaves of *F. hispida*.

Constituents of Steam Distilled Oils. Large numbers of volatile compounds were obtained through steam distillation (Table 1). Fifty-five compounds from steam distilled oil of male receptive figs were isolated, and 44 of them were identified by GC-MS. Among them, palmitic oil (30.74%), linalool (18.77%), and 9,12-octadecadienoic acid (5.43%) were the major constituents. *cis*-Linalool oxide, *trans*-linalool oxide, 2,6-dimethyl-3,7-octadiene-2,6-diol, and some other compounds were found as well. In the oil of postparasitized male figs, 21 compounds were isolated and 19 were identified. Dibutyl phthalate (41.07%), palmitic oil (27.80%), 9,12 -octadecadienoic acid (9.31%), and heptadecane (3.24%) were the major compounds found. From the oil of female receptive figs, 31 compounds were isolated and 21 were identified. Linalool (19.83%), β-pinene (11.65%), α-terpeneol (9.90%), 3-phenyl-2-propenal (7.83%), sabinene (7.76%), α-pinene (6.85%), terpene-4-ol (6.77%), geraniol (6.00%), 2,6-dimethyl-3,7-octadiene- 2,6-diol (4.90%), and γ-terpene (4.80%) were the major compounds. 1-Hydroxy-linalool, 6-methyl-(*E*)-3,5-heptadien-2-one, and 2,6-dimethyl-3,7-octadiene-2,6-diol were not found in oils of other fig materials. From the oil of postpollinated female figs, 13 compounds were isolated, and two were identified. The major compound was dibutyl phthalate (72.71%).

According to the data, a large change occurred in the volatile composition of figs from the receptive stage to the postpollinated or postparasitized stage. Forty of the 44 identified compounds from male receptive figs were not detected from the oil of postparasitized figs, while 15 compounds from postparasitized figs were not found in the oil of receptive figs. Similarly, 20 compounds from female receptive figs were not found in the oil of female postpollinated figs, which, in turn, have their own special compounds. However, female and male receptive figs share some volatile compounds together. After pollination or parasitization, some volatile compounds in the figs disappeared or were present in reduced amounts (such as linalool and α-terpeneol), while other compounds occurred newly or in increased amount (such as dibutyl phthalate, which is a known insect repellent).

TABLE 1. CONSTITUENTS OF STEAM-DISTILLED OILS FROM DIFFERENT FIG TYPES OF *Ficus hispida*[a]

No.	Compound	Male receptive figs	Male postparasitized figs	Female receptive figs	Female postpollinated figs
1	hexanal	0.83			
2	3-hexen-1-ol	0.89			
3	1-hexanol	3.95			
4	2,4,6-trimethy l-1-nonene	0.30			
5	benzaldehyde	0.29			
6	*cis*-linalool oxide	0.87			
7	*trans*-linalool oxide	0.76			
8	linalool	18.77		19.83	
9	2,6-dimethyl-3, 7-octadiene-2, 6-diol	0.42			
10	(*Z*) -2-decenal	0.24			
11	1-dodecyne	0.31			
12	borneol	0.19			
13	α-terpeneol	3.90		9.90	
14	2,7-dimethyl-2, 6-octadien-1-ol	1.18			
15	geraniol	1.83		6.00	
16	(*E*)-2-tridecen-1-ol	0.19			
17	3-phenyl-2-propenal	0.40			
18	5-(2-propenyl)-1, 3-benzodioxole	0.15			
19	2,4-decadienal	0.23			
20	1-(2,6,6-trimethyl-1,3 -cyclohexadienyl)-2-buten-1-one	0.24			
21	β-elemene	0.60			
22	zingiberene	0.54			
23	12-methyl-(E,E)-1,5,9, 11-tridecatetraene	0.17			
24	β-caryophyllene	0.36			
25	3-phenol-2-propen-1-ol, acetate	0.70			
26	β-farnesene	0.56			
27	curcumene	0.44			
28	β-bisabolene	0.94			
29	elemol	1.38			
30	nerolidol	1.55			
31	caryophyllene oxide	1.00			
32	guaiol	1.02			
33	farnesol	5.83			
34	11-octadecenal	0.60			
35	octyl-oxirane	0.62			
36	cyclopentadecanone	0.42			
37	6-octadecenoic acid methyl ester	0.53			
38	dibutyl phthalate	4.59	41.07		72.71

TABLE 1. CONTINUED

No.	Compound	Male receptive figs	Male postparasitized figs	Female receptive figs	Female postpollinated figs
39	hexadecanoic acid	30.74	27.8		
40	12,15-octadecadienoic acid, methyl ester	0.09			
41	9,12,15-octadecatrienoic acid, methyl ester (*Z*,*Z*,*Z*)	0.34			
42	1,7,11-trimethyl-4-l (1-methylethyl)-cyclotetradecanol	0.94			
43	9,12-octadecadienoic acid	5.43	9.31		
44	octadecanoic acid	0.40	0.82		
45	6-ethyltetrahydro-2,2, 6-trimethyl-2H-pyran-3-ol		0.34		
46	dodecaboic acid		0.81		
47	1-chloro-octadecane		0.67		
48	heptadecane		3.24		
49	octadecanal		2.32		
50	8,11-octadecadiynoic acid, methyl ester		1.58		
51	octadecanol		1.16		
52	2,6-dimethyl-heptadecane		1.25		
53	2,3-dimethyl-heptadecane		1.64		
54	nonadecane		0.86		
55	11-dodecen-2-one		0.86		
56	17-octadecenoic acid, methyl ester		1.75		
57	(*Z*)-9-octadecenoic acid, methyl ester		0.59		
58	9-octadecenal		1.10		
59	(*Z*)-9-tricosene		0.83		
60	α-pinene			6.85	
61	1-tetradecen-3-yne			3.70	
62	sabinene			7.76	
63	β-pinene			11.65	
64	α-terpene			2.72	
65	1-methyl-4-(1-methylethyl)-benzene			2.74	
66	limonene			3.88	
67	eucalyptol			2.78	
68	γ-terpene			4.80	
69	6-methyl-(*E*)-3, 5-heptadien-2-one			2.89	
70	terpene-4-ol			6.77	
71	2,6-dimethyl-3,7-octadiene-2,6-diol			4.90	
72	2-ethenyl-2,5-dimethyl-4-hexen-1-ol			2.86	
73	3-phenyl-2-propenal			7.83	
74	1-hydroxylinalool			2.88	
75	*trans*-2-tridecenal			2.97	

TABLE 1. CONTINUED

No.	Compound	Male receptive figs	Male postparasitized figs	Female receptive figs	Female postpollinated figs
76	pentadecane			1.76	
77	camphene			2.88	
78	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester				5.24
	unidentified compounds	11[b], 4.30[c]	2[b], 1.99[c]	10[b], 11.38[c]	11[b], 21.05[c]

[a] Values are the percentage of each compound in whole steamed oil.
[b] Number of unidentified compounds.
[c] Combined percentage of unidentified compounds.

Constituents of Solvent Extracts. Sixteen compounds were isolated from petroleum ether extracts of male receptive figs (Table 2). Eight were identified. Palmitic oil (51.90%) and 9,12-octadecadienoic acid (39.39%) were the major constituents. Fifteen compounds were isolated from petroleum ether extracts of male postparasitised figs, and 10 were identified. Hexadecanoic acid ethyl ester (29.69%) and hexadecanoic methyl ester (22.26%) were the major constituents. Thirteen compounds were isolated from petroleum ether extracts of female receptive figs, and four were identified. Palmitic oil (68.73%) and 9,12-octadecadienoic acid (25.99%) were the major constituents. Eleven compounds were isolated from female postpollinated figs, and five were identified. Major compounds were hexadecanoic acid ethyl ester (34.32%), hexadecanoic methyl ester (24.01%), and palmitic acid (22.35%). Sixteen compounds were isolated from petroleum ether extracts of leaves, and seven were identified. Major constituents were 9,12-octadecadienoic acid (43.39%) and palmitic acid (28.82%). In comparison with steam distillation, petroleum ether extraction yielded fewer volatile compounds. That female and male receptive figs were different in volatile composition from female postpollinated or male postparasitized figs was confirmed by petroleum extraction. Female and male receptive figs have their own special volatiles, characterized by a greater proportion of 9,12-octadecadienoic acid and palmitic acid. The main constituents of leaf extracts were similar to those of female and male receptive figs.

In dichloromethane extracts of male receptive figs (Table 3), 17 compounds were detected, and eight were identified. The major constituents were linalool (51.20%), 1-hydroxylinalool (11.45%), bicyclo[2,2,1]hepta-2,5-dien-7-ol (11.46%), and 2,6-dimethyl-1,7-octadiene-3,6-diol (5.22%). Fifteen compounds were isolated from dichloromethane extract of male postparasitized figs, and 11 were identified, with 1-hydroxylinalool (33.00%), linalool (22.32%), and benzyl alcohol (20.56%) being the main constituents. In dichloromethane extracts of female receptive figs, 20 compounds were detected, and 13 were identified. The

TABLE 2. COMPOUNDS FROM PETROLEUM ETHER EXTRACTS OF FIGS AND LEAVES OF *Ficus hispida*[a]

No.	Compound	Male receptive fig	Male postparasitized fig	Female receptive fig	Female postpollinated fig	Leaves
1	α-methylstyrene	0.64				
2	1-ethyl-2-methylbenzene	0.22				
3	2,6-dimethyl-2,7-octadiene-1,6-diol	0.17				
4	butanone		0.34			
5	dibutyl phthalate	4.33		1.78		4.88
6	hexadecanoic acid methyl ester	0.35	22.26		24.01	1.5
7	hexadecanoic acid ethyl ester		29.69	1.61	34.32	
8	palmitic acid	51.90	15.91	68.73	22.35	28.82
9	9,12-octadecadienoic acid	39.39	4.84	25.99		43.39
10	octadecanoic acid	1.16	1.3			2.19
11	9-octadecenoic acid methyl ester		6.53			
12	octadecanoic acid methyl ester		2.46			
13	octadecanoic acid ethyl ester		7.53			
14	9,15-octadecadienoic acid methyl ester				4.12	
15	9,12,15-octadecatrienoic acid methyl ester					9.82
16	9,12,15-octadecatrienoic acid ethyl ester					6.43
17	oleic acid ethyl ester		7.03			
18	linoleic acid ethyl ester				8.82	
	unidentified compounds	8^b, 1.84[c]	5^b, 2.09[c]	9^b, 1.89[c]	6^b, 6.37[c]	9^a,2.96[c]

[a] Values are the percentage of each compound in whole extract.
[b] Number of unidentified compounds.
[c] Combined percentage of unidentified compounds.

main constituents were linalool (33.64%), 1-hydroxylinalool (12.15%), benzyl alcohol (11.20%), 4-methyl-2-pentadecyl-1,3-dioxolane (10.43%), and α-farnesene (6.98%). In dichloromethane extracts of female postpollinated figs, 20 compounds were detected, and 16 were identified. The major compounds were 1-hydroxylinalool (23.77%), benzyl alcohol (19.55%), linalool (16.55%), and methyl-(3-methoxy- 4-hydroxy-benzyl)-ether (8.90%). The number of compounds recovered from dichloromethane extraction was less than that from steam distillation,

TABLE 3. COMPOUNDS FROM DICHLOROMETHANE EXTRACTS OF *Ficus hispida*[a]

No.	Compound	Male receptive fig	Male postparasitized fig	Female receptive fig	Female postpollinated fig
1	benzyl alcohol		20.56	11.20	19.55
2	benzeneacetaldehyde		3.80	1.05	3.86
3	linalool	51.20	22.32	33.64	16.55
4	4-methyl-2-pentadecyl-1,3-dioxolane			10.43	
5	(*Z*)butanoic acid 2-hexenyl ester			4.89	
6	1-(2,2-dimethylcyclopentyl)-ethanone			1.00	
7	1-(dichloromethyl)-3-methyl-benzene			0.71	
8	trans,trans-2,6-dimethyl-2,6-octadiene-1,8-diol			0.84	
9	dodecyl-oxirane			1.08	
10	1-hydroxylinalool	11.45	33.00	12.15	23.77
11	β-farnesene	4.00	1.78	2.90	
12	α-farnesene	2.67	1.88	6.98	0.87
13	methyl-(3-methoxy-4-hydroxy-benzyl)-ether			1.80	8.90
14	1-(1-cyclohexen-1-yl)ethanone		1.05		2.95
15	1-undecyne		2.12		0.97
16	tetradecanal				1.73
17	vanillin				0.79
18	caryophyllene				1.55
19	4-ethyl-1,4-dimethyl-2-cyclohexen-1-ol				1.00
20	nerolidol				0.88
21	4-hydroxy-β-ionone				3.67
22	allyl undecylenate				2.00
23	bicyclo[2,2,1]hepta-2,5-dien-7-ol	11.46			
24	2-amino-4-nitro-phenol	4.50			
25	2,6-dimethyl-1,7-octadiene-3,6-diol	5.22			
26	decyl-oxirane	2.60			
27	phenylethyl alcohol		1.08		0.90
28	2,5-dimethyl-1,5-hexadien-3-ol		1.15		
29	dodecanal		2.83		
	unidentified compounds	9[b],6.90[c]	4[b], 8.43[c]	7[b], 11.33[c]	4[b], 10.06[c]

[a] Values: the percentage of each compound in whole extract.
[b] Number of unidentified compounds.
[c] Combined percentage of unidentified compounds.

but more than that from petroleum ether extraction. The chemical constitution of dichloromethane extracts was similar to that of distilled oil; however, it was rather different from that of petroleum ether extracts. For dichloromethane extracts, female and male receptive figs were similar, while both were different from female postpollinated figs and male postparasitized figs. Female and male receptive figs have their own special volatile compounds. The content of linalool was higher than that for female postpollinated figs and male postparasitized figs. On the other hand, the content of 1-hydroxylinalool and benzyl alcohol of female postpollinated figs and male postparasitized figs was higher than that for receptive figs.

Attractiveness of Volatiles of F. hispida to C. solmsimarchali. Bioassays using sticky traps showed that there were significantly more *C. solmsimarchali* trapped by dichloromethane extracts of female and male receptive figs and by petroleum ether extract of female receptive figs ($P < 0.05$) and that there were no significantly more fig wasps trapped by petroleum ether extract of leaves than by solvent controls (Table 4). Moreover, there were more wasps trapped by petroleum ether extracts of female receptive figs than by those of postpollinated figs ($P < 0.05$). As for dichloromethane extracts, those of receptive figs attracted significantly more wasps than those of postpollinated or postparasitized figs, either female or male ($P < 0.05$). However, there were no differences in fig wasp numbers trapped by either dichloromethane or petroleum ether extracts of female postpollinated and male postparasitized figs and solvent control ($P > 0.05$). Wasps trapped by dichloromethane extracts of female and male receptive figs were not different either ($P > 0.05$). The sticky plate trapped many other insects but no other fig wasp species.

DISCUSSION

Results of bioassays indicate that receptive figs are attractive but postpollinated and postparasitized figs are not attractive to the pollinating wasp in the case of *F. hispida*. Different responses of wasps to receptive figs, postpollinated figs, postparasitized figs, and leaves of the *Ficus* tree were caused by the varying volatile constitutions of these plant materials. The unique volatile constituents and special volatile blend-up are the chemical basis of the receptive figs of *F. hispida* to attract *C. solmsimarchali*.

Field bioassay results of fig volatile extracts of *F. hispida* confirmed conclusions drawn from previous studies on other fig species (Ware and Compton, 1994). Figs are attractive to their wasp pollinator only at the receptive stage.

To identify chemical cues for fig wasps, we adopted a comparative approach. Figs changed in their volatile constituents from receptive to postpollinated or postparasitized states. Some compounds decreased in concentration or disappeared,

TABLE 4. ATTRACTION OF VOLATILE EXTRACTS OF *Ficus hispida* TO *Ceratosolen solmsimarchali*

Treatment	Traps (N)	Mean Fig wasps trapped ($\pm$SE)	Newman-Keuls multiple comparison (F values)			
			Against control	Against A	Against E	Against G
A. Petroleum ether extract of female receptive figs	6	14.3 ± 4.2	4.2*		2.9 NS	3.4*
B. Petroleum ether extract of female postpollinated figs	6	0.5 ± 0.8	NS	4.5*	4.4*	4.2*
C. Petroleum ether extract of male receptive figs	6	1.5 ± 1.4	NS	4.0*	3.8*	3.4*
D. Petroleum ether extract of male postparasitized figs	6	0.3 ± 0.5	NS	4.6*	4.5*	4.4*
E. Dichloromethane extract of female receptive figs	6	12.5 ± 1.4	4.0*	2.9 NS		2.9 NS
F. Dichloromethane extract of female postpollinated figs	6	0.5 ± 0.5	NS	4.4*	4.2*	4.0*
G. Dichloromethane extract of male receptive figs	6	11.0 ± 2.6	3.8*	3.4*	2.9 NS	
H. Dichloromethane extract of male postparasitized figs	6	0.2 ± 0.4	NS	4.7*	4.6*	4.5*
I. Petroleum ether extract of leaves	6	2.7 ± 0.8	NS	3.8*	3.4*	2.9*
J. Control	6	0.7 ± 0.8		4.2*	4.0*	3.8*

*$P < 0.05$, significant difference; NS: no significant difference at $P > 0.05$.

while others increased in concentration or several additional chemicals appeared. The compounds that disappeared or decreased in amount after pollination or parasitization were linalool, linalool oxide, α-terpeneol, and 2,6-dimethyl-1,7-octadiene-3,6-diol, which may act as attractants for the wasps. Compounds that increased in amount or newly occurred were dibutyl phthalate, 1-hydroxylinalool, and benzyl alcohol, which could be deterrents to the wasps.

The composition of volatiles of receptive figs produced by steam distillation and dichloromethane extraction was similar to that from the headspace collection (Grison et al., 1999) and pentane extraction (Gibernau et al., 1997) used in investigations of other fig species. The essential chemicals suggested to attract fig

wasps, such as linalool, linalool oxide, and benzyl alcohol, were detected in this investigation also. Linalool and linalool oxide were found in the distilled oil, and linalool, benzyl alcohol, and 1-hydroxylinalool (rather than linalool oxide) were found in dichloromethane extracts. However, benzyl alcohol did not appear to act as a fig wasp attractant but rather as a repellent, because its content in figs increased after pollination or parasitization. Characteristic or major volatile compounds and special blends of volatiles may be important in attractiveness of receptive figs to wasps. Some volatile compounds found in trace amounts may also play a role in this attraction. A much greater amount and number of volatile compounds were produced from steam distillation and dichloromethane extraction compared with previous methods (Gibernau et al., 1997; Grison et al., 1999). It is interesting that dibutyl phthalate, a known insect repellent, was found in large quantity in postpollinated and postparasitized figs. This indicates that figs may, in fact, be repellent to fig wasps after losing attractiveness following pollination or parasitization. Compounds yielded by petroleum ether extraction were largely fatty acid derivatives. Only the extract from female receptive figs showed activity in fig wasp attraction. Extraction with petroleum ether is not ideal for obtaining fig volatiles in large quantity.

Although volatile compositions of receptive figs, male or female, proved much different from those of postpollinated or postparasitized figs, compositions of male and female receptive figs proved similar. They differed only in the quantity of some major compounds; this corresponds to findings on other fig species (Grison et al., 1999). That dichloromethane extracts of male and female receptive figs showed similar activities in attracting fig wasps indicates that receptive figs of both sexes are similarly attractive to fig wasps. This is further supported by their similar volatile composition. Pollinating fig wasps should favor male receptive figs and avoid female figs because they can lay eggs only in male figs, not in female ones, where they pollinate and die. On the other hand, in order to attract wasps for pollination, female receptive figs could imitate the odor of male receptive figs. There could be an intersexual mimicry of odor in the case of *F. hispida*.

Leaf extracts did not show significant activity in the wasp attraction. This suggests that the canopy of fig trees may not play a role in long distance odor attraction of fig wasp. Compounds in leaf extracts were mainly fatty acid derivatives; this is similar to previous findings on other fig species (Buttery et al., 1986).

The species-specific mutualism between a fig tree and its pollinating wasps is based on some unique volatile compounds and special volatile blends of receptive figs. Our bioassays showed that volatile extracts of receptive figs of *F. hispida* were attractive only to one species of fig wasp, its pollinator *C. solmsimarchali*. They were not attractive to other fig wasps, although the sticky plates baited with the extracts trapped some other insects. Furthermore, only *C. solmsimarchali* were found in more than 10,000 dissected receptive figs.

About 900 species of *Ficus* (Janzen, 1979) are widespread in tropical ecosystems. They not only provide food to many diverse animals and microorganisms, but also act as habitats for epiphytes, saprophytes, parasites, and shade-requiring plants. They are keystone species in tropical ecosystems (Wiebes, 1979; Xu, 1994; Yang et al., 1997, 1999). In Xishuangbanna, the tropical area of China, *Ficus* species (such as *F. hispida*, *F. semicordata*, and *F. tinctoria* subsp. *gibbosa*) grow quickly as pioneer woody plants after deforestation. Inside or under the canopy of these plants, a community with rich animal, plant, and microorganism diversity is likely to build and expand gradually. Thus, *Ficus* trees are important in the restoration of tropical ecosystems. The species-specific mutualism between *Ficus* trees and their pollinating wasps makes their reproduction extremely efficient. However, this special relationship is vulnerable. The decrease or extinction of any partner in the mutualism could lead to a decrease or extinction of the corresponding other partner. The study of chemically based mechanisms of species-specific pollination of *Ficus* trees might be significant in the conservation of these keystone species and the whole tropical forest ecosystem. The collection, isolation, identification, and bioassay of the fig wasp attractants from *Ficus* trees deserve further attention.

Acknowledgments—The authors thank Ms. Yuanfen Yi for GC/MS operation, Mr. Yaowu Wang and Mr. Tinzhou Zhao for partial field works. This research was supported by grants from the Ninth Five-Year Key Program of Chinese Academy of Sciences, National Natural Science Foundation of China and Science Foundation of Yunnan.

REFERENCES

BAIJNATH, H., NAICKER, S., and RAMCHARUN, S. 1986. The interaction of figs and fig wasps, pp. 348–349, *in* B. E. S. Juniper and T. R. E. Southwood (eds.). Insect and the Plant Surface. Edward Arnold, London.

BARKER, N. P. 1985. Evidence of a volatile attractant in *Ficus ingens* (Moraceae). *Bothalia* 15:607–611.

BRONSTEIN, J. L. 1987. Maintenance of species specificity in a neotropical fig-pollinator wasp mutualism. *Oikos* 48:39–46.

BRONSTEIN, J. L. 1992. Seed predators as mutualists: ecology and evolution of the fig/pollinator interaction, pp. 1–44, *in* E. Bernays (ed.). Insect–Plant Interactions, Vol. IV. CRC Press, Boca Raton, Florida.

BUTTERY, R. G., FLATH, R. A., MON, T. R., and LING, L. C. 1986. Identification of germacrene D in walnut and fig leaf volatiles. *J. Agric. Food Chem.* 34:820–822.

CARDÉ, R. T. 1984. Chemo-orientation in flying insects, pp. 111–124, *in* W. J. Bell and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

CONDIT, I. J. 1947. The Fig Chronica Botanica. Waltham.

GALIL, J. 1977. Fig biology. *Endeavour* 1:52–56.

GIBERNAU, M., BUSER, H. R, FREY, J. E., and HOSSAERT-MCKEY, M. 1997. Volatile compounds from extracts of figs of *Ficus carica*. *Phytochemistry* 46:241–244.

GRISON, L., EDWARDS, A. A., and HOSSAERT-MCKEY, M. 1999. Interspecies variation in floral fragrances emitted by tropical *Ficus* species. *Phytochemistry* 52:1293–1299.

HILLS, H. G., WILLIAMS, N, H., and DOBSON, C. H. 1972. Floral fragrances as isolating mechanisms in the genus *Catasetum* (Orchidaceae). *Biotropica* 4:61–76.

JANZEN, D. H. 1979. How to be a fig. *Annu. Rev. Ecol. Syst*. 10:13–51.

MILLER, J. R. and STRICKLER, K. L. 1984. Finding and accepting host plants, pp. 127–155, *in* W. J. Bell and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

OWENS, E. D. and PROKOPY, R. J. 1986. Relationship between reflectance spectra of host plant surfaces and visual detection of host fruit by *Rhagoletis pomonella*. *Physiol. Entomol*. 11:297–307.

RAUSHER, M. D. 1978. Search image for leaf shape in a butterfly. *Science* 200:1071–1073.

RAMIREZ, B. W. 1970. Host specificity of fig wasps (Agaonidae). *Evolution* 24:680–691

RAMIREZ, B. W. 1974. Coevolution of *Ficus* and Agaonidae. *Ann. Mo. Bot. Gard*. 61:770–780.

VAN NOORT, S., WARE, A. B., and COMPTON, S. G. 1989. Pollinator specific volatile attractants released from the figs of *Ficus burtt-davyi*. *S. Afr. J. Sci*. 85:323–324.

WARE, A. B. and COMPTON, S. G. 1994. Responses of fig wasps to host plant volatile cues. *J. Chem. Ecol.* 20:785–802.

WARE, A. B., COMPTON, S. G., KAYE, P. T., and VAN NOORT, S. 1993. Fig volatiles: Their role in attracting pollinators and maintaining pollinator specificity. *Plant Syst. Evol*. 186:147–156.

WIEBES, J. T. 1979. Co-evolution of figs and their insect pollinators. *Annu. Rev. Evol. Syst*. 10:1–12.

XU, Z. 1994. *Ficus* trees: Keystone species of tropical rain forest ecosystem in southern Yunnan, China. *Chin. Biodivers.* 2:21–23 (in Chinese with English abstract).

YANG, D., LI, C., and YANG, B. 1997. Studies on the diversity and structure of animal community living on the *Ficus* trees in Xishuangbanna tropical rain forest. *Zool. Res.* 18:189–196 (in Chinese with English abstract).

YANG, D., LI, C., HAN, D., and YAO, R. 1999. The impact of the fragmentation of tropical rain forest on fig wasps and *Ficus* species. *Zool. Res.* 20:126–130 (in Chinese with English abstract).

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

VOLATILE COMPOUNDS FROM *Salix* spp. VARIETIES DIFFERING IN SUSCEPTIBILITY TO THREE WILLOW BEETLE SPECIES

L. PEACOCK,* M. LEWIS, and S. POWERS

IACR-Long Ashton Research Station
Department of Agricultural Science
University of Bristol
Long Ashton, Bristol, BS41 9AF, UK

(Received January 23, 2001; accepted June 20, 2001)

Abstract—The volatile compounds emitted by leaves of 10 willow varieties that differ in their susceptibility to damage by blue (*Phratora vulgatissima*), brassy (*P. vitellinae*), and brown (*Galerucella lineola*) willow beetles were examined both before and after mechanical damage and correlated with feeding preferences of these beetles determined under laboratory conditions. Three compounds were identified from intact undamaged leaves of six willow varieties, namely *cis*-3-hexenyl acetate, *cis*-3-hexenol, and benzaldehyde. After mechanical damage, the yield and number of volatile compounds increased for all varieties. There were significant differences among willow varieties for both the concentration of *cis*-3-hexenyl acetate and the relative proportion of this compound to *cis*-3-hexenol (green leaf volatile ratio). The 10 varieties collectively showed a significant negative correlation between the relative resistance of each variety to blue and brown willow beetles and the yield of *cis*-3-hexenyl-acetate from damaged plants. The green leaf volatile ratio of damaged plants was also negatively correlated with the relative resistance of willow variety to these two beetle species.

Key Words—Attractant, *Galerucella*, green leaf volatiles, *Phratora*, semiochemicals, short-rotation coppice.

INTRODUCTION

The blue [*Phratora vulgatissima* (L.)], brassy [*P. vitellinae* (L.)] and brown [*Galerucella lineola* (L.)] (Coleoptera: Chrysomelidae) willow beetles are the

*To whom correspondence should be addressed; e-mail: lori.peacock@bbsrc.ac.uk

0098-0331/01/1000-1943$19.50/0

major insect pests of short-rotation coppice willows grown as a potential source of renewable energy. Willow beetles have an aggregated distribution on preferred hosts in willow plantations (Peacock et al., 1999). The combined effects of the presence of conspecifics and beetle-feeding induced plant kairomones are suggested as factors affecting this aggregative behavior (Peacock et al., 2001). This agrees with data from many Coleoptera that have been shown to use feeding-induced odors as aggregation kairomones (Harari et al., 1994; Loughrin et al., 1995; Bolter et al., 1997; Landolt et al., 2000; Kalberer et al., 2001). Under laboratory conditions, three volatile compounds were recorded from undamaged *Salix dasyclados* Wimm. plants [benzaldehyde and the green leaf volatiles (GLVs) *cis*-3-hexenyl acetate and *cis*-3-hexenol] (Peacock et al., 2001). After beetle-feeding damage, the yield and number of volatiles increases. GLVs are thought to be used by plants for allelopathy or to attract or repel insects (Hatanaka, 1996). Thus, plant resistance could be influenced by the overall characteristics of individual or blends of volatiles by either increasing or decreasing damage (see references in Loughrin et al., 1996, 1997a).

Willow varieties differ in their susceptibility to willow beetles (Kendall and Wiltshire, 1996) due to differences in feeding preferences of the beetles. The willow beetle species considered here tend to have different feeding preferences, with *P. vulgatissima* and *G. lineola* preferring willows low in phenylglucosides (Kelly and Curry, 1991; Kendall et al., 1996), while *P. vitellinae* prefers poplars and willow varieties high in phenylglucosides (Pasteels and Rowell-Rahier, 1992). It is unclear, however, how willow beetles locate their host plants. Due to differences in host preference, a major insecticide-free pest management strategy is a mixture of willows that differ in their susceptibility to the willow beetle (Peacock and Herrick, 2000). A better knowledge of the mechanisms of host resistance, especially as regards potential attractants, such as volatile chemicals, is necessary for effective deployment of varieties in mixtures.

Salix dasyclados Wimm., the variety shown by Peacock et al. (2001) to emit volatile compounds, is the willow variety most preferred by *P. vulgatissima* in a feeding preference assay of 106 different willow varieties (Kendall and Wiltshire, 1996). It is unknown if the types and levels of volatile emissions collected from *S. dasyclados* Wimm. are specific to this willow or if they differ among varieties. Therefore, we examined the volatile compounds emitted by intact undamaged and damaged leaves of willow varieties. The aims of this study are: (1) to compare and contrast the volatile emission profile of different willow varieties; (2) to assess whether varieties differ in their volatile emission response to mechanical damage; and (3) to determine whether volatile compounds correlate with feeding preferences of willow beetles under laboratory conditions for different willow varieties.

METHODS AND MATERIALS

Plants. Five cuttings (25 cm length each) of 10 willow varieties were taken from cold storage in February 2000, planted individually in 10-cm-diameter pots, and maintained in a greenhouse until required for volatile analysis (between March 6 and April 12, 2000). The following year, cuttings for the beetle feeding preference tests were grown under similar conditions, but planted in March 2001 and used May 22, 2001. The 10 varieties were *Salix dasyclados* Wimm., *S. viminalis* Bowles Hybrid, *S.* x *sericans* Coles, *S. burjatica* Germany, *S. viminalis* 79046, *S.* x 445 de Biardii, *S. purpurea* 012, *S. triandra* Black Maul, *S. burjatica* Korso, and *S. eriocephala*.

Volatile Compound Collection and Analysis. The emissions of volatile compounds from individual plants were collected and measured following the procedure described in Peacock et al. (2001). Briefly, the terminal 20 cm of a plant was placed in an inverted glass beaker (155 $\times$ 90 mm) held by a retort stand, and the opening loosely sealed with aluminium foil. After 90 min, the trap was desorbed in a SGE Unijector fitted to a Carlo Erba Mega GC into a Superox II column that was connected to a Kratos MS80RFA mass spectrometer. After collection, the plant was removed and, to simulate beetle damage, five of the leaves contained within the beaker space had five holes punched into each one with a standard hole-punch. The volatile emissions were collected for 90 min and treated as previously described. All 10 varieties were assessed in this way, with the procedure being replicated on three plants for each variety.

Mechanical damage, as opposed to beetle damage, was chosen to standardize the amount and timing of damage, as it has been shown that different leaf volatiles may be released at different times or even follow a diurnal pattern of emission (Loughrin et al., 1997b; Turlings et al., 1998). Standardization was essential, as the main emphasis of the study was to detect whether there were any differences in the quantity of volatiles among varieties, which could be affected by any variations in the timing and amount of damage produced.

Compound identities were confirmed by a library search against the Wiley/NBS Registry of Mass Spectral Data, and the area under each peak was estimated. To estimate the concentration of compounds detected (in nanograms), stock standard solutions of the two major volatiles previously detected (*cis*-3-hexenol and *cis*-3-hexenyl acetate) (both Sigma) were made up in ethyl acetate. Serial dilutions of these solutions were then used to prepare the calibration graphs to convert area to approximate concentration.

Beetle Feeding Preferences. Standard sized leaf disks (110 mm diameter) of each willow variety were cut from 2-month-old (500 cm tall) potted willows. One disk of each variety was placed in a Petri dish (14 cm diameter) lined with

a damp filter paper to form a circle, and secured into position with a stainless steel entomological pin. This was replicated six times (for each beetle species), each dish with a different random arrangement of willow varieties. Ten beetles of one species were put in the center of each dish and maintained at 15°C. Blue and brown willow beetles had been collected the previous day from a planting of *S. viminalis* Bowles Hybrid, and brassy willow beetles from a planting of poplars at Long Ashton Research Station; they were not fed for seven hours before the start of the experiment. After 18 hr, beetles were removed, and the leaf disks were transferred to acetates and mounted flat under clear adhesive tape. The remaining leaf area of each disk was measured with an Optomax Image Analyser (cf. Kendall et al., 1996). This was done for each of the three beetle species separately.

Statistical Methods. Taking the feeding preference experiments for the three species of beetle separately, analysis of variance (ANOVA) was performed on the measurements of leaf area remaining, taking account of variation due to dishes and position as dictated by the experimental design. For each of the three volatiles detected in undamaged plants (*cis*-3-hexenyl acetate, *cis*-3-hexenol, and benzaldehyde), ANOVA was applied separately to assess any difference in quantity emitted by undamaged compared with damaged plants across the different varieties. ANOVA was also used to consider any differences in quantity of each compound emitted by the 10 varieties after mechanical damage. The ratio of *cis*-3-hexenyl acetate to *cis*-3-hexenol (referred to as the GLV ratio) emitted after damage was also analyzed in this way. The correlation between mean emission of volatile and mean amount of leaf area remaining across pairs of these measurements for all the varieties was made for each volatile separately and for the GLV ratio. A significant negative correlation implied that willow varieties with less leaf area remaining (i.e., relatively susceptible) tended to have a high concentration of volatile compound and GLV ratio, and vice versa.

RESULTS

Volatile Compounds. Three volatile compounds were emitted from undamaged plants (*cis*-3-hexenyl acetate, *cis*-3-hexenol, and benzaldehyde), which increased in yield after they were damaged, as did the number of different volatile compounds detected (Table 1). There were differences among undamaged willow varieties for the yield of compounds: four gave no detectable volatile compounds, while relatively low levels were collected from six. Of the three compounds tested, the yield difference between undamaged and damaged plants was significant for only *cis*-3-hexenyl acetate and *cis*-3-hexenol ($F = 10.97$ and $F = 4.57$, $df = 10, 38$; $P < 0.001$) and for certain varieties (Table 1, undamaged). There were also significant differences among varieties after damage for certain compounds

TABLE 1. VOLATILES EMITTED FROM WILLOW[a]

	Compound	Yield (ng ± SE)									
		S. dasyclados Wimm.	*S. viminalis* Bowles Hybrid	*S.* x *sericans* Coles	*S. burjatica* Germany	*S. viminalis* 79046	*S.* x 445 de Biardii	*S. purpurea* 012	*S. triandra* Black Maul	*S. burjatica* Korso	*S. eriocephala*
Undamaged											
1	3-hexenal	—[b]	—	—	—	—	—	—	—	—	—
2	2-hexenal	—	—	—	—	—	—	—	—	—	—
3	*cis*-3-hexenyl acetate	7.3 ± 3.2[c]	—	1.2 ± 0.9[c]	0.8 ± 0.2[c]	—	0.2 ± 0.2[c]	—	0.3 ± 0.3	—[c]	—
4	*trans*-2-hexenyl acetate	—	—	—	—	—	—	—	—	—	—
5	*cis*-3-hexenol	2.2 ± 1.9[c]	—	1.0 ± 1.0[c]	1.2 ± 0.9	—	—	—	0.2 ± 0.2[c]	—[c]	—[c]
6	*trans*-2-hexenol	—	—	—	—	—	—	—	—	—	—
7	a hexenyl butyrate	—	—	—	—	—	—	—	—	—	—
8	a hexenyl pentanoate	—	—	—	—	—	—	—	—	—	—
9	benzaldehyde	0.3 ± 0.2	—	0.2 ± 0.2	—	—	0.3 ± 0.3	0.8 ± 0.6	0.3 ± 0.3	—	—
Damaged[d]											
1		7.0 ± 4.6a	1.0 ± 1.0a	7.0 ± 3.8a	9.0 ± 2.1a	4.7 ± 0.3a	4.7 ± 2.4a	9.3 ± 2.4a	10.7 ± 2.0a	14.3 ± 7.4a	14.3 ± 5.2a
2		6.7 ± 3.2b	0.2 ± 0.2b	18.3 ± 9.2a	4.3 ± 0.9b	9.0 ± 2.1ab	3.5 ± 2.3b	5.7 ± 1.8b	5.7 ± 1.2b	5.0 ± 2.6b	18.3 ± 6.1a
3		128.0 ± 24.0d	0.3 ± 0.2c	53.7 ± 27.2a	46.7 ± 19.6ab	20.7 ± 11.3ac	32.0 ± 15.6ac	11.7 ± 10.7bc	10.7 ± 3.8c	34.0 ± 16.7ac	9.5 ± 4.7c
4		4.0 ± 1.0a	—	3.7 ± 2.7a	—	0.3 ± 0.2b	—	—	—	—	—
5		57.7 ± 26.4a	13.0 ± 7.0a	42.3 ± 24.4a	32.7 ± 18.1a	24.0 ± 11.9a	24.7 ±11.7a	29.7 ± 12.2a	39.3 ± 18.5a	56.0 ± 37.3a	56.0 ± 28.3a
6		6.3 ± 2.4a	—	14.0 ± 6.7b	—	5.2 ± 2.7a	—	—	—	3.8 ± 3.6a	6.3 ± 3.2a
7		1.3 ± 0.9bc	—	0.3 ± 0.2c	0.2 ± 0.2c	—	2.8 ± 1.6ab	—	3.8 ± 1.7a	1.2 ± 0.4bc	—
8		—	—	0.2 ± 0.2a	1.7 ± 1.7a	—	0.3 ± 0.2a	—	—	0.3 ± 0.3a	2.5 ± 2.3a
9		1.8 ± 0.7a	—	0.2 ± 0.2a	0.2 ± 0.2a	1.0 ± 0.6a	0.8 ± 0.2a	0.5 ± 0.3a	1.2 ± 0.9a	0.2 ± 0.2a	0.2 ± 0.2a

[a] The mean quantity (ng ± SE) of volatile compounds emitted in 90 min from the top 20 cm of stem from three plants of 10 willow varieties before and after mechanical damage (numbers related to compounds for undamaged plants). Different letters indicate significant difference ($P < 0.05$) between varieties for the yield of each compound separately.

[b] — indicates compound was not detectable.

[c] Yields are significantly different ($P < 0.05$) between undamaged and damaged plants for individual variety.

[d] For compound name see a) undamaged above.

TABLE 2. RATIO OF TWO MAJOR VOLATILES EMITTED FROM DAMAGED WILLOW VARIETIES ($N = 3$)

Willow variety	Ratio (±SE) of *cis*-3-hexenyl acetate to *cis*-3-hexen-1-ol
Salix dasyclados Wimm.	3.03 ± 0.98 c[a]
Salix viminalis Bowles Hybrid	0.03 ± 0.03 a
Salix x *sericans* Coles	1.20 ± 0.66 ab
Salix burjatica Germany	1.72 ± 0.78 b
Salix viminalis 79046	0.73 ± 0.17 ab
Salix x 445 de Biardii	1.32 ± 0.23 ab
Salix purpurea 012	0.26 ± 0.19 a
Salix triandra Black Maul	0.31 ± 0.04 a
Salix burjatica Korso	0.73 ± 0.26 ab
Salix eriocephala	0.13 ± 0.08 a

[a] Ratios with different letters are significantly different at $P < 0.05$.

(Table 1, damaged), with *S. viminalis* Bowles Hybrid having the lowest number of compounds and yield of all the varieties.

There were differences among willow varieties for the GLV ratio (*cis*-3-hexenyl acetate to *cis*-3-hexenol) from damaged plants ($F = 3.5$, $df = 8, 18$; $P < 0.01$). *Salix dasyclados* had the ($P < 0.05$) highest ratio of all varieties, with *S. viminalis* Bowles Hybrid having the lowest (Table 2).

Beetle Feeding Preferences. There were differences for all beetle species in their feeding preferences of the 10 willow varieties (*P. vulgatissima* $F = 5.9$, $df = 9,54$; $P < 0.001$; *P. vitellinae* $F = 15.2$, $df = 9,54$; $P < 0.001$; *G. lineola* $F = 2.5$, $df = 9,54$; $P < 0.02$). *Salix dasyclados* was the most preferred variety of both *P. vulgatissima* and *G. lineola* (Table 3), whereas *P. vitellinae* preferred *S. burjatica* Germany and *S. purpurea*.

Correlations. For both *P. vulgatissima* and *G. lineola*, there were negative correlations between the amount of leaf area remaining and the absolute concentration of *cis*-3-hexenyl acetate (*P. vulgatissima* $r = -0.88$, $P < 0.001$; *G. lineola* $r = -0.84$, $P < 0.01$). Two other compounds showed no correlation: *trans*-2-hexenyl acetate (*P. vulgatissima* $r = -0.57$, $P = 0.08$; *G. lineola* $r = -0.52$, $P = 0.12$) and benzaldehyde (*P. vulgatissima* $r = -0.59$, $P = 0.07$; *G. lineola* $r = -0.54$, $P = 0.10$). In comparison, for *P. vitellinae*, there were no significant correlations between leaf area remaining and any of the emitted compounds.

Likewise, there was a negative correlation between the GLV ratio and the remaining leaf area after feeding by *P. vulgatissima* ($r = -0.95$, $P < 0.001$) and *G. lineola* ($r = -0.84$, $P < 0.01$), with none for *P. vitellinae*.

TABLE 3. FEEDING PREFERENCES OF *Phratora vulgatissima*, *P. vitellinae*, AND *Galerucella lineola* FOR 10 POTTED WILLOW VARIETIES

Willow variety	Leaf area remaining[a] (mm)		
	P. vulgatissima	*P. vitellinae*	*G. lineola*
Salix dasyclados Wimm.	81.4 ± 7.8	105.1 ± 1.0	89.4 ± 6.7
Salix viminalis Bowles Hybrid	100.7 ± 2.9	107.5 ± 1.1	99.8 ± 4.0
Salix x *sericans* Coles	98.6 ± 1.8	105.9 ± 1.1	102.5 ± 1.4
Salix burjatica Germany	92.1 ± 2.7	77.9 ± 6.3	99.8 ± 3.2
Salix viminalis 79046	98.1 ± 2.2	105.7 ± 0.2	104.8 ± 0.9
Salix x 445 de Biardii	92.9 ± 2.5	102.7 ± 0.2	99.4 ± 0.8
Salix purpurea 012	105.2 ± 0.9	85.5 ± 4.1	103.3 ± 0.5
Salix triandra Black Maul	104.4 ± 0.6	104.2 ± 0.7	103.0 ± 1.4
Salix burjatica Korso	101.8 ± 0.9	100.7 ± 1.6	98.6 ± 2.8
Salix eriocephala	104.0 ± 0.5	102.9 ± 0.7	104.0 ± 0.8
SED (*df*)	4.3 (54)	3.6 (54)	4.0 (54)

[a] Values represent the mean amount of leaf area remaining (from a 110-mm diameter leaf disk) 18 hr after beetles were placed in dishes containing a disk of each variety ($N = 7$).

DISCUSSION

The present findings for the willow *S. dasyclados* corroborate previous results for this variety (Peacock et al., 2001), with only *cis*-3-hexenyl acetate, *cis*-3-hexenol, and benzaldehyde emitted from undamaged plants, increasing in yield after damage, as did the number of different volatile compounds detected. While this pattern of volatile emission was similar for the other willow varieties tested, there were differences among varieties in the concentration of compounds and GLV ratio. Correlation analysis indicated a significant relationship between susceptibility to feeding damage by *P. vulgatissima* and *G. lineola* and a relatively high concentration of both *cis*-3-hexenyl acetate and GLV ratio (*cis*-3-hexenyl acetate to *cis*-3-hexenol).

These two green leaf volatiles have previously been shown to be insect attractants (Schütz et al., 1997; Rojas, 1999; Coghlan, 2000). The relationship found for the GLV ratio is of note, as it has been suggested that specialized herbivores (like the Colorado potato beetle and, in the present case, the willow beetle) would likely be attracted to a blend of compounds in a definite ratio, in contrast to generalist insects (like the Japanese beetle) (see references in Loughrin et al., 1996).

The two varieties of *S. burjatica* tested differed significantly in their susceptibility to *P. vulgatissima* and *P. vitellinae. Salix burjatica* Germany, the more susceptible variety, appeared to have a higher GLV ratio than the relatively resistant *S. burjatica* Korso (although not significant at $P > 0.05$). This is of interest as

S. burjatica Germany is one of only a small number of willow varieties that is host to both these *Phratora* species. Usually the adult feeding preferences are different between these two beetle species; this is thought to be due to differences in the phenylglucoside content of the leaves. *Salix burjatica* has relatively high levels of these chemicals (Kelly and Curry, 1991) and, thus, should be a suitable host for *P. vitellinae* but relatively unsuitable for *P. vulgatissima*. However, if there were a higher proportion of *cis*-3-hexenyl-acetate to *cis*-3-hexenol after damage, and if it had an attractant effect on *P. vulgatissima*, any difference in GLV ratio between *S. burjatica* varieties might help explain the anomaly of the similar feeding preference. Under field conditions, more *P. vulgatissima* were found on *S. dasyclados* Wimm. plants that had conspecifics and beetle-feeding damage than on undamaged plants, suggesting the occurrence of a feeding induced plant kairomone (Peacock et al., 2001). These authors found that the GLV ratio had increased from 0.54 in undamaged plants to 2.18 after 24 hr of beetle feeding.

While significant differences among damaged varieties for some volatile compounds and correlations between these quantities and susceptibility to willow beetles were found, green leaf volatiles may not necessarily be the key components in host selection because they are emitted by almost all damaged plants. Volatile collections were made immediately after damage, yet it may be that other, induced compounds associated with the presence of these green leaf volatiles are involved in host plant choice. Many green leaf volatiles are assumed to "bleed" from damaged sites, whereas *cis*-3-hexenyl acetate is known to be partially induced in response to herbivory (Loughrin et al., 1994; Turlings et al., 1998). Therefore, further studies are needed to determine the behavioral responses of beetles to induced leaves and to compare the volatile chemicals after mechanical and beetle damage.

Acknowledgments—Thanks to Judy Harris for the initial contact, David Glen and two anonymous referees for helpful comments on the manuscript, Carmen Ruiz-Martinez for the use of the willow plants, and The Ministry of Agriculture, Fisheries and Food (project code NF0406) for funding this work. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

REFERENCES

BOLTER, C. J., DICKE, M., VAN LOON, J. J. A., VISSER, J. H., and POSTHUMUS, M. A. 1997. Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. *J. Chem. Ecol.* 23:1003–1023.

COGHLAN, A. 2000. Double-crossed. A potato pest's favourite fragrance could be its undoing. *New Sci.* 165:14.

HARARI, A. R., BEN-YAKIR, D., and ROSEN, D. 1994. Mechanism of aggregation behaviour in *Maladera matrida* Argaman (Coleoptera: Scarabeidae). *J. Chem. Ecol.* 20:361–371.

HATANAKA, A. 1996. The fresh green odor emitted by plants. *Food Rev. Int.* 12:303–350.

KALBERER, N. M., TURLINGS, T. C. J., and RAHIER, M. 2001. Attraction of a leaf beetle (*Oreina cacaliae*) to damaged host plants. *J. Chem. Ecol.* 27:647–661.

KELLY, M. T. and CURRY, J. P. 1991. The influence of phenolic compounds on the suitability of three *Salix* species as hosts for the willow beetle *Phratora vulgatissima*. *Entomol. Exp. Appl.* 61:25–32.

KENDALL, D. A. and WILTSHIRE, C. W. 1996. An applied study of clonal resistance to willow beetle attack in SRC willows. Report for ETSU (DTI) Biofuels Study B/M4/00532/27/REP.

KENDALL, D. A., HUNTER, T., ARNOLD, G. M., LIGGITT, J., MORRIS, T., and WILTSHIER, C. W. 1996. Susceptibility of willow clones (*Salix* spp.) to herbivory by *Phyllodecta vulgatissima* (L.) and *Galerucella lineola* (Fab.) (Coleoptera, Chrysomelidae). *Ann. Appl. Biol.* 129:379–390.

LANDLOT, P. J., TUMLINSON, J. H., and ALBORN, D. H. 2000. Attraction of the Colorado potato beetle (Coleoptera: Chrsomelidae) to damaged and chemically induced potato plants.*Environ. Entomol.* 28:973–978.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., TURLINGS, T. C. J., and TUMLINSON, J. H. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton. *Proc. Nat. Acad. Sci. USA* 91:11836–11840.

LOUGHRIN, J. H., POTTER, D. A., and HAMILTON-KEMP, T. R. 1995. Volatile compounds induced by herbivory act as aggregation kairomones for the Japanese Beetle (*Popillia japonica* Newman). *J. Chem. Ecol.* 21:1457–1467.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON-KEMP, T. R., and BYERS, M. E. 1996. Volatile compounds from crabapple (*Malus* spp.) cultivars differing in susceptibility to the Japanese beetle (*Popillia japonica* Newman). *J. Chem. Ecol.* 22:1295–1305.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON-KEMP, T. R., and BYERS, M. E. 1997a. Response of Japanese beetles (Coleoptera: Scarabaeidae) to leaf volatiles of susceptible and resistant maple species. *Environ. Entomol.* 26:334–342.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON-KEMP, T. R., and BYERS, M. E. 1997b. Diurnal emission of volatile compounds by Japanese Beetle-damaged grape leaves. *Phytochemistry* 45:919–923.

PASTEELS, J. M. and ROWELL-RAHIER, M. 1992. The chemical ecology of herbivory on willows. *Proc. R. Soc. Edinburgh* 98B:63–73.

PEACOCK, L. and HERRICK, S. 2000. Responses of the willow beetle *Phratora vulgatissima* to genetically and spatially diverse *Salix* spp. plantations. *J. Appl. Ecol.* 37:821–831.

PEACOCK, L., HERRICK, S., and BRAIN, P. 1999. Spatio-temporal dynamics of willow beetle (*Phratora vulgatissima*) in short-rotation coppice willows grown as monocultures or a genetically diverse mixture. *Agric. For. Entomol.* 1:287–296.

PEACOCK, L., LEWIS, M., and HERRICK, S. 2001. Factors influencing the aggregative response of the blue willow beetle. *Entomol. Exp. Appl.* 98:195–201.

ROJAS, J. C. 1999. Electrophysiological and behavioral responses of the cabbage moth to plant volatiles. *J. Chem. Ecol.* 25:1867–1883.

SCHÜTZ, S., WEISSBECKER, B., KLEIN, A., and HUMMEL, H. E. 1997. Host plant selection of the Colorado Potato Beetle as influenced by damage induced volatiles of the potato plant. *Naturwissenchaften* 84:212–217.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L., and WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta* 207:146–152.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

EFFECT OF CONGENERIC CHEMICAL SIGNALS OF DIFFERENT AGES ON FORAGING RESPONSE AND FOOD CHOICE IN THE FIELD BY GOLDEN SPINY MICE (*Acomys russatus*)

ALEXANDRE DOBLY,[1,*] FRANCINE M. ROZENFELD,[1] and ABRAHAM HAIM[2]

[1]*Behavioural Biology of Mammals*
Free University of Brussels (ULB) - CP 160/12
50, Av. Franklin Roosevelt
B-1050 Bruxelles, Belgium

[2]*Department of Biology*
University of Haifa at Oranim
Kiryat Tivon 36006, Israel

(Received July 14, 2000; accepted June 8, 2001)

Abstract—The common spiny mice *Acomys dimidiatus* and golden spiny mice *Acomys russatus* coexist in the extreme warm and dry parts of the Rift Valley in Israel. However, they are temporally segregated in that the former is nocturnal, whereas the latter is diurnal. Daily rhythms of physiological and behavioral variables in *A. russatus* responded to semiochemical signals released by *A. dimidiatus* (in the urine and feces). Both species feed upon the same food items but at different times of the 24-hr cycle. The main aim of the present study was to test under field conditions the foraging response of *A. russatus* to odors of different ages released by *A. dimidiatus*. Various feeding and behavioral variables were compared in three groups of *A. russatus*. The results show that fresh semiochemical signals released by *A. dimidiatus* decrease the feeding efficiency and increase the rate of smelling from a distance in *A. russatus*. These results support the idea that temporal segregation between the two coexisting species is at least partly through semiochemicals present in the urine and feces.

Key Words—Heterospecific chemical signals, feeding behavior, urine and feces, *Acomys*, spiny mouse, arid environment.

*To whom correspondence should be addressed; e-mail: adobly@ulb.ac.be

0098-0331/01/1000-1953$19.50/0

INTRODUCTION

The coexistence of spiny mice of the genus *Acomys*, the common spiny mouse *A. dimidiatus* (Harrison and Bates, 1991) and the golden spiny mouse *A. russatus*, has been shown to be through exclusion of the latter from nocturnal activity by the former (Shkolnik, 1971). Attempts have been made in the last decade to understand the mechanisms for such temporal segregation. Under laboratory conditions when *A. russatus* is kept separately from *A. dimidiatus*, it manifests body temperatures and oxygen consumption daily rhythms typical of nocturnal species (Rubal et al., 1992). Body temperature and oxygen consumption daily rhythms of *A. russatus*, exposed to indirect chemical signals released by *A. dimidiatus*, showed a phase shift of approximately 2 hr compared with controls (Fluxman and Haim, 1993; Haim and Fluxman, 1996). However, when studying the response of the daily activity rhythms of *A. russatus* to direct contact with urine and feces released by *A. dimidiatus*, a shift of approximately 7 hr into diurnal activity was observed (Haim and Rozenfeld, 1993). From these studies and others, it was concluded that semiochemicals released from the urine and feces of *A. dimidiatus* are important in maintaining the temporal segregation between the two species.

Furthermore, the hypothesis that the nest site (resting place) could be a source of competition between the congeners was tested under laboratory conditions (Haim and Rozenfeld, 1995). The results supported the hypothesis, and it was also noted that *A. russatus* keeps its resting place only by being active during the daytime when *A. dimidiatus* is inactive. Recently, Van Aarde and Haim (1999) have shown that semiochemical signals released by *A. dimidiatus* may affect aspects of reproduction in *A. russatus*.

Both species utilize the same diet (Shkolnik, 1966), but it is eaten at different times of the 24-hr cycle. However, food is probably the source of competition between the two species. From this, we asked the following question: do semiochemicals of different age released by *A. dimidiatus* have an effect on feeding behavior of *A. russatus*? We recorded feeding behavior of *A. russatus* in the field in relation to the size and quality of food items. We studied the change in frequency of feeding sessions and of behavioral acts in response to semiochemicals released by *A. dimidiatus*.

METHODS AND MATERIALS

Study Area. Field observations were carried out at the foot of Masada cliff, 3 km from the Dead Sea shores in the Jehudean desert, which is a part of the Rift Valley, in Israel. The study area was located close to the ruins of a Roman camp, at about 340 m below sea level. The climate is extremely dry and hot. The average annual precipitation is 47 mm with large year-to-year fluctuations. The mean minimum temperature, recorded in January, was 11°C, whereas the mean

maximum temperature, recorded in July, reached 39°C (Jaffe, 1988). Petri dishes containing different food items were used (see below). The dishes were set close to walls made of rough stones, 2 m wide at their base and 1.2 m high. The walls were built some 2000 years ago and are inhabited by different rodent species; however, the dominant species in the area are the spiny mice *A. russatus* and *A. dimidiatus* (Dobly et al., 1999). Along these walls, garbage has accumulated, some of which is presumably consumed by the mice inhabiting the walls. In order to observe marked individuals, *A. russatus* were trapped before the current study (Dobly et al., 1999) and individually marked with an animal-marking crayon (Raidi-Raider, Ottenbach, Germany).

Feeding Observations. Clean Petri dishes (liquid dish-washing soap, water, and then ethanol 70%) were set on a white piece of filter paper (28 × 23 cm). Each dish contained five different items: (1) an intact grilled peanut ($N = 1$); (2) half a peanut seed ($N = 5$); (3) corn seeds ($N = 10$); (4) wheat seeds ($N = 15$); and (5) oat flakes ($N = 15$). The position of the different food items in the Petri dishes as well as mass and energy contents are presented in Figure 1. Two Petri dishes were positioned at a distance of 1.5 m from each other in each session, which lasted 1 hr. A total of 28 sessions were performed.

Preliminary observations indicated that mice were not disturbed by the observer (Dobly et al., 1999). Direct visual observations were carried out from an exposed spot at a distance of 3 m from 8:45 to 16:00 hr. We chose daytime in order to observe *A. russatus*. Different behavioral variables were recorded (see Dobly et al., 1999): emerging from shelter, watching, approaching a Petri dish, smelling from

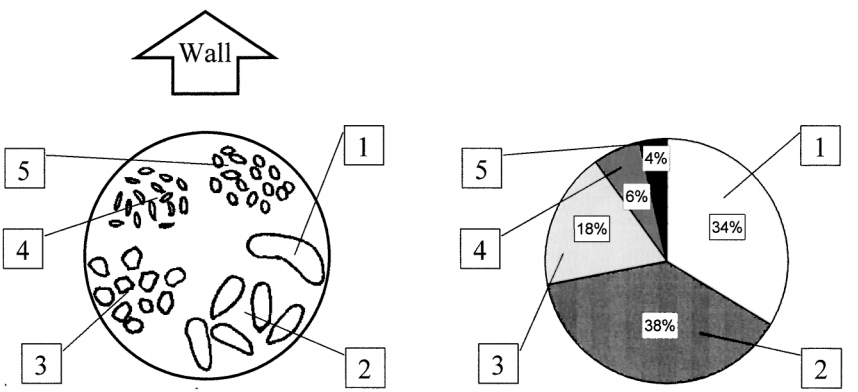


FIG. 1. Presentation and composition of dietary items offered in each Petri dish used as an artificial feeding site. The types, numbers, energy contents, and total weights were as follows: (1) intact grilled peanut (ca. 2 seeds, 1 × 14,000 cal, 2 g), (2) half a peanut seed (5 × 3200 cal, 2.5 g), (3) corn seeds (10 × 750 cal, 1.6 g), (4) wheat seeds (15 × 175 cal, 0.65 g), and (5) oats (15 × 100 cal, 0.35 g). The total energy percentages are given in the pie chart.

a distance, and close sniffing. When more than one mouse was active, behavioral patterns were recorded by using a video camera (Sony Hi8 CCD-TRV70E). Data were analyzed frame by frame. At the end of each session, the food items left over were counted and the Petri dishes cleaned. We also used the number of food items touched, i.e., the number of food categories with at least one item eaten. The energy values of the different food items were determined by using a bomb calorimeter (Semimicro Calorimeter 1425, Parr).

Exposure to Heterospecific Odors. In 15 of 28 sessions, the observed mice were exposed to a type of odor obtained from *A. dimidiatus*. Toilet paper and feces, obtained from traps in which *A. dimidiatus* were successfully trapped, were used as a source of the heterospecific odors. All the collected odorous items were kept in a sealed plastic bag. In eight cases, *A. russatus* were offered, in each session and in each dish, a combination of fresh urine (on one piece of toilet paper) with three fecal pellets collected on the same day (we used three pellets according to the average number available per day). In seven cases, only three fecal pellets were used for testing the response of *A. russatus* to feces alone, 24 hr after collection. Paper was put below the Petri dishes, and the feces were put close to the dishes on the side of the wall. Therefore, three different groups were studied: (1) control (no odor), (2) heterospecific feces only, collected one day before exposure (old odor) and (3) fresh heterospecific urine and feces (fresh odor). We did not compare heterospecific odor to homospecific odor as other laboratory and field studies have shown that only heterospecific odor has a repellent effect on *A. russatus* (Haim and Rozenfeld, 1993; A. Haim and E. Baert, unpublished data). Beyond this identified effect of homospecific versus heterospecific odors, our main purpose was to test the unknown difference between fresh and old heterospecific odor.

Data Analyses. Results are presented as frequency, percentage, mean $\pm$ standard deviation, or median with range. In order to establish differences among studied groups, the following statistical tests were used: chi-square test, Mann-Whitney *U* test, and Kruskal-Wallis one-way ANOVA. All were one-tailed due to the hypothesis of a negative influence of heterospecific odor on feeding. To test the food preference inside one treatment, we used two-tailed Friedman two-way ANOVA. To determine which pairs were the sources of the detected difference in ANOVA tests, we calculated the critical difference necessary for significance in each compared pair.

RESULTS

A total of eight golden spiny mice (five females and three males) were marked and observed. At least one mouse appeared in 22 of the 28 sessions. The six sessions with no mouse were from groups with old [two of seven (29%)] or fresh [four of eight (50%)] odors. This distribution of appearance among the three groups is different (chi-square test, $P < 0.01$, Table 1). Similarly, in all sessions with at

TABLE 1. FEEDING BEHAVIOR OF *Acomys russatus* UNDER THREE ODOR TREATMENTS OF ARTIFICIAL FEEDING SITES

Measurement	Types of heterospecific odors[a]			Statistical tests
	None	Old	Fresh	
Number of sessions with mice	100% (13/13)	71% (5/7)	50% (4/8)	$P < 0.01$, chi-square test
Number of mice per session	1.54 (SD = 0.97) (20/13)	1.71 (SD = 1.70) (12/7)	0.88 (SD = 1.13) (7/8)	$P = 0.045$ between none and fresh odor, Mann-Whitney U test
Median latency to appear (min, sec)	20′27″ [2′20″–52′45″] ($N = 20$)	15′05″ [2′00″–53′00″] ($N = 12$)	29′45″ [1′50″–47′15″] ($N = 7$)	NS, Kruskal-Wallis one-way ANOVA, $df = 2$
Median latency to eat (min, sec)	1′10″ [0′05″–38′25″] ($N = 19$)	1′10″ [0′10″–8′30″] ($N = 9$)	1′07″ [0′25″–04′40″] ($N = 4$)	NS, Kruskal-Wallis one-way ANOVA, $df = 2$
Relative frequency of smelling from a distance	13.3% ($N = 14$)	14.5% ($N = 8$)	28.0% ($N = 6$)	$P = 0.041$, Kruskal-Wallis one-way ANOVA, $df = 2$
Relative frequency of close sniffing	11.1% ($N = 14$)	13.4% ($N = 8$)	9.2% ($N = 6$)	NS, Kruskal-Wallis one-way ANOVA, $df = 2$
Percentage and number of mice that ate	95% (19/20) 1.46 (SD = 0.88)	75% (9/12) 1.29 (SD = 1.11)	57% (4/7) 0.50 (SD = 0.76)	$P = 0.017$ between none and fresh odor, Mann-Whitney U test
Mean food touched (out of 10)	3.5 (SD = 2.7) ($N = 13$)	2.6 (SD = 2.6) ($N = 7$)	1.1 (SD = 1.8) ($N = 8$)	$P = 0.031$, Kruskal-Wallis one-way ANOVA, $df = 2$

[a] None: no odor (control), old: one-day-old heterospecific feces; fresh: fresh heterospecific urine and feces. Range of median is given in square brackets. SD = standard deviation.

least one animal observed, there were, on average, more individuals per session in the control group than in the fresh odor group; the old odor group was intermediate (Mann-Whitney U test, $N = 21$, $P = 0.045$, Table 1).

No significant difference was noted among the three groups in regards to the median time to appear or to eat (Table 1). Moreover, in three cases (two controls and one old odor, 15% and 14%, respectively), an *A. dimidiatus* was detected; all of them appeared only once and took either an entire or half a peanut in a single trip lasting less than 10 sec.

Not all observed *A. russatus* ate from the Petri dish. Table 1 shows, for the three groups studied, the number of mice that ate food, as well as the number of food types that were touched by mice. Mice from the control group ate more frequently than mice confronted with fresh odor (95% vs 57%, Mann-Whitney U test, $N = 27$, $P = 0.017$). Similarly, with regard to number of food types touched, a significant difference was noted only between control mice and those exposed to fresh odor (Kruskal-Wallis one-way ANOVA, $df = 2$, $N = 28$, $P = 0.031$, Table 1).

Mice of all groups preferred the larger items with a high-energy content per gram. The item most often taken on a first visit was the whole peanut, and then the half peanuts. In total, a larger amount of grilled intact peanuts was eaten than oats, corn, or wheat seeds (Friedman two-way ANOVA, $df = 4$, $N = 28$, $P = 0.017$). The energy context of the five food types is presented in Figure 2. The relationship then became clearer. The energy value of whole peanuts eaten is higher than that of corn, wheat, or oats (Friedman two-way ANOVA, $df = 4$, $N = 28$, $P = 0.008$).

The analysis of behavioral patterns revealed that mice exposed to fresh odor showed more smelling from a distance than control mice (Kruskal-Wallis one-way ANOVA, $df = 2$, $N = 28$, $P = 0.041$, Table 1). However, no difference was detected either in close sniffing or in any other recorded behavior (e.g., while the maximum number of mice approaching a Petri dish decreased among the three treatments, the medians were not different: no odor: 2.5 (range 1–30), old: 2.5 (1–19) fresh: 3.0 (1–10), Kruskal-Wallis one-way ANOVA, $df = 2$, $N = 28$, NS).

DISCUSSION

Food and energy consumption in *A. russatus* have been studied under short and long photoperiod regimes (Haim et al., 1994). Under acclimation to a short day, *A. russatus* consumed significantly more food compared with acclimation to a long day. The energetic demands of *A. russatus* are low in relation to its body mass, compared with mesic species. Field metabolic rates (FMR) were compared between the two *Acomys* species by using the doubly labeled water technique (Degen, 1994). Daily metabolic rates of *A. russatus* were 27% lower than those for a similar sized *A. dimidiatus*. The energy content of the items offered in

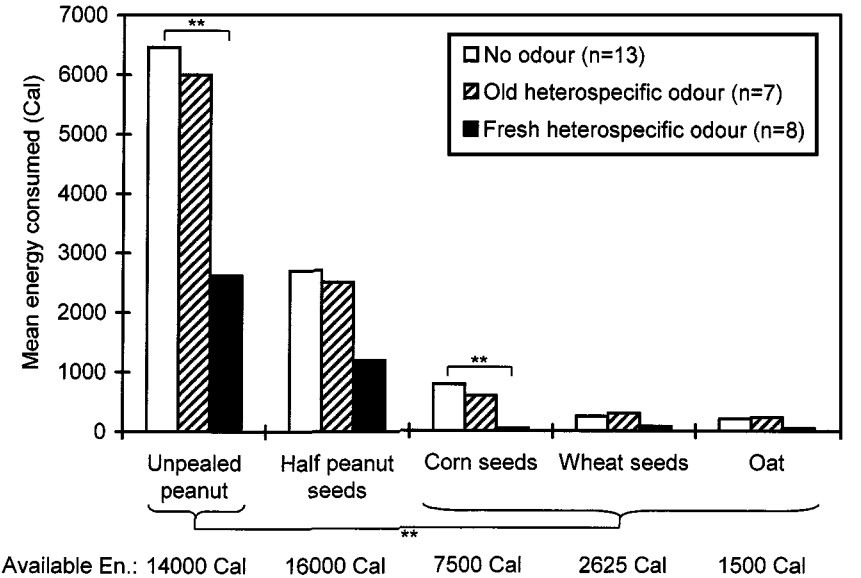


FIG. 2. Mean energy taken from the five food types by the mice under three different treatments. The total energy available is given below each food type (Available En.). Two-tailed Friedman two-way ANOVA, one-tailed Kruskal-Wallis one-way ANOVA, and one-tailed Mann-Whitney U tests were used ($^{*}P < 0.05$, $^{**}P < 0.025$).

the Petri dish exceeded the energy demands, even during the winter season when experiments were carried out.

Our results show that *A. russatus* selected the largest food item (intact peanut), which contained the highest energetic value. Such selection would be advantageous to a diurnal animal by minimizing the time spent foraging and reducing vulnerability to predation or overheating. These requirements are consistent with the high number of distant smelling bouts and the small number of close sniffing bouts recorded in the presence of fresh odor. The results of food selection support the conclusion of Kerley and Erasmus (1991) that rodent preference for seeds is related to the net rate of energy intake provided by the seed.

However, our results revealed a significant decrease in food and energy intake as a result of exposure to fresh urine and feces released by *A. dimidiatus*. In a laboratory study, *A. russatus* marked its food with an oral secretion (Rozenfeld et al., 1994). Oral marking by *A. russatus* may serve to mask the original odor of *A. dimidiatus*. The number of sessions in which mice appeared upon exposure to fresh heterospecific odor and the number of mice per session were significantly lower compared with control sessions. This suggests that odor can act from a distance. However, it seems that under dry desert conditions, odor sources are

not so effective. Therefore, we conclude that, at least partly, scent marking of the habitat by *A. dimidiatus* contributes to the temporal segregation of the two species. As *A. russatus* does not accumulate food in its resting place, the marking of food items supports the hypothesis that such marking has a heterospecific role (Rozenfeld et al., 1994).

From the results of this and previous studies, we conclude that heterospecific odors released in the urine and/or feces of *A. dimidiatus* have an effect on the behavior of *A. russatus*. Activity in *A. dimidiatus* is confined to the early hours of the dark phase (Shkolnik, 1966). Hence, we assume that odors released at night during the activity period of *A. dimidiatus* decline with time, so that when *A. russatus* is active, the odor effect is of little significance.

As the temporal segregation between the two species is maintained, at least partly, by chemicals released by *A. dimidiatus* (Haim and Rozenfeld, 1993), we assume that this depends on fresh urine and feces. The chemicals involved appear to be volatile. Semiochemicals produced in the urine and feces of *A. dimidiatus* can bring about competitive exclusion of *A. russatus* from nocturnal activity and can be used to exclude them from access to food and habitat resources. The isolation of the chemical component of this signal will be important for future studies, involving the segregation of coexisting species.

Acknowledgments—This project was financed by the Israeli Ministry of Sciences and the Belgian "Commisariat Général aux Relations Internationales de la Communauté Française de Belgique" (CGRI). A. Dobly benefited from a FRIA grant from Belgian government ("Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture"). We thank all the people at Ein-Gedi for the use of their facilities. We also thank Allina Neuman for her help.

REFERENCES

DEGEN, A. A. 1994. Field metabolic rates of *Acomys russatus* and *Acomys cahirinus*, and a comparison with other rodents. *Isr. J. Zool*. 40:127–134.

DOBLY, A., ROZENFELD, F. M., and HAIM, A. 1999. Lack of neophobic response to traps in a diurnal rodent the golden spiny mouse (*Acomys russatus*) from an arid environment. *Mammalia* 63:244–249.

FLUXMAN, S. and HAIM, A. 1993. Daily rhythms of body temperature in *Acomys russatus*—the response to chemical signals released by *Acomys cahirinus. Chronobiol. Int.* 10:159–164.

HAIM, A. and FLUXMAN, S. 1996. Daily rhythms of metabolic rates: The role of chemical signals in coexistence of the spiny mice of the genus *Acomys. J. Chem. Ecol.* 22:223–229.

HAIM, A. and ROZENFELD, F. M. 1993. Temporal segregation in coexisting *Acomys* species: The role of odour. *Physiol. Behav.* 54:1159–1161.

HAIM, A. and ROZENFELD, F. M. 1995. Temporal segregation in coexisting *Acomys* species. The possible role of nest site. *J. Arid Environ.* 29:505–509.

HAIM, A., YEDIDIA, I., HAIM, D., and ZISAPEL, N. 1994. Photoperiodicity in daily rhythms of body temperature, foods and energy intake of the golden spiny mouse *Acomys russatus. Isr. J. Zool.* 40:145–150.

HARRISION, D. L. and BATES P. J. 1991. The Mammals of Arabia. Harrison Zoological Museum, Sevenoaks, Kent, UK.

JAFFE, S. 1988. Climate of Israel, pp. 79–94, *in* Y. Yom-Tov and E. Tchernov (eds.). Zoogeography of Israel: The Distribution and Abundance at Zoogeographical Crossroad. W. Junk Publishers, Dordrecht, The Netherlands.

KERLEY, G. I. H. and ERASMUS, T. 1991. What do mice select for in seeds? *Oecologia* 86:261–267.

ROZENFELD, F. M., RASMONT, R., and HAIM, A. 1994. Home site scent marking with urine and oral secretion in the golden spiny mouse (*Acomys russatus*). *Isr. J. Zool*. 40:161–172.

RUBAL, A., CHOSHNIAK, I., and HAIM, A. 1992. Daily rhythms of metabolic rate and body temperature of two murids from extremely different habitats. *Chronobiol. Int.* 9:341–349.

SHKOLNIK, A. 1966. Studies in the comparative biology of Israel's two species of spiny mice (genus *Acomys*). PhD dissertation. Hebrew University of Jerusalem, Jerusalem (English summary).

SHKOLNIK, A. 1971. Diurnal activity in a small desert rodent. *Int. J. Biometeorol.* 15:115–120.

VAN AARDE, R. and HAIM, A. 1999. The influence of urinary and faecal odours on ovarian function in coexisting *Acomys* species. *Isr. J. Zool*. 45:261–265.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

PERCEPTION OF ASPEN AND SUN/SHADE SUGAR MAPLE LEAF SOLUBLE EXTRACTS BY LARVAE OF *Malacosoma disstria*

M. PANZUTO,[1] F. LORENZETTI,[1] Y. MAUFFETTE,[1] and P. J. ALBERT[2,*]

[1]*Département des Sciences Biologiques*
Université de Québec à Montréal
C.P. 8888 Succ. A.
Montréal, Québec, Canada H3C 3P8

[2]*Department of Biology*
Concordia University
1455 De Maisonneuve Blvd W.
Montréal, Québec, Canada H3G 1M8

(Received March 27, 2001; accepted May 29, 2001)

Abstract— We investigated the behavioral feeding preference and the chemoreception of leaf polar extracts from trembling aspen, *Populus tremuloides*, and from sun and shade sugar maple, *Acer saccharum*, by larvae of the polyphagous forest tent caterpillar, *Malacosoma disstria*, a defoliator of deciduous forests in the Northern Hemisphere. Three polar extracts were obtained from each tree species: a total extract, a water fraction, and a methanol fraction. *M. disstria* larvae were allowed *ad libitum* access to an artificial diet from eclosion to the fifth instar. Two-choice cafeteria tests were performed comparing the mean (±SE) surface area eaten of the total extracts, and the following order of preference was obtained: aspen > sun maple > shade maple. Tests with the other fractions showed that *M. disstria* larvae preferred the total aspen extract to its water fraction, and the latter to its methanol fraction. The response to sun maple was similar to aspen. However, for the shade maple experiment, there was no difference between the total extract and its water fraction. Electrophysiological recordings for aspen showed that the sugar-sensitive cell elicited more spikes to the water fraction, followed by the total extract, and finally the methanol fraction. Spike activity to stimulations of sun and shade maple extracts revealed a similar trend, where methanol fraction > water fraction > total extract. Our findings are discussed in light of previously known information about this insect's performance on these host plants.

*To whom correspondence should be addressed at Department of Biology, Concordia University, 1455 De Maisonneuve Blvd. W., Montréal, Québec, Canada H3G 1M8. Tel: (514) 848-8729; Fax: (514) 848-2881; e-mail: albert@alcor.concordia.ca

0098-0331/01/1000-1963$19.50/0

Key Words—*Malacosoma disstria*, *Populus tremuloides*, *Acer saccharum*, sun and shade leaves, polar extracts, feeding preference, cellulose discs, styloconic sensilla, chemoreception, electrophysiology.

INTRODUCTION

The primary feeding host of the forest tent caterpillar (*Malacosoma disstria* HBn.) is trembling aspen (*Populus tremuloides* Michx.), but in Québec during epidemics, larvae are known to feed on sugar maple (*Acer saccharum* Marsh.). Aspen is widespread throughout North America (Hodson, 1941), whereas sugar maple forests occur predominantly in the northeastern portion of the continent (Rowe, 1972). Larvae of *M. disstria* develop better on aspen compared to maple foliage (Lorenzetti, 1993; Nicol et al., 1997). Aspen contains twice the amount of soluble sugars of sugar maple, and these were the only constituents of the foliage that could be correlated with the insect's performance (Lorenzetti, 1993). This is consistent with the work of Lindroth et al. (1993), who showed that in aspen leaves, contents of sucrose and hexose (glucose and fructose) and total nonstructural carbohydrates (all expressed in percent dry mass) were more than three times higher than in the leaves of sugar maple. Other common chemical constituents of aspen include tremulacin and salicortin, which constitute 85% of the total phenolic glycosides (Lindroth et al., 1987). Long-chain fatty alcohols and α-tocopherylquinone (α-TQ) have also been identified (Lin et al., 1998a,b). Lindroth and Hemming (1990) reported that poor performance (prolonged duration of instars and reduced growth) of the polyphagous gypsy moth (*Lymantria dispar* L.) can be attributed to levels of tremulacin that are $>3\%$ in poplar leaves. Some maples (*Acer palmatum*, *A. platanoides*, and *A. rubrum*) are known to contain galloylcyanidin glycosides (Shi-Bao, et al., 1992; Abou-Zaid and Nozzolillo, 1999). Sugar maple is characterized by high levels of hydrolyzable (ellagitannins, gallic acid, gallotannins) and condensed tannins (Baldwin et al., 1987; Lindroth et al., 1993). In contrast, aspen leaves possess significantly lower levels of condensed tannins and no traces of hydrolyzable tannins (Lindroth et al., 1987, 1993).

Differences in foliar concentrations of sugars can be found within a tree, depending on vertical distribution (Futuyama and Saks, 1981; Coley, 1983; Lincoln and Mooney, 1984; Ellsworth and Reich, 1992; Dudt and Shure, 1994). Fortin (1994) observed that when larvae of *M. disstria* were fed foliage that had been exposed to the sun versus exposed to shade, there were differences in pupal weights and in larval development time. Recently, Fortin and Mauffette (in preparation) demonstrated that in four consecutive years, the biological performance of *M. disstria* was superior on foliage located on the crown of mature sugar maple compared to that found at the base of the tree. They argue that this might be due to higher amounts of total nitrogen and soluble sugars present at the summit of the crown, which would result in a more nutritious diet for the insect.

Many phytophagous caterpillars perceive sugars, in particular sucrose, as a cue for the selection of a favorable food plant, and sugar-sensitive chemoreceptors are ubiquitous in all lepidopteran larvae that have been studied to date (Schoonhoven, 1967, 1987). In current behavioral experiments with *M. disstria* larvae, we found that sucrose was preferred over water at all concentrations tested (0.1–500 mM). We also found that sucrose elicited a high firing frequency from cell 1 (sugar cell) on the medial styloconic sensillum of the galea at ecologically relevant concentrations. It is known that neural input from these sensilla on the mouthparts of lepidopteran caterpillars is necessary for unimpaired food selection (Ma, 1972; Hanson and Dethier, 1973; Schoonhoven and Blom, 1988; De Boer, 1993). However, there is limited information available on the sensory perception of plant extracts by maxillary styloconic sensilla, probably because the neurophysiological results are difficult to interpret, due to their considerable variability (Schoonhoven et al., 1991), and to changes in spike shape of the same cell, especially at high frequencies (Frazier and Hanson, 1986). Despite these obstacles, unambiguous results have been obtained in several studies where foliage extracts were used as stimuli in electrophysiological (Ohsugi et al., 1978; Toufexis et al., 1996) and behavioral studies (Cobbinah et al., 1982; Albert and Parisella, 1988; Guertin and Albert, 1992; del Campo and Renwick, 1999).

Since there is more information available on the responses of caterpillars to single compounds, and we know that an insect is exposed to a mixture of compounds when feeding on leaves, we examined the responses of *Malacosoma disstria* larvae to extracts from two host plants. The specific objectives were to assess behavioral preferences of *M. disstria* to polar extracts from aspen and from sun and shade sugar maple foliage and to perform feeding and electrophysiological tests with the constituents obtained from each polar extract and from further fractions of these extracts. We hypothesized that larvae would prefer the total extracts in the following order: aspen > sun maple > shade maple since aspen contains higher sugar levels, followed by sun maple, and then by shade maple. We also hypothesized that the methanol fraction whould be the least preferred since it contains mostly phenolic compounds, such as condensed tannins, which are generally believed to act more as deterrents than as phagostimulants (Feeny, 1976; Rhoades and Cates; 1976; Swain; 1979). Finally, we expected that the electrophysiological data would concur with the behavior.

METHODS AND MATERIALS

Insects. Malacosoma disstria larvae were obtained from the Great Lakes Forest Research Center in Sault-St-Marie, Ontario, Canada, and reared on an artificial diet (Addy, 1969) that was slightly modified as follows: raw linseed oil was replaced by wheat germ oil, and whole wheat germ was replaced by whole

wheat flour. We used 5 instead of 8.4 g of cholesterol, 50 instead of 84 g of alphacel, and 42 instead of 70 g of agar. We also added 1.7 g of methyl paraben, a preservative. Larvae were kept in Petri dishes (100 × 15 mm) in an incubator at 24°C and on a 16L:8D cycle. The Petri dishes were cleaned every two days and fresh diet was provided.

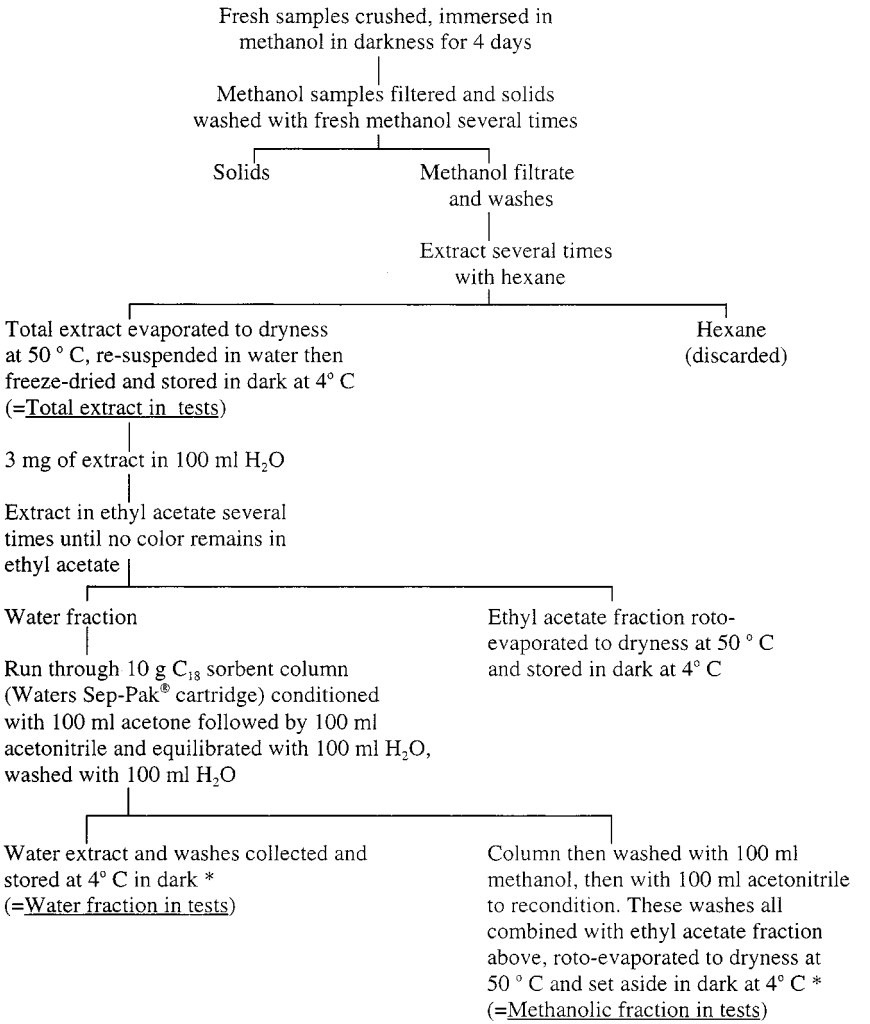
Foliage. Trembling aspen (*Populus tremuloides* Michx.) and sugar maple (*Acer saccharum* Marsh.) foliage was collected on May 18, 1999, at three different locations in southwestern Québec (the region of collection is delimited by the municipalities of Ste-Barbe, Dundee, and Huntington, approximately 60 km south of Montréal). Although collected at an early date, the foliage of both species was well developed and close to full expansion in the case of maple. The spring of 1999 was especially early. It was estimated that leaf age was at least two weeks, but leaves could have been as old as three weeks for some trees. Under natural conditions, leaves of that age are generally fed upon by fourth instar *M. disstria* larvae.

Aspen trees were either growing in small stands adjacent to the sampled maple stands, or they were growing directly at the edge of these. At each location, foliage was collected from two or three trees of each species. Sugar maple foliage was collected from both the top (sun leaves) and the bottom (shade leaves) of the crown. Samples of sun and shade leaves were kept separate. Foliage collected from aspen trees consisted of only sun leaves. In addition to the aspen trees sampled as above, other samples were also collected from an aspen stand in the Montréal area (Rivière-des-Prairies). In all cases, sampling was conducted between morning and early afternoon, and leaves were immediately placed on ice in a cooler. Upon arrival in the laboratory, small subsamples were weighed and placed in an airflow dryer, left to dry completely, and reweighed to calculate the water content (Table 1). The remainder of each sample was weighed and placed overnight in an −80°C freezer. The next day samples were retrieved and crushed into small bits while still frozen, and immediately placed to thaw in pure HPLC grade methanol (1:10 w/w). The extraction flasks were sealed and kept in the dark for four days, with frequent gentle stirring over this period.

Polar constituents from the foliage were obtained as shown in Figure 1. The samples in methanol were filtered, the solids were washed with fresh methanol several times, and the whole volume of methanol (filtrate and wash) was extracted several times with hexane until no appreciable change in color was visible in

TABLE 1. WET AND DRY WEIGHTS OF THREE TYPES OF FOLIAGE

	Aspen	Shade maple	Sun maple
Fresh weight (g)	821.8	768.4	853.6
Dry weight (g)	267.4	223.9	280.9
% Water content	67.5	70.9	67.1



* Note: these last steps were repeated 6 times.

FIG. 1. Procedure for the preparation of polar extracts from the three host plants.

the methanol. The hexane was discarded, and the methanol containing the polar constituents was roto-evaporated to complete dryness under reduced pressure at a temperature of 50°C. The dry extracts were dissolved in a minimal amount of bidistilled demineralized water (H_2O_{bddm}), and freeze-dried. The powdered extracts were collected, weighed, placed in brown-glass bottles, and stored at 4°C.

Extracts were further separated to obtain two fractions, one mainly containing soluble sugars, amino acids, and other highly polar constituents, and the other containing less polar constituents, such as tannins and flavonoids. First, a known amount of extract (ca. 3 mg) was dissolved in 100 ml H_2O_{bddm} and extracted with ethyl acetate as many times as was necessary to see no significant coloration in the ethyl acetate. All volumes of ethyl acetate were pooled, roto-evaporated to dryness at 50°C, and kept in the dark at 4°C. This procedure was performed to reduce the amount of constituents dissolved in water. The extracted volume of water was then run by gravity through a conditioned (100 ml acetone followed by 100 ml acetonitrile) and equilibrated (100 ml H_2O_{bddm}) column packed with 10 g of C_{18} sorbent (Waters Sep-Pak cartridge). The column was washed with 100 ml H_2O_{bddm}, which was collected and pooled with the previously collected volume of water containing the polar extract, and set aside in the dark at 4°C. The column was washed with 100 ml of methanol, followed by 100 ml of acetonitrile to recondition the column. These volumes were collected in the flask from the ethyl acetate extraction performed earlier, pooled, and set aside in the dark at 4°C. The column was once more equilibrated by running 100 ml H_2O_{bddm}, and the water fraction was run through the column again, followed by all the steps previously described. This procedure was repeated six times. At the end, the fraction containing the less polar constituents (the methanol fraction) was roto-evaporated to dryness at 50°C, resuspended in the appropriate volume of H_2O_{bddm}, and freeze-dried. The powdered methanol fraction was collected, weighed, placed in brown glass bottles, and stored at 4°C. The fraction containing the more polar constituents, (the water fraction) was roto-evaporated at 50°C to the final volume necessary to arrive at the concentration (w/w on a dry matter basis) present in the foliage at the time of collection. The water fraction was placed into a small opaque vial and stored at −20°C.

For the behavioral experiments, total extracts and their fractions were reconstituted in distilled water to attain a final concentration equivalent to that found in the foliage at the time of collection (dry weight). A drop of methanol was then added to the extracts and their fractions to increase solubilization. For the electrophysiology, concentrations were adjusted to foliar concentrations on a fresh weight basis, and all extracts and their fractions were prepared in a solution of potassium chloride (10 mM) to ensure adequate electrical conductance.

Behavior. All experiments were conducted in the morning, over a four-day period. For each day, 10–15 larvae were selected from the Petri dishes as mid-fifth instars and starved for one day prior to bioassays to ensure maximum response. They were transferred into an arena consisting of a two-choice situation arranged in the following manner: four Millipore HA 0.045-μm cellulose disks, 9 mm in diameter, were suspended individually on pins 0.5 cm above the floor of the arena. A 20-cm × 5-cm Petri dish served as a cover to the whole setup to keep the larvae from roaming about. Disks were wetted with 20-μl aliquots of extracts or

their fractions and arranged in an alternating manner with the water controls or the other extract solutions. This amount was just sufficient to saturate the whole disk. For every treatment, a minimum of 20 disks were used to determine the average dry weight of each disk containing a specific extract in water, or water alone, after all the water had evaporated. These average weights were used as the initial disk weight prior to feeding by larvae. The setup was transferred to an incubator maintained at the same growth conditions that the larvae had been exposed to, and larvae were allowed to feed for 4 hr, then removed from the arenas, after which time the disks were allowed to dry completely and weighed on a Sartorius single-pin balance to a precision of ± 0.001 mg. The amount of each disk (eaten) was calculated as the difference between the initial and final dry weights of the disk, and this value was used to express the mean surface area eaten of the test and control disks for each experiment. Only insects that ate $>50\%$ of the total disk area, control disks included, were considered for analysis. This cafeteria test was a modification of that of Jermy et al. (1968), and a similar setup is also seen in Albert and Jerrett (1981) and Albert and Parisella (1985). Statistical analysis was done with Number Cruncher Statistical Software (J. L. Hintze, 865 East North, Kaysville, Utah 84037, USA). Parametric tests were used since the data were normally distributed. Comparisons of the treatments versus. water or treatments among themselves were done by using a randomized two-sample *t* test ($P < 0.05$) to examine whether there was a preference between the two choices of the extracts tested. Data also were subjected to a one-way ANOVA ($P < 0.05$) to verify whether there were any differences among the three extracts, i.e., aspen and sun and shade maple.

Electrophysiology. All experiments were performed in the morning. About 10–15 mid-fifth instar larvae were selected from the Petri dishes prior to an experiment. Standard tip-recording was used (see Panzuto and Albert, 1997). The head and the first three thoracic segments were severed from the rest of the body of the larvae, and a glass microcapillary pipet containing insect saline (Schnuch and Hansen, 1989) was fitted into the opening and subsequently into the walls of the hypopharynx to expose the mouthparts. Special care was taken to avoid damaging the gustatory nerve. This preparation was placed onto a reference electrode under a compound microscope, with the larva ventral side up, to facilitate viewing and contact. A second glass micropipet was filled with a test solution. In preliminary experiments, we determined that the lateral styloconic sensilla of the galea did not respond to our extracts, while the medial styloconic sensilla consistently responded to all extracts. Action potentials were recorded for 1 sec on digital audio tape, digitized by using the SAPID Tools program (Smith et al., 1990), and then visually analyzed by counting the number of neural impulses from cell 1 (sugar-sensitive neuron), cell 2 (amino acid-sensitive neuron), and cell 3 (salt-sensitive neuron) in the traces. There were no cell 4 (water-sensitive neuron) responses to the stimuli used in our study. The physiological responses of cell 1 were analyzed

TABLE 2. RESPONSE (SURFACE AREA EATEN) IN TWO-CHOICE TESTS BY FIFTH-INSTAR *M. disstria* REARED ON ARTIFICIAL DIET TO THREE TOTAL EXTRACTS VS WATER

Extract	Surface area eaten (mm^2, mean ± SE)		P^a	N
	Treatment	Water		
Shade maple	268.5 ± 14.8	11.8 ± 7.7	0.0022	6
Sun maple	254.2 ± 15.7	1.4 ± 0.82	<0.0001	9
Aspen	287.2 ± 14.7	16.4 ± 12.16	<0.0001	14

[a] Two-sample *t* test.

statistically with a one-way ANOVA within the various extracts and their fractions as main effect. Another one-way ANOVA was performed to compare sugar cell response to the three extracts (i.e., aspen and sun and shade maple) and to their fractions (e.g., aspen and sun and shade water fractions). In addition, *a posteriori* tests (Newman-Keuls multiple comparison) were run to separate further the means. Statistical significance was accepted at $P < 0.05$ throughout.

RESULTS AND DISCUSSION

In two-choice feeding tests of total extracts of aspen, sun maple, or shade maple, larvae of *M. disstria* strongly and consistently preferred the disks containing extracts over those containing only water (Table 2). However, there was no difference in the responses to the three extracts (one-way ANOVA, $df = 2,26$; $F = 1.25$; $P = 0.3018$). When total extracts of the different hosts were tested against each other, sun maple was preferred over shade maple, and aspen was preferred over both sun and shade maple (Table 3), thus confirming our first prediction: aspen > sun maple > shade maple. These findings are consistent with our current knowledge of *M. disstria*'s performance on aspen compared to sugar maple foliage (Lorenzetti, 1993; Lindroth et al., 1993; Nicol et al., 1997). Aspen leaves have high soluble sugar levels during the larval growth period of *M. disstria*; these constitute about 8.5% of the dry matter of aspen leaves compared to <3.5% for sugar maple (Lorenzetti, 1993). Aspen also has fewer (3% of dry weight) condensed tannins (Bryant et al., 1987), compared to mature sugar maple (30% of dry weight) (Schulz, 1983), which the larvae would normally encounter while in their fifth instar in mid-June. Phenolic compounds, particularly phenolic glycosides, salicin, salicortin, tremuloidin, and tremulacin are common in aspen and constitute 2–6% of fresh leaf weight (Lindroth, 1991). Although sugar maple is not known to contain such compounds, 1-*O*-galloyl-α-L-rhammose has been identified in red maple, (Abou-Zaid and Nozzolillo, 1999). Nicol et al. (1997)

TABLE 3. RESPONSE (SURFACE AREA EATEN) IN TWO-CHOICE TESTS FOR FIFTH-INSTAR *M. disstria* REARED ON ARTIFICIAL DIET TO TOTAL EXTRACTS OF ONE HOST SPECIES VS ANOTHER

Extract	Surface area eaten (mm^2, mean ± SE) A	B	P^a	N
Shade maple (A) vs sun maple (B)	109.7 ± 22.8	223.3 ± 25.0	0.0021	10
Sun maple (A) vs aspen (B)	100.8 ± 23.2	253.1 ± 19.7	0.0007	9
Shade maple (A) vs aspen (B)	89.1 ± 19.2	239.2 ± 12.1	0.0001	13

[a] Two-sample *t* test.

measured the performance of *M. disstria* on aspen and on sugar and red maple foliage. They found that larvae reared on aspen ate smaller amounts of foliage than those reared on sugar maple, yet they grew 22.5 times the size of the latter. They also found that larvae refused to eat foliage of red maple, and all starved in less than two weeks. They concluded that red maple foliage contains one or more strong antifeedants and that sugar maple does not contain such antifeedants but has a "physiological growth inhibiting component." They argue that the effect of this component on reduced growth leads to compensatory feeding by larvae, thus accounting for the greater amounts of feeding on sugar maple foliage. A similar study in our lab also showed better growth, survival, and fecundity for larvae of *M. disstria* reared on aspen compared to sugar maple (Lorenzetti, 1993). We suggest that the higher amounts of condensed tannins in sugar maple and of soluble sugars in aspen could explain these observations through a combination of chemosensory as well as physiological effects. The sugar-sensitive cell in the medial styloconic sensillum of the galea gave the highest firing frequency to the water fraction from aspen, an intermediate response to the total extract, and the lowest response to the methanol fraction (Figure 2). During our extraction process, the first sugars to come out of the separation column in sugar maple are sucrose, then glucose, galactose, and finally fructose (Gougeon, personal communication). The methanol fraction should contain the aglycones, monoglycosides, condensed tannins, etc., and the latter, in particular, are known to reduce the digestibility of proteins in many insects (Swain, 1979). This is consistent with the argument of Nicol et al. (1997) that larvae show compensatory feeding on sugar maple and develop poorly compared to those fed on aspen foliage. Our electrophysiological results show that the water fraction from aspen provides the best stimulus for the sugar-sensitive cell in the medial styloconic sensillum, and the methanol fraction is the least stimulative.

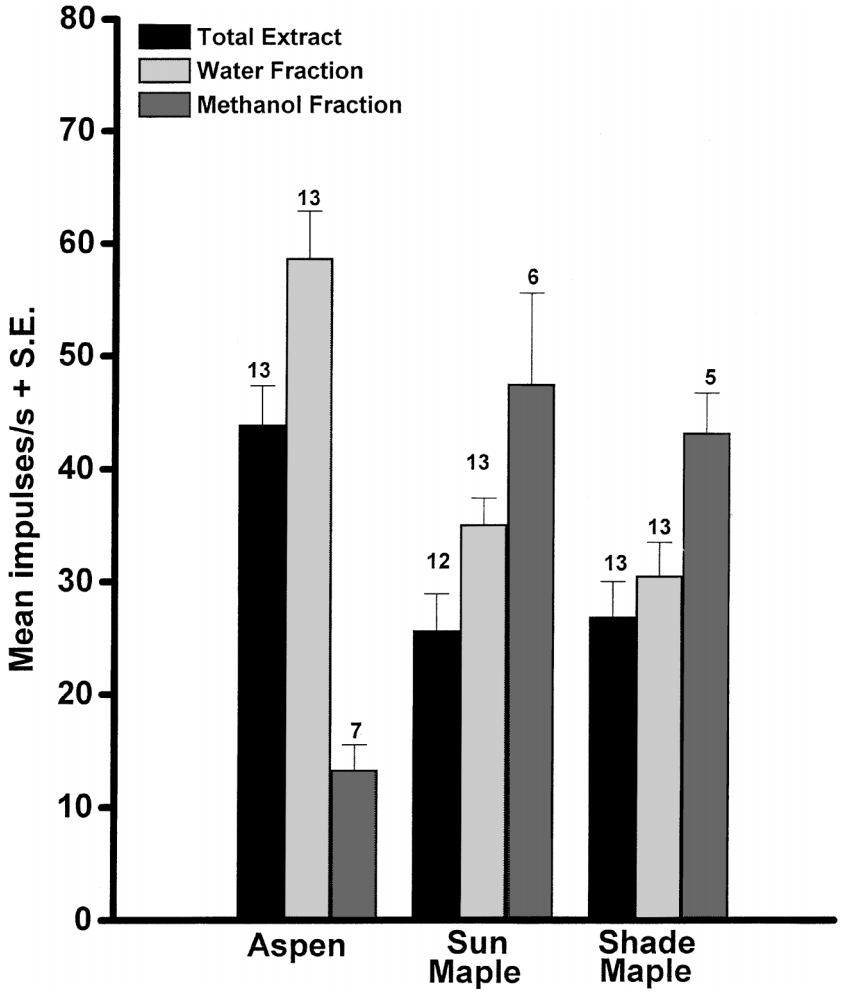


FIG. 2. Mean impulses/sec + SE for cell 1 in medial styloconic sensilla of fifth-instar *M. disstria* larvae in response to stimulation with aspen total extract and its water and methanol fractions (numbers above histograms represent sample size for each stimulus).

When we compared the responses of larvae to the total extract, water, and methanol fractions of the three types of foliage, the following trends were observed. Total extract of a given foliage was always preferred over the methanol fraction (Table 4). The water fraction was usually preferred over the methanol fraction, although not significantly so in the case of aspen. The total extract was preferred over the water fraction for aspen and for sun maple, but not for shade maple. The

TABLE 4. RESPONSE IN (SURFACE AREA EATEN) IN TWO-CHOICE TESTS FOR FIFTH-INSTAR *M. disstria* REARED ON ARTIFICIAL DIET TO TOTAL EXTRACTS AND THEIR RESPECTIVE FRACTIONS

Extract	Surface area eaten (mm^2, mean ± SE) A	B	P[a]	*N*
Aspen total extract (A) vs water fraction (B)	56.5 ± 14.1	29.6 ± 16.4	0.0123	10
Aspen total extract (A) vs methanol fraction (B)	101.6 ± 7.5	8.8 ± 5.0	<0.0001	12
Aspen methanol fraction (A) vs aspen water fraction (B)	6.6 ± 2.3	24.0 ± 7.3	0.1259	9
Sun maple total extract (A) vs sun maple water fraction (B)	57.2 ± 8.1	26.6 ± 11.3	0.0079	12
Sun maple total extract (A) vs sun maple methanol fraction (B)	40.4 ± 8.2	2.3 ± 1.3	<0.0001	15
Sun maple methanol fraction (A) vs sun maple water fraction (B)	6.9 ± 2.3	56.9 ± 5.4	<0.0001	15
Shade maple total extract (A) vs shade maple water fraction (B)	40.1 ± 4.6	36.1 ± 8.3	0.4792	11
Shade maple total extract (A) vs shade maple methanol fraction (B)	38.3 ± 5.6	6.0 ± 1.6	<0.0001	14
Shade maple methanol fraction (A) vs shade maple water fraction (B)	8.9 ± 1.8	40.0 ± 5.6	<0.0001	15

[a] Two-sample *t* test.

preference for total extracts over methanol suggests that the more polar compounds are most important in stimulating ingestion, supporting our second hypothesis. There are some subtle differences in the insects' responses to sun versus shade maple extracts, notably that shade maple total extract is not preferred over the water fraction. This foliage is presumably lacking, or has reduced levels, some of the phagostimulants such as sugars, amino acids, etc. compared to sun maple. When *M. disstria* larvae are reared on shade maple versus sun maple foliage, the result is lower pupal weights and longer developmental times (Fortin, 1994). These differences are attributed to the presence of higher levels of soluble sugars, that are responsible for the insect's better performance on sun maple foliage (Fortin et al., 1997; Fortin and Mauffette, in preparation). It is known that sun and shade maple leaves differ in biochemical properties (Lincoln and Mooney, 1984; Ellswoth and Reich, 1992; Dudt and Shure, 1994). Sun/shade differences in herbivory have also been observed in other insects (Janzen, 1975; Coley, 1983; Louda and Rodman, 1983; Louda et al., 1987; Fitzgerald, 1995). For example, the congener eastern tent caterpillar, *M. americanum*, will rarely attack black cherry leaves that are grown in shade (Fitzgerald, 1995). In fact, larvae reared on these leaves produce

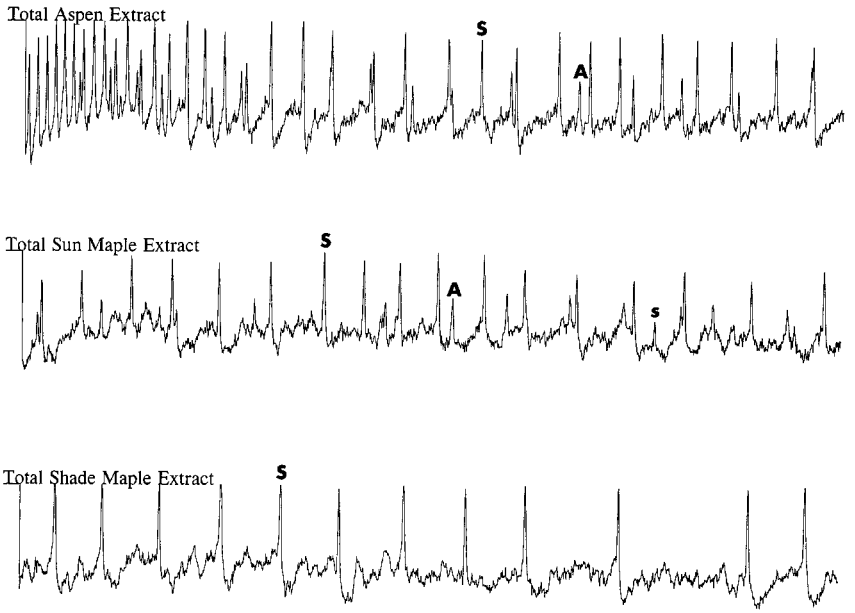


FIG. 3. Representative traces from stimulation of a medial styloconic sensillum of *M. disstria* with total aspen, sun maple and shade maple extracts. A = amino acid cell (cell 2); S = sugar cell (cell 1); s = salt cell (cell 3). All traces durations are 500 msec.

pupae that weigh only 67% of those reared on sun leaves (Futuyama and Saks, 1981).

Samples of the electrophysiological responses of the medial styloconic sensillum to the total extracts are shown in Figure 3. These reveal a response from three of the four neurons innervating this sensillum. These are cell 1 (sugar), cell 2 (amino acid), and cell 3 (salt), which can be differentiated from each other on the basis of their characteristic spike shapes. Cell 1 is monophasic (no prominent negative flank) and has a large amplitude, whereas cell 2 has a lower amplitude. Cell 3 spikes are biphasic, i.e., possess a steep negative flank, and have small amplitudes (Figure 3). In all of our tests, we found no effect of the extracts on the responses of cell 3 (salt), which fluctuated between 1 and 10 impulses/sec and were likely the result of stimulation by the 10 mM KCl solvent present in all solutions. A similar effect was seen for the responses of cell 2 (amino acid), whose means were only 4–8 impulses/sec for all aspen extracts, 8–13 impulses/sec for maple total extract and water fractions, and 19 to 23 impulses/sec for the maple methanol fractions. In only one case, the shade methanol fraction, did cell 2 respond with a higher firing frequency that was different from total extract and water fractions of that foliage; but when we compared the responses of cell 2 for each of the sun and shade maple

extracts, they were not different. However, the responses from cell 1 (sugar) were consistently much higher, ranging from 13 to 59 impulses/sec, and accounted for most of the sensillum's response.

The highest firing frequency (59 impulses/sec) from cell 1 (sugar) was obtained with the aspen water fraction (Figure 2), and the total extract and methanol fraction from aspen produced intermediate and mimimum firing rates, respectively. These responses were all different from each other (one-way ANOVA; $df = 2, 30$; $F = 29.15$; $P = <0.001$ and Newman-Keuls multiple comparison). For sun maple, maximal responses from cell 1 (49 impulses/sec) were obtained with the methanol fraction; intermediate and minimum responses were obtained with the water fraction and the total extract, respectively. The response to the methanol fraction differed from the others (one-way ANOVA; $df = 2, 28$; $F = 6.37$; $P = 0.0052$). An a posteriori Newman-Keuls test showed no difference between the responses to the total sun maple extract and the water fraction. A similar response was found for shade maple with the methanol fraction eliciting maximal firing from cell 1. Again this differed from the responses to total extract and water fraction (one-way ANOVA; $df = 2, 28$; $F = 4.29$; $P = 0.0237$), while the latter two did not differ from each other.

As stated earlier, cell 1 response is lowest to the methanol fraction for aspen, and this concurs with the feeding behavior. However, cell 1 response is highest for the aspen water fraction, and this differs from the behavior where the insect preferred the aspen total extract over the water fraction. This suggests that cell 1 is contributing important information, but that a sensory input other than cell 1 in the medial styloconic sensillum is also responsible for modulating this response. The latter is even more probable in the case of the electrophysiological responses to sun versus shade maple. The lower responses of cell 1 to the total extracts and the water fractions of both sun and shade maple compared to aspen is consistent with the behavioral data, i.e., that larvae prefer total extracts from aspen over the others. This is consistent with the literature, which shows that larvae perform best on aspen. However, we cannot explain the high firing frequencies of cell 1 to both maple methanol fractions, and we are not aware of any compounds in these fractions that might have the required binding properties necessary to stimulate this sugar-sensitive cell.

Both behavioral and physiological experiments indicate that aspen is the preferred choice for *M. disstria* larvae. We find it intriguing that cell 1 in the medial styloconic sensillum of *M. disstria* larvae was the only chemosensory neuron to consistently show differences in response to the various aspen extracts, since these extracts must contain a wide variety of chemicals. Larvae also distinguished between sun and shade maple extracts in the feeding tests, yet the electrophysiological responses from cell 1 to the extracts from these two types of foliage were almost identical. It is generally assumed that the sensory input from both lateral and medial styloconic sensilla of caterpillars is the most important in informing the

animal as to its correct food plant, as was clearly shown in the tobacco hornworm, *Manduca sexta* (De Boer, 1993). This is not the case in the forest tent caterpillar; the preference for sun over shade maple, for example, is likely to be mediated by another chemosensory field such as the epipharyngeal sensilla or the maxillary palps. More work is needed before we can fully understand the effects of these host plant chemicals on chemosensory inputs and the feeding behavior of *M. disstria* larvae.

REFERENCES

ABOU-ZAID, M. M. and NOZZOLILLO, C. 1999. 1-*O*-Galloyl-α-L-rhammose from *Acer rubrum. Phytochemistry* 52:1629–1631.

ADDY, N. D. 1969. Rearing of the forest tent caterpillar on an artificial diet. *J. Econ. Entomol.* 62:270–271.

ALBERT, P. J. and JERRETT, P. A. 1981. Feeding preferences of spruce budworm (*Choristoneura fumiferana* Clem.) larvae to some host plant chemicals. *J. Chem. Ecol.* 7:391–402.

ALBERT, P. J. and PARISELLA, S. 1985. Tests for induction of feeding preferences in larvae of eastern spruce budworm using extracts from three host plants. *J. Chem. Ecol.* 11:809–817.

ALBERT, P. J. and PARISELLA, S. 1988. Physiology of a sucrose-sensitive cell on the galea of the eastern spruce budworm larvae, *Choristoneura fumiferana. Physiol. Entomol.* 13:243–247.

BALDWIN, I. T., SCHULTZ, J. C., and WARD, D. 1987. Patterns and sources of leaf tannin variation in yellow birch (*Betula allengheniensis*) and sugar maple (*Acer saccharum*). *J. Chem. Ecol.* 13:1069–1078.

BRYANT, J. P., CLAUSEN, T. P., REICHARDT, P. B., and WERNER, R. A. 1987. Effect of nitrogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix [*Choristoneura conflictana* (Walker)]. *Oecologia* 73:513–517.

COBBINAH, J. R., MORGAN, F. D., and DOUGLAS, T. J. 1982. Feeding responses of the gum leaf skeletoniser *Uruba lugens* Walker to sugars, amino acids, lipids, sterols, salts, vitamins and certain extracts of eucalyptus leaves. *J. Aust. Entomol. Soc*. 21:225–236.

COLEY, P. D. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 53:209–233.

DE BOER, G. 1993. Plasticity in food preference and diet-induced differential weighting of chemosensory information in larval *Manduca sexta. J. Insect Physiol.* 39:17–24.

DEL CAMPO, M. L. and RENWICK, J. A. A. 1999. Dependence on host constituents controlling food acceptance by *Manduca sexta* larvae. *Entomol. Exp. Appl.* 93:209–215.

DUDT, J. F. and SHURE, D. J. 1994. The influence of light and nutrients on foliar phenolics and insect herbivory. *Ecology* 75:86–98.

ELLSWORTH, D. S. and REICH, P. B. 1992. Water relations and gas exchange of *Acer saccharum* seedlings in contrasting natural light and water regimes. *Tree Physiol.* 10:1–20.

FEENY, P. 1976. "Biochemical Interactions Between Plants and Insects." *Recent Adv. Phytochem.* 10:1–40.

FITZGERALD, T. D. 1995. The Tent Caterpillars. The Cornell Series in Arthropod Biology. G. C. Eickwort (ed.). Cornell University Press, Ethaca, New york, 303 p.

FORTIN, M. 1994. Les stress environnementaux: Effets indirects sur la biologie et le comportement alimentaire de la livrée des forêts (*Malacosoma disstria* Hbn.). Master's thesis. UQÀM, Montreal.

FORTIN, M., MAUFFETTE, Y., and ALBERT, P. J. 1997. The effects of ozone-exposed sugar maple seedlings on the biological performance and the feeding preference of the forest tent caterpillar (*Malacosoma disstria* Hbn.). *Environ. Pollut.* 97:303–309.

FRAZIER, J. L. and HANSON, F. E. 1986. Electrophysiological recording and analysis of insect chemosensory responses, pp. 286–330, *in* J. R. Miller and T.A. Miller (eds.). Insect–Plant Interactions. Springer-Verlag, New York.

FUTUYAMA, D. J. and SAKS, M. E. 1981. The effect of variation in host plant on the growth of an oligophagous insect, *Malacosoma americanum* and its polyphagous relative, *Malacosoma disstria*. *Entomol. Exp. Appl.* 30:163–168.

GUERTIN, C. and ALBERT, P. J. 1992. Feeding preferences and feeding rate of two instars of spruce budworm (Lepidoptera: Tordtricidae) in response to pure sucrose and sugar extracts of white spruce. *For. Entomol.* 85:2317–2322.

HANSON, F. E. and DETHIER, V. G. 1973. Role of gustation and olfaction in food plant discrimination in the tobacco hornworm *Manduca sexta*. *J. Insect Physiol.* 19:1019–1034.

HODSON, A. C. 1941. An ecological study of the forest tent caterpillar, *Malacosoma disstria* Hbn., in northern Minnesota. Tech. Bull. Univ. Minn. Agric. Exp. Stn. No. 148.

JANZEN, D. H. 1975. Intra- and interhabitat variations in *Guazuma ulmifolia* (Sterculiaceae) seed predation by *Amblycerus cistelinus* (Bruchidae) in Costa Rica. *Ecology* 56:1009–1013.

JERMY, T., HANSON, F. E., and DETHIER, V. G. 1968. Induction of specific food preferences in lepidopterous larvae. *Entomol. Exp. Appl.* 11:211–230.

LIN, S., BINDER, B. F., and HART, E. R. 1998a. Chemical ecology of cottonwood leaf beetle adult feeding preferences on *Populus*. *J. Chem. Ecol.* 24:1791–1902.

LIN, S., BINDER, B. F., and HART, E. R. 1998b. Insect feeding stimulants from the leaf surface of *Populus*. *J. Chem. Ecol.* 24:1781–1790.

LINCOLN, D. E. and MOONEY, H. A. 1984. Herbivory on *Diplacus aurantiacus* shrubs in sun and shade. *Oecologia* 64:173–176.

LINDROTH, R. L. 1991. Biochemical ecology of aspen-lepidoptera interactions. *J. Kans. Entomol. Soc.* 64:372–380.

LINDROTH, R. L. and HEMMING, J. D. C. 1990. Responses of the gypsy moth (Lepidoptera: Lymantriidae) to tremulacin, an aspen phenolic glycoside. *Environ. Entomol.* 19:842–847.

LINDROTH, R. L., HSIA, M. T. S., and SCRIBER, J. M. 1987. Characterization of phenolic glycosides from quaking aspen (*Populus tremuloides*). *Biochem. Syst. Ecol.* 15:677–680.

LINDROTH , R. L., KINNEY, K. K., and PLATZ, C. L. 1993. Responses of deciduous trees to elevated atmospheric CO_2 : Productivity, phytochemistry, and insect performance. *Ecology* 74:763–777.

LORENZETTI, F. 1993. Performances relatives de la livrée des forêts *Malacosoma disstria* Hbn. sur l'érable à sucre *Acer saccharum* Marsh. sain et dépéri et sur le peuplier faux-tremble *Populus tremuloides* Michx. en relation avec la chimie foliare. Master's thesis. UQÀM, Montreal.

LOUDA, S. M. and RODMAN, J. E. 1983. Variation in glucosinolate concentration in relation to habitat and insect herbivory for the native crucifer, *Cardamine cordifolia* (bittercress). *Biochem. Syst. Ecol.* 11:199–208.

LOUDA, S. M., HUNTLY, N., and DIXON, P. M. 1987. Insect herbivory across a sun/shade gradient: response to experimentally-induced in situ plant stress. *Acta Oecol.* 8:357–363.

MA, W. C. 1972. Dynamics of feeding responses in *Pieris brassicae* Linn. as a function of chemosensory input: A behavioural, ultrastructural and electrophysiological study. *Meded. Landbouwhogesch. Wageningen* 72:1–162.

NICOL, R. W., ARNASON, J. T., HELSON, B., and ABOU-ZAID, M. M. 1997. Effect of host and non-host trees on the growth and development of the forest tent caterpillar, *Malacosoma disstria* (Lepidoptera: Lasiocampidae). *Can. Entomol.* 129:991–999.

OHSUGI, T., HIDAKA, I., and IKEDA, M. 1978. Taste receptor stimulation and feeding behaviour in the puffer, *Fugu pardalis.* II. Effects produced by mixtures of constituents of clam extracts. *Chem. Senses Flavour* 3:355–368.

PANZUTO, M. and ALBERT, P. J. 1997. Different sensitivities of the sugar receptor of the lateral styloconic sensillum in fourth- and sixth-instar larvae of the spruce budworm *Choristoneura fumiferana. Entomol. Exp. Appl.* 82:335–340.

RHOADES, D. F. and CATES, R. 1976. Towards a general theory of plant antiherbivore chemistry. *Recent Adv. Phytochem.* 10:168–231.

ROWE, J. S. 1972. Forest Regions of Canada. Dept. Environ., Can. For. Serv. Publ. No. 1300, 172 p.

SCHNUCH, M. and HANSEN, K. 1989. Sugar reception by the salt receptor of the taste hairs of the fly *Protophormia terraenovae*, p. 7, *in* N. Elsner and W. Singer (eds.). Dynamics and Plasticity in Neuronal Systems. Proceedings 17th Gottingen Neurobiology Conference. Verlag, Stuttgart.

SCHULTZ, J. C. 1983. Tree tactics. *Nat. Hist.* 92:12–25.

SCHOONHOVEN, L. M. 1967. Chemoreception of mustard oil glucosides in larvae of *Pieris brassicae. Proc. K. Ned. Akad. Wet. C.* 70:556–568.

SCHOONHOVEN, L. M. 1987. What makes a caterpillar eat? The sensory code underlying feeding behaviour, pp. 69–97 *in* R. F. Chapman, E. A. Bernays, and J.G. Stoffolano, Jr. (eds.). Proceedings in Life Sciences. Perspectives in Chemoreception and Behaviour. Springer Verlag, Berlin.

SCHOONHOVEN, L. M. and BLOM, F. 1988. Chemoreception and feeding behaviour in a caterpillar towards a model of brain functioning in insects. *Entomol. Exp. Appl.* 49:123–129.

SCHOONHOVEN, L. M., SIMMONDS, M. S. J., and BLANEY, W. M. 1991. Changes in the responsiveness of the maxillary styloconic sensilla of *Spodoptera littoralis* to inositol and sinigrin correlate with feeding behaviour during the final larval stadium. *J. Insect Physiol.* 37:271–268.

SHI-BAO, J., SAITO, N., YOKOI, M., SHIGIHARA, A., and HONDA, T. 1992. Galloylcyanidin glycosides from *Acer. Phytochemistry* 31:655–657.

SMITH, J. J. B., MITCHELL, B. K., ROLSETH, B. M., WHITEHEAD, A. T., and ALBERT, P. J. 1990. Sapid Tools: Microcomputer programs for analysis of multi-unit nerve recordings. *Chem. Senses* 15:253–270.

SWAIN, T. 1979. Tannins and lignins, pp. 657–682 *in* Herbivores. Their Interaction with Secondary Plant Metabolites. G. A. Rosenthal and D. H. Janzen (eds.). Academic Press, San Diego, California.

TOUFEXIS, D. J., ALBERT, P. J., and BAUCE, E. 1996. Age-related responses from the maxillary sensilla styloconica of *Choristoneura fumiferana* to foliage extracts from balsam fir hosts. *Entomol. Exp. Appl.* 78:129–133.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

TRADE-OFF BETWEEN CHEMICAL AND BIOTIC ANTIHERBIVORE DEFENSE IN THE SOUTH EAST ASIAN PLANT GENUS *Macaranga*

GERO ECK,[1] BRIGITTE FIALA,[2] KARL EDUARD LINSENMAIR,[2] ROSLI BIN HASHIM,[3] and PETER PROKSCH[1,*]

[1]*Institut für Pharmazeutische Biologie*
Heinrich-Heine-Universität Düsseldorf
Universitätsstrasse 1, Geb. 26.23
D-40225 Düsseldorf, Germany

[2]*Lehrstuhl für Tierökologie und Tropenbiologie*
Bayrische Julius-Maximilians-Universität Würzburg
Biozentrum, Am Hubland
D-97074 Würzburg, Germany

[3]*Department of Biology*
University of Malaya
50603 Kuala Lumpur, Malaysia

(Received January 24, 2001; accepted June 13, 2001)

Abstract—The plant genus *Macaranga* is known for its manifold mutualistic associations with ants. The plants provide food for the ants and in turn get protection from herbivores. Depending on the strength of the plant–ant interaction, the plant's investment in ants and the biotic defense derived from them is more or less effective. We conducted a comparative study on tannin content in 12 *Macaranga* species that were selected based on their associations with ants (three nonmyrmecophytes and nine myrmecophytes, three of which start their ontogeny as nonmyrmecophytes). Different developmental stages were investigated in three *Macaranga* species. Extracts of every individual plant analyzed for tannins were also tested for their effects on larval growth employing larvae of the common cutworm (*Spodoptera littoralis*). The studied *Macaranga* species differed significantly in their tannin contents as well as in the effects of their leaf extracts on the growth of *S. littoralis* larvae. A correlation analysis shows a connection between tannin contents and larval growth. High tannin contents and, thus more effective chemical defense, were observed in nonmyrmecophytic *Macaranga* species associated only facultatively with ants as compared to obligate myrmecophytes. Our study supports the hypothesis of a trade-off between chemical and biotic defense in the genus *Macaranga*.

Key Words—Ant–plant associations, biotic defense, chemical defense, herbivory, *Macaranga*, tannins.

*To whom correspondence should be addressed.

INTRODUCTION

Plants face pronounced biotic pressure from phytophagous animals (e.g., Marquis and Braker, 1994) and have, therefore, evolved effective biotic and/or abiotic (physical and chemical) means to counter these pressures in order to enhance their fitness (e.g., Adler, 2000). The chemical defenses of plants are highly diverse (e.g., Rosenthal and Berenbaum, 1991). A widespread form of indirect (biotic) defense is mutualistic interactions with ants (Hölldobler and Wilson, 1990; Davidson and McKey, 1993) that defend plants from herbivores (e.g., Schupp, 1986; Fiala et al., 1989, 1991; Gaume et al., 1997; Gaume and McKey, 1999), from plant pathogens such as fungi (Letourneau, 1998; Heil et al., 1999, 2000, 2001) and, in some cases, even from other competing neighboring plants (Fiala et al., 1989, 1991). The ants, in turn, benefit from food [extrafloral nectar (for an overview see: Beattie, 1985; Huxley and Cutler, 1991)] or food bodies produced by the plants (e.g., Rickson, 1980; Fiala and Maschwitz, 1992a; Heil et al., 1997).

The genus *Macaranga* (Euphorbiaceae) consists of approximately 250 mainly pioneer species that occur in the tropics of the Old World (Whitmore, 1969, 1975) with the center of distribution in (sub-)tropical Asia and the Pacific (Whitmore, 1969, 1975; Fiala, 1996). This genus is known for a wide range of mutualistic associations with ants, ranging from facultative to strictly obligate ones (Fiala et al., 1989; Fiala and Maschwitz, 1990). In the nonmyrmecophytic (but myrmecophilic) species, different opportunistic ant species visit the plants and forage on food bodies and extrafloral nectar. The plants in turn may gain protection due to the ants' foraging behavior. Myrmecophytic species not only produce food but also permanently house specialized ants in their internodes (so-called domatia) (Fiala and Maschwitz, 1992b). Here the interaction is obligate for both partners. The ants protect the plants from phytophagous insects and probably from plant pathogens (Fiala et al., 1989, 1994; Heil et al., 1999, 2001). A few *Macaranga* species change their life-style from myrmecophilic to myrmecophytic at later ontogenetic stages. This change is accompanied by morphological and physiological alterations. Small plants with thin, solid stems mainly secrete extrafloral nectar that attracts different ant species. These species can be colonized by their specific ants only when the stem diameters increase (e.g., in the case of *M. hosei*, at a height of approximately 70 cm). Colonizable plants subsequently start to produce food bodies and usually reduce the production of extrafloral nectar (Fiala, 1996; Fiala et al., 1999). The efficacy of the biotic defense of these different life styles seems to be most pronounced in myrmecophytic species (Fiala et al., 1994; Heil et al., 2001).

Both means of antiherbivore defense (abiotic defense with physical or chemical means and the biotic defense via mutualistic partners) are metabolically costly for the plants. For maintenance of the biotic defense, especially the production of food bodies with their high content of lipids (Rickson, 1980), amino acids and energy-rich carbohydrates (Heil et al., 1998) have to be provided by the plants.

Consequently, the number, biomass, and quality of food bodies differs among *Macaranga* species and is highest in the myrmecophytic species (Heil et al., 1998). For example, Heil et al. (1997) found an investment in food bodies that accounts for up to 5% of the total assimilation of myrmecophytic *Macaranga triloba* plants. The production of Müllerian bodies in the neotropical ant-plant *Cecropia* also causes significant costs to the plants (Janzen, 1973).

Costs involved in the production of defensive compounds can not be accurately quantified. It is reasonable to assume, however, that the production of secondary metabolites reduces the amounts of nutrients and photosynthetic products that otherwise could promote growth and reproduction (Simms and Rausher, 1989). Rhoades (1979) postulates a balance between the costs for defense and the ecological benefit of reduced herbivory. Plants with effective biotic defense are expected to reduce their investment in abiotic (chemical) defense mechanisms since an investment in both would be an unnecessary metabolic burden on the plant (Janzen, 1966; Gershenzon, 1994). Some studies provide support for this assumption. In neotropical acacias, Rehr et al. (1973) found lower contents of cyanogenic glycosides in myrmecophytic species in comparison to nonmyrmecophytic ones (but see also Seigler and Ebinger, 1987). Another potential trade-off between chemical and biotic defenses in neotropical myrmecophytic acacias has been demonstrated by Heil et al. (2000), who reported reduced chitinase activity in the myrmecophytes *M. triloba* and *M. hosei* compared to other plant taxa. In addition, leaves of the myrmecophytic *Leonardoxa africana* had lower tannin contents than those of the nonmyrmecophytic *L. gracicaulis* (Bidal, 1997). According to Janzen (1973), in the neotropical *Cecropia*, the lack of latex-bearing ducts is probably one of the reasons for the herbivore susceptibility following ant removal experiments.

In the genus *Macaranga*, there is almost no information regarding a possible reduction of chemical defense as a result of the symbiotic relationship with ants. However, regarding resource allocation by the plant into effective means of biotic defense, such as food bodies or extrafloral nectar, a high investment of the nonmyrmecophytes into chemical defense should be expected. Turner (1995), for example, found lower contents of condensed tannins in two myrmecophytes (*M. hypoleuca* and *M. triloba*) as compared to three nonmyrmecophytic species employed in this investigation (*M. conifera, M. gigantea*, and *M. heynei*). Nomura et al. (2000) reported a less pronounced abiotic defense in three myrmecophytic species compared to two nonmyrmecophytes, as indicated by the growth performance of cutworm larvae on an artificial diet spiked with the respective leaf powders. In the same publication, the authors also determined the intensity of physical defense of *Macaranga* species by measuring leaf toughness of selected species. Again, the nonmyrmecophytes had tougher leaves than the myrmecophytes. In a feeding experiment with fresh leaves, only two of the myrmecophytes allowed the development of pupae (Nomura et al., 2000). The diet

containing plant material of the other *Macaranga* species analyzed proved lethal to the larvae. These data support the hypothesis of a more pronounced abiotic defense in the nonmyrmecophytes compared to myrmecophytic species of the genus *Macaranga*. However, no broad comparative phytochemical analyses exist within the genus *Macaranga*. For the first time, in this study we tested for secondary compounds with relevance to antiherbivore defense using 12 *Macaranga* species, which included three nonmyrmecophytes and nine myrmecophytes (three of which started their ontogeny as nonmyrmecophytes). In this study, we focused on tannins, which have previously been shown to be among the secondary compounds accumulated by *Macaranga* species (Lin, 1993, 1994; Lin et al., 1990a,b).

METHODS AND MATERIALS

Sampling Sites and Selected Species. Leaves for phytochemical investigation were collected in Malaysia between September and December 1998. Six species were collected in the area around the Ulu Gombak Field Studies Centre of the University of Malaya northeast of Kuala Lumpur (3°17′ N, 101°44′ E): the nonmyrmecophytes *M. gigantea*, *M. heynei*, *M. tanarius*, and the myrmecophytic *M. hullettii*, *M. triloba*, and *M. hosei* (the latter being colonized only later in its ontogeny). (*M. triloba* probably has to be renamed *M. bancana*, T. C. Whitmore and S. J. Davies, personal communication.) Plants were mainly found along former logging roads in secondary forest and along the road to Genting Highlands. *M. pruinosa*, which also becomes inhabited by ants at a later stage, was obtained from a peat swamp forest in the north of Kuala Selangor (3°24′ N, 3°44′E). In the eastern part of Malaysia in the Mount Kinabalu park in Sabah, Borneo (6°5′N, 160°33′E), four obligate myrmecophytic species [*M. angulata* (Davies, 1999), *M. hypoleuca*, *M. indistincta*, *M. winkleri*] as well as one species with changing life-style (*M. pearsonii*) were collected. Plants were identified following Whitmore (1975). We used the youngest two to six leaves of 1.5- to 2.5-m-high plants. In three species, additional ontogenetical comparisons were carried out. From the species with the changing life-style (*M. hosei* and *M. pruinosa*), we also analyzed young saplings that were not yet inhabited by ants and young trees (*M. pruinosa* only). In addition, we studied trees (6–12 m high) of *M. tanarius*, which at that developmental stage produce no more extrafloral nectaries. For the collected species, leaves from at least 10 individuals (with the exception of the grown-up trees and not colonizable plants of *M. pruinosa* where five individuals were collected) were combined for subsequent phytochemical studies.

Myrmecophytes that start their ontogeny as nonmyrmecophytes and are, therefore, colonized later by ants are referred to as late myrmecophytes, compared to the early myrmecophytes, which are already colonized as small saplings.

Processing of Plant Material. Fresh plant material was dried in a drying oven (40–45°C) on the peninsula and under a fan in Borneo. It was subsequently stored in air-tight Ziploc bags over silica gel. Dried leaf material was ground with a mill and sieved. Nerves of bigger leaves were removed prior to grinding.

Tests for Alkaloids, Cyanogenic Glycosides, and Saponins. To test for other classes of secondary compounds that might interfere with development of insects, quick tests for alkaloids, cyanogenic glycosides, and saponins were conducted. For alkaloids, dried and powdered plant material of one individual of each collected species was extracted with 20 ml sulfuric acid over night. After filtration, the extract was alkalized with 25% NH_3 (pH 10–11). Extrelut 3 columns (Merck) were used for eluation of alkaloids with dichloromethane. Thin-layer chromatography (silica gel 60 F254, 0.2 mm; Merck) with Dragendorff spray reagent (German Pharmacopoea, DAB 9) was used to detect alkaloids. To test for cyanogenic glycosides, the sodium picrate test (Brinker and Seigler, 1989) was used on fresh plant material of one individual of each species. To test for hemolytic saponins, a blood agar test was used on three individuals of each species.

Tannin Quantification. For tannin quantification, the skin-powder method of the German Pharmacopoea (DAB 7) in combination with photometric quantification using Folin's reagent was used in a modified way as follows: 0.75 g plant powder of each individual was extracted with 150 ml hot water (in a waterbath at 90°C) for 30 min. After cooling to room temperature, the extract was diluted to 250 ml and filtered. The first 50 ml of each filtrate was rejected in order to allow for saturation of the filters with tannins. For the determination of total phenolics, 5 ml of the remaining filtrate was diluted with water to 25 ml. To 0.5 ml of this solution, 100 μl wolframium phosphoric acid was added, and the mixture was diluted to 5 ml with sodium carbonate solution (15%). After 1.5 min, the absorption of the solution was measured at λ 715 nm. In the same way, the absorption of the remaining phenolics was measured after binding the tannins to skin powder. 10 ml extract was shaken with 0.1 g skin powder CRS (European Directorate for the Quality of Medicines, Strasbourg, France) for 1 hr. For each extract, the content of total phenolics and phenolics, after removal of tannins, was determined three times. Pyrogallol was used as reference. Pyrogallol (50 mg) was dissolved in 100 ml water. 5 ml of this solution were diluted to 100 ml with water, all under light exclusion due to the light sensitivity of the compound. To 5 ml of the resulting solution, 1 ml wolframium phosphoric acid and sodium carbonate solution was added to give a total of 50 ml. After 1.5 min, the absorption at λ 715 nm was measured. Absorption of pyrogallol was measured three times for each determination of tannin content.

The content of the leaf tannin ($T = 13.12 * (A_1 - A_2)/A_R * m$) was calculated from the absorption of the total phenolics (A_1), the remaining phenolics (A_2), the reference substance (A_R), and the mass of plant material (0.75 g leaf powder) (m).

In addition, the content of tannins present in two methanolic plant extracts was measured. Two plants (one *M. heynei* and one *M. triloba* with tannin contents of 30.17% and 2.83%, respectively, as determined in the water extract) were used. From each plant, 0.75 g powdered leaf material was extracted with an adequate amount of methanol over 12 hr. The dried methanolic extract was processed by using the regular skin-powder method for tannin quantification (see above). With this method the *M. heynei* plant specimen showed a content of 23.69%, whereas virtually no tannins were detected in the extract of *M. triloba*.

To prove the linearity of the applied method for tannin quantification, the water extract of one plant individual was dissolved with water to 25, 50, and 75% of the original concentration. Each concentration was tested in triplicate with the skin-powder method as described above. This dilution experiment showed a linear correlation between tannin content and the result of the quantification in the whole range of results between less than 3% and more than 30% tannins (linear Spearman Rank correlation; $r = 0.99877$; $N = 4$; coefficient: 1.0; $P < 0.01$) and thereby proved the suitability of the method.

Extraction for Bioassay. Depending on the amount of plant powder available, 0.5, 1.0, 2.0, or 5.0 g of plant material was extracted three times with 20, 40, 50, or 100 ml methanol, respectively. The leaf material of several *M. hosei* saplings had to be combined to get enough crude extract for the bioassay.

Bioassay. For the bioassay, newly hatched caterpillars of the common cutworm *Spodoptera littoralis* (so-called neonates) were used. These polyphagous pest insects were reared in the laboratory on an artificial diet under controlled conditions (see Srivastava and Proksch, 1991). A known amount of artificial diet was treated with the equivalent of 10 mg of crude extract. Each plant individual was tested with 20 neonates, which fed on the prepared food for eight days. Afterwards, the surviving caterpillars were counted and weighed. The survival and growth rates of the test caterpillars were compared with control larvae that had been exposed to diet treated with the solvent (methanol) only. Of the seedlings of *M. hosei*, only a pool of the collected leaves could be tested with *Spodoptera* due to the small amount of plant material. The same pool of dried leaf powder was extracted three times, and each of the extracts was also tested three times.

In one experiment, the tannin content of the extracts of two plants with a relatively strong effect on the growth rates of the caterpillars (one *M. triloba* and *M. winkleri*) was removed with skin powder prior to incorporation into the diet. The crude extract equivalent to 0.3 g plant powder was extracted with 60 ml hot water (following the protocol of the skin-powder method with a smaller amount). After centrifugation, the water fraction was filled with water to give 100 ml, and 1 g skin powder was added and shaken for 60 min. After filtration the residue was combined with the remaining methanol residue and freeze dried. For the bioassay with *Spodoptera* larvae, 10 mg of each of the two extracts were used and tested three times.

As positive control, amounts of 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 mg pure tannic acid were tested (three times each) with the bioassay. As expected, the pure tannic acid in the artificial diets (used instead of plant extracts) also had an negative effect on the growth of *Spodoptera*. The effect was dependent on the concentration ($r = 0.98626$) with a nonlinear course.

Statistical Analysis. The tannin contents of the *Macaranga* plants and the growth rates of the *Spodoptera* larvae in the bioassay were compared by using a univariate ANOVA with the LSD post-hoc test. Correlations between the measured tannin content and the effects of the plant extracts on the growth of the larvae were analyzed with Spearman Rank correlation.

RESULTS

Tannin Quantification. Analysis revealed significant differences among the various species analyzed with regard to tannin contents (Figure 1, univariate ANOVA: $N = 149$, $P < 0.001$). The nonmyrmecophytic *M. heynei*, in particular, had strikingly high tannin contents (up to 33% of the dry weight). However, the

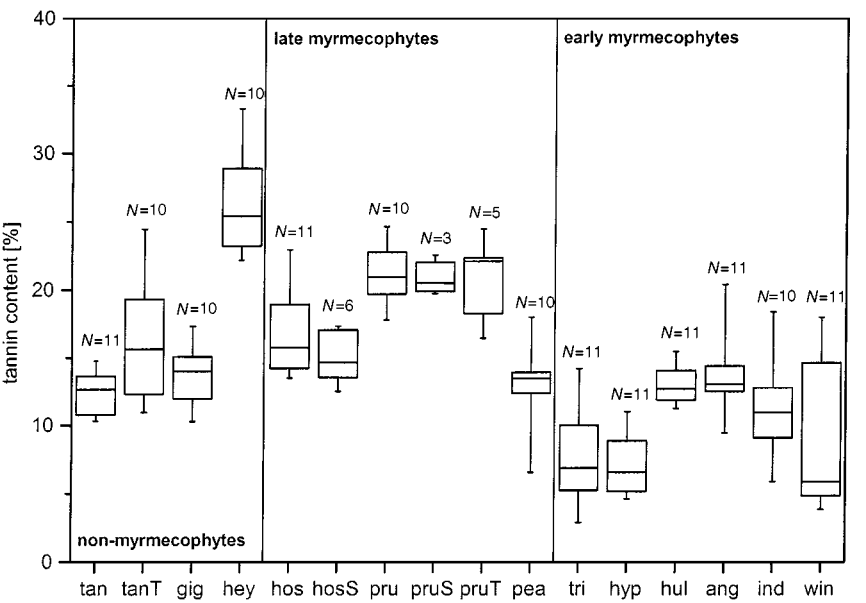


FIG. 1. Tannin contents of the investigated *Macaranga* species: tan = *M. tanarius*; gig = *M. gigantea*; hey = *M. heynei*; hos = *M. hosei*; pru = *M. pruinosa*; pea = *M. pearsonii*; tri = *M. triloba*; hyp = *M. hypoleuca*; hul = *M. hullettii*; ang = *M. angulata*; ind = *M. indistincta*; win = *M. winkleri*; T = adult trees; S = saplings, not colonizable plants.

myrmecophytic species that start their ontogeny as nonmyrmecophytes (*M. hosei* less than *M. pruinosa*) had high tannin contents (up to 25% of dry weight) as well. These proved to be even higher than those from most of the nonmyrmecophytes (except *M. heynei*). The tannin content of *M. pearsonii* from the same section was comparable to that of the majority of the nonmyrmecophytes analyzed (except *M. heynei*) and higher than the tannin contents observed for some of the "early" myrmecophytes (Figure 1). In general, a tendency for lower tannin contents was observed for the early myrmecophytes when compared to nonmyrmecophytes. Significant differences in tannin contents ($P < 0.05$), however, were only found for the early myrmecophytes *M. triloba, M. hypoleuca*, and *M. winkleri*, compared to the remaining species analyzed (non-myrmecophytes as well as myrmecophytes). Within the group of early myrmecophytes, which are already colonized at juvenile stages of their life cycle, striking differences in tannin contents were found: *M. triloba, M. hypoleuca*, and *M. winkleri* had lower tannin contents when compared to other early myrmecophytes analyzed (univariate ANOVA; $P < 0.05$ for each combination, except *M. winkleri* and *M. indistincta*; $P = 0.270$), whereas they did not differ from each other ($P > 0.05$).

When comparing different ontogenetic stages of *Macaranga* species (*M. tanarius, M. hosei*, and *M. pruinosa*), differences of tannin contents were only found among juvenile plants of *M. tanarius* (2 m height) and adult trees (univariate ANOVA; $P = 0.011$). No such differences were observed among small saplings of *M. hosei* and already colonizable plants of this species (univariate ANOVA; $P = 0.278$) or among the different ontogenetical stages analyzed for *M. pruinosa* (univariate ANOVA; $P > 0.05$ for each combination).

Bioassay. Most *Macaranga* extracts had a negative effect on the growth of *Spodoptera littoralis* larvae as judged from the weights of the treated larvae compared to controls. However, the bioassays also showed differences among the species analyzed with regard to the extent of reduction of larval growth when compared to controls (Table 1; Figure 2; univariate ANOVA: $N = 153$, $P < 0.001$). These differences are in most cases, paralleled by similar differences in tannin contents.

Some of the *Macaranga* species analyzed (*M. tanarius, M. pruinosa*, *M. hullettii*, and *M. angulata*) were particularly active in reducing larval growth. However, the feeding experiments did not reveal a clear correlation between the influence on larval growth and life form (myrmecophytes versus nonmyrmecophytes). For example, within the group of nonmyrmecophytes, the extract of *M. gigantea* caused a lower reduction of larval growth than that observed for the extracts of *M. tanarius* and *M. heynei*. The myrmecophytes with late colonization showed similar effects on larval growth compared to *M. tanarius* and *M. heynei*. Among the myrmecophytes with late colonization, *M. pruinosa* caused the strongest reduction of larval growth. The highest growth rates of larvae were found when treated with extracts of the myrmecophytic species, especially with those of *M. triloba* and

TABLE 1. SIGNIFICANCE VALUES FOR DIFFERENCES IN EFFECT ON *Spodoptera* GROWTH RATES OF EXTRACTS FROM DIFFERENT *Macaranga* SPECIES[a,b]

	tan T	gig	hey	hos	pru	pru S	pru T	pea	tri	hyp	hul	ang	ind	win
tan	0.583	0.002**	0.667	0.583	0.530	0.633	0.722	0.132	0.000***	0.133	0.812	0.623	0.924	0.000***
tan T		0.000***	0.907	0.270	0.938	0.975	0.930	0.046*	0.000***	0.045	0.751	0.944	0.529	0.000***
gig			0.001**	0.007**	0.000**	0.004**	0.005**	0.116	0.260	0.099	0.001**	0.000***	0.004**	0.195
hey				0.327	0.846	0.899	0.995	0.059	0.000***	0.059	0.843	0.961	0.607	0.000***
hos					0.236	0.360	0.429	0.301	0.000***	0.308	0.426	0.290	0.664	0.000***
pru						0.975	0.880	0.038*	0.000***	0.037	0.692	0.881	0.479	0.000***
pru S							0.917	0.095	0.000***	0.097	0.772	0.929	0.585	0.000***
pru T								0.121	0.000***	0.123	0.867	0.974	0.670	0.000***
pea									0.007**	0.968	0.083	0.048*	0.168	0.004**
tri										0.005**	0.000***	0.000***	0.000***	0.862
hyp											0.083	0.047*	0.170	0.003**
hul												0.800	0.743	0.000***
ang													0.565	0.000***
ind														0.000***

[a] Univariate ANOVA; post-hoc test (LSD); $N = 149$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$.
[b] Abbreviations as in Fig.1 legend.

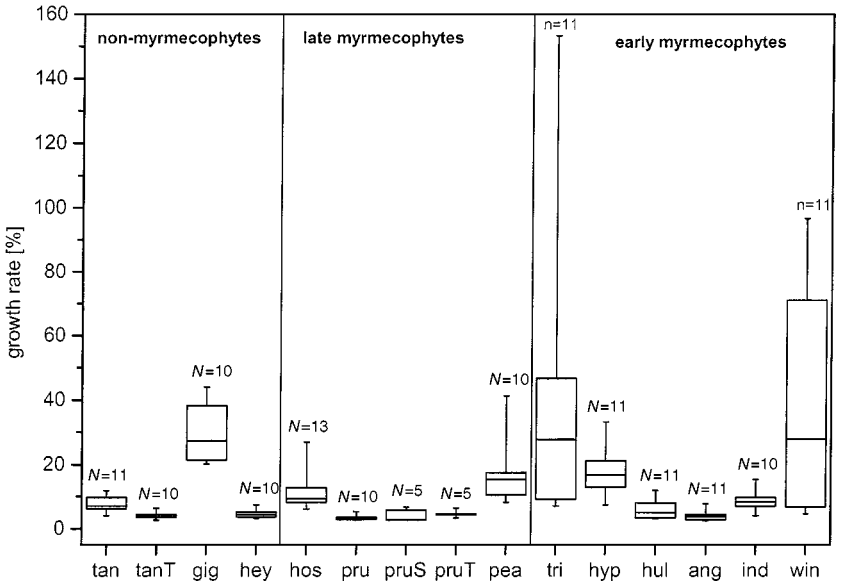


FIG. 2. Growth rates of *Spodoptera littoralis* larvae grown on artificial diet treated with extracts of different *Macaranga* species (mean growth rate of controls is set to 100%). Abbreviations as in Figure 1 legend.

M. winkleri. Surprisingly, high growth reduction was caused by extracts of the myrmecophytes *M. angulata, M. hullettii*, and *M. indistincta*.

For some of the *Macaranga* species (e.g., *M. triloba* and *M. winkleri*), considerable intraspecific variability of tannin contents as well as of their effects on larval growth were found. No significant differences with regard to larval growth were found when testing extracts derived from different ontogenetic stages of *M. tanarius, M. hosei*, and *M. pruinosa* (univariate ANOVA; $P > 0.05$).

Correlation Between Tannin Contents and Influence on larval Growth. There was a general correlation between the amounts of tannin *contents* in the different *Macaranga* species and the extent to which larval growth was reduced (Figure 3; $N = 136$; $P < 0.001$). This correlation (Figure 4), however, was statistically significant only for *M. pruinosa* saplings that were not colonizable and for larger plants of *M. pruinosa* ($N = 5$; $P < 0.05$), *M. triloba* ($N = 11$; $P < 0.001$), *M. indistincta* ($N = 10$; $P < 0.05$), and *M. winkleri* (up to 2 m height) ($N = 11$; $P < 0.001$). A relatively strong correlation of tannin contents and larval growth was also observed for young trees of *M. pruinosa* ($N = 11$; $P = 0.066$) and *M. tanarius* ($N = 11$; $P = 0.066$). When a tannin-free extract of *M. triloba* (following removal of tannins with skin powder) was incorporated into the artificial diet and offered to neonate larvae, the larval growth rate was similar to that of the controls

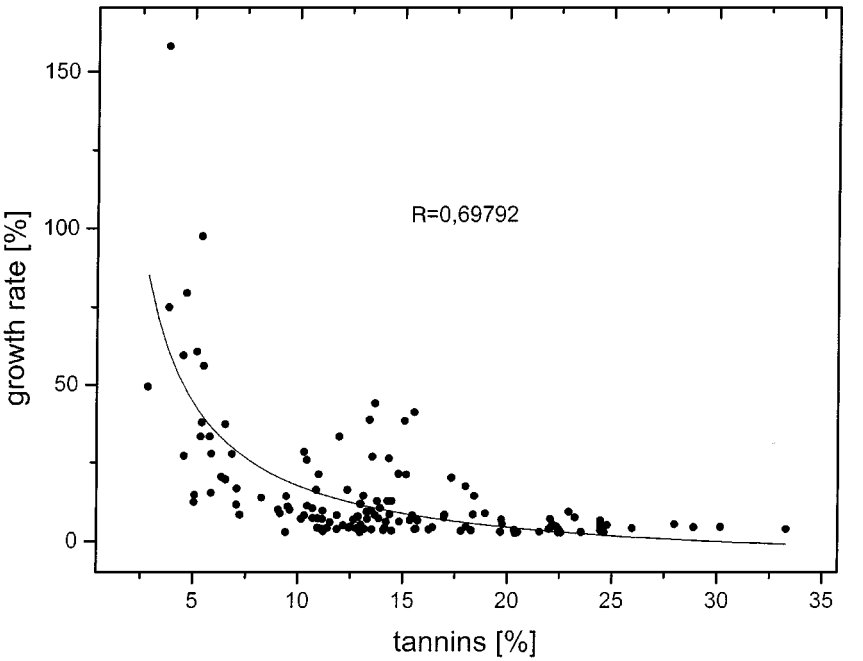


FIG. 3. Connection between tannin contents of individual *Macaranga* plants and growth rates of *Spodoptera littoralis* larvae.

(100%) compared to a growth rate of only 8.4% for the respective extract prior to tannin removal. A similar result was obtained for *M. winkleri* (larval growth rate of 4.5% compared to 100% following removal of tannins). Both chosen plants originally had the strongest effect on larval growth of the respective species.

Tests for Presence of Alkaloids, Cyanogenic Glycosides and Saponins. Besides tannins, *Macaranga* species were also tested for alkaloids, cyanogenic glycosides, and saponins. A weak indication for the presence of alkaloids (following spraying of TLC plates with Dragendorff reagent) was only found for *M. tanarius* and *M. winkleri*, whereas no hints for the presence of cyanogenic glycosides or saponins could be obtained for any of the species analyzed.

DISCUSSION

Consumption of tannins by herbivores is generally associated with depressed growth rates and reduced efficiency of food utilization (Hagerman and Butler, 1991). In Coley's (1986) studies of *Cecropia peltata*, leaf tannin concentrations were negatively correlated with herbivory rates on seedlings and also with the

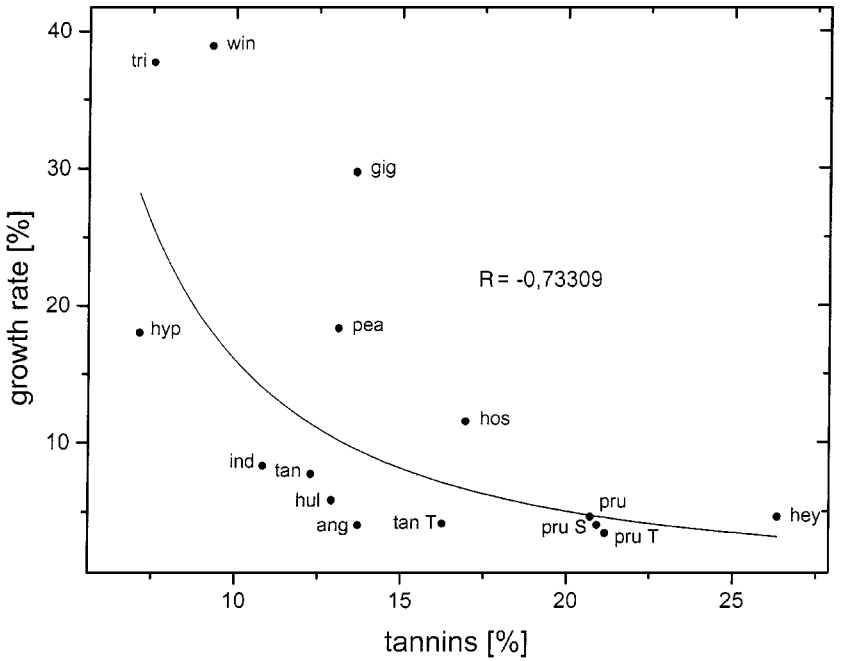


FIG. 4. Connection between mean tannin contents of analyzed *Macaranga* species and mean growth rates of *Spodoptera littoralis* larvae: Abbreviations as in Figure 1 legend.

number of newly produced leaves. High tannin levels also reduced herbivory in *Psychotria horizontalis* and were negatively correlated with growth, suggesting metabolic costs for tannin production (Sagers and Coley, 1995). It was also suggested that tannins play an important role in the defense of plants against bacterial or fungal infections (Bernays et al., 1989).

Some of the *Macaranga* species investigated in our study are extremely rich in tannins, exhibiting tannin contents $\geq$30% dry weight of leaves (Figure 1). Tannin-rich extracts of *Macaranga* species severely depressed the growth of larvae of *Spodoptera littoralis* that were used as experimental insects in this study despite their well-known tolerance of many plant secondary compounds (Figure 2). This effect could be reversed by the removal of tannins from the extracts, leading to larval growth rates comparable to those of control larvae.

In general, there was a negative correlation between larval growth rates and the tannin contents of *Macaranga* species (Figure 3), even though this negative correlation was not significant for every species studied (Figure 4). For some species (e.g., *M. angulata*), the reduction of larval growth was stronger than originally expected based on the analyzed tannin contents. Even though tannins play a major role in the chemical defense of *Macaranga* species (as shown in the tannin removal

experiment), it can not be excluded that other secondary compounds contribute to their defense. Tests for the presence of alkaloids, cyanogenic glycosides, and saponins with hemolytic activity in *Macaranga* consistently gave negative or inconclusive results. For a second group of *Macaranga* species (e.g., *M. gigantea*), larval growth rates were higher than expected from their respective tannin contents. It is possible that qualitative differences in the different species are responsible for this unexpected result. In this context, it is interesting to note that Ayres et al. (1997) reported differences in tannin structure among congeneric plant species that were responsible for differences of antiherbivore activity of the respective tannins.

When the tannin contents of the various *Macaranga* species were analyzed with regard to their respective life forms, it became obvious that most of the early myrmecophytes had lower tannin contents than those observed for the nonmyrmecophytes (Figure 1) although these differences were not significant for every pair of species studied. Superior growth performance of *Spodoptera* larvae on an artificial diet spiked with *Macaranga* leaf powder from myrmecophytic species compared to leaf powder from nonmyrmecophytes was also reported by Nomura et al. (2000), who studied three myrmecophytic and two nonmyrmecophytic species. Two of them, *M. winkleri* and *M. gigantea*, were also treated in our study. Unfortunately, our results are not directly comparable to those of Nomura et al. (2000), since they used different amounts of dried leaf powder (based on leaf area rather than on dry weight) in the artificial diets. The highest amounts of leaf powder incorporated into artificial diet originated from the nonmyrmecophytic species (Nomura et al., 2000), which might have biased the results obtained (higher growth rates on extracts from myrmecophytic species compared to extracts from nonmyrmecophytic species). Nomura et al. (2000) correlated their cutworm survival results on artificial diet treated with one of the three myrmecophytic species analyzed with the intensity of the biotic defenses of the respective species. In another study (Itioka et al., 2000), the myrmecophytic species recorded to be most intensively defended by ants (*M. winkleri*) had the weakest effect on larval survival. In our investigation, which covers several species with the same ant inhabitants, a more complex pattern than that reported by Nomura et al. (2000) emerges. We found high tannin content and indications for more intense chemical defense in *M. hypoleuca*, a sister species to *M. beccariana* used in the studies mentioned above. Both plants are inhabited by the same morphospecies [Fiala et al., 1999 (probably *Crematogaster decamera*)]. However, other *Macaranga* species analyzed in our study and inhabited by the same ant species (Fiala et al., 1999) show different tannin contents and vary with regard to their influences on larval growth. Thus, our study, with its large sample sizes of 10 or 11 plants from 13 species, revealed a more differentiated picture, even within the group of myrmecophytes.

The myrmecophytes from our study that are colonized by ants only at later stages of their ontogeny (with the exception of *M. pearsonii*) had strikingly high

contents of tannins compared to the early myrmecophytes and to the nonmyrmecophytic species (Figure 1). The former species apparently do not rely as heavily on biotic defense by ants as compared to the early myrmecophytes. *M. pruinosa*, which belongs to the group of late myrmecophytes and has an exceptionally high tannin content, features a rather low percentage of ant inhabitation even at later ontogenetic stages, when its stem structure would, in principle, allow colonization (Fiala et al., 1999). *M. hosei* and *M. pearsonii*, however, which had a lower tannin content than *M. pruinosa*, are usually inhabited by ants as soon as colonization is possible. Interestingly, investment into ant food (nutrient value of food bodies) was lowest in *M. pruinosa* when compared to other late myrmecophytes (Heil et al., 1998).

The theory of optimal defense postulates a protection of plant parts or tissues in relation to their value to the plant (Rhoades, 1979). The resource availability hypothesis (Coley et al., 1985) predicts a pattern of defense corresponding to the supply of nutrients and light at the plants' location. At locations rich in resources (e.g., light and/or nutrients), plants are able to compensate for tissue losses caused by herbivores more easily. Furthermore, following Herms and Mattson (1992), there is competition between biosynthetic pathways involved in growth (primary metabolism) and the production of secondary metabolites. According to these theories, plants pursue different strategies: depending on resource availability and growth rates, plants may be able to compensate for tissue losses caused by herbivory by increased biomass production or by protection through physical means and/or secondary metabolites or, as in the case of ant plants, by mutualistic ants. In a comparative study of three pairs of pioneer and forest gap species in *Cecropia* myrmecophytes, Folgarait and Davidson (1994) report faster plant growth and higher investment in biotic defense in pioneer species than in gap species, which possess longer-lived and tougher leaves. These considerations complicate the interpretation of results obtained in our study on *Macaranga*. Interspecific differences with regard to plant growth rates and with regard to the amounts of macronutrients present in leaves (N, S, P, C) were found among three *Macaranga* species from one study site: for example, *M. tanarius* has a growth rate that is two times higher than that of *M. triloba* and three times higher than that of *M. hosei* (Heil, 1998). *M. gigantea* also grows fast (Davies, 1996; Fiala, unpublished results). These findings could serve as an adequate explanation for the rather low tannin contents in the high disturbance pioneers *M. tanarius* and *M. gigantea* compared to myrmecophytic species such as *M. angulata, M. hullettii*, and *M. indistincta*, which grow in less disturbed forests.

In conclusion, our results indicate a tendency for a trade-off between chemical and biotic defense in *Macaranga* species featuring different life forms. As Heil et al. (1997, 1998) demonstrated, myrmecophytic plants invest more of their photosynthetic assimilates, i.e. sugars, into nutrition for ants and thus into biotic defense. Metabolic costs of nutrition of defending ants can be quite high (Heil et al., 1997),

but, on the other hand, defense by specific, obligate ant inhabitants seems to be efficient and fulfills a number of different defense functions for the plants. Thus, metabolic costs for additional chemical defense can be reduced. The differences in tannin contents of juvenile and mature trees of *M. tanarius* (Figure 1) also point to a change of resource allocation. Leaves become smaller and tougher as the plants mature. Nitrogen and phosphate contents decrease but carbohydrates increase with age (Heil, 1998). In larger trees of *M. tanarius*, the development of extrafloral nectaries ceases and the opportunistic ants no longer visit these plants (Fiala and Maschwitz, 1991). The investment of these plants seems to shift from biotic towards abiotic defense.

In some of the analyzed species, especially in *M. triloba* and *M. winkleri*, we found remarkable intraspecific variation of tannin contents (Figure 1), which might be due to different resource qualities but may also reflect genetic differences. Perhaps these species are particularly euryotopic, i.e., they can be found at locations differing widely with regard to their resources. As predicted by the C/N balance hypothesis (Bryant et al., 1983; Folgarait and Davidson, 1994, 1995), intraspecific differences in defenses can be caused by different resource qualities since an excess of carbon (caused by a high light regime at the plants location) compared to other nutrients (mainly nitrogen) can promote the carbon-based defense (like the tannins investigated in this study) of the plant.

Macaranga is a genus with a remarkable diversity of ecophysiological and life-history attributes, with variation even among sympatric species (Davies, 1998). This diversity strongly contrasts with the lack of molecular differentiation, especially in the group of obligate myrmecophytes (section *Pachystemon* s. stricto) (Davies, 1996; Blattner et al., 2001). Apparently, these species share close phylogenetic relationships but have diverged ecologically. Our study shows a great deal of intrageneric variation among the defensive mechanisms in this genus. Mutualistic relationships with ants may have contributed to this diversity.

Acknowledgments—The Department of Biology, University of Malaya, granted permission to use their field station in Ulu Gombak. We are grateful to the former Director of Sabah Parks, Datuk Lamri Ali, as well as to Francis Liew, Dr. Jamili Nais, and Maklarin bin Lakim for providing facilities and opportunities to work in Kinabalu Park. We thank EPU for permission to conduct research in Malaysia. Thomas Fendert, Andrea Hilpert, and Dr. Bambang Nugroho provided skilled assistence in the analyses. We gratefully acknowledge financial support by the DFG to K. E. L. (Li 150/13-4 and SFB 251) and P. P. (SFB 251 and Fonds der Chemischen Industrie).

REFERENCES

ADLER, L. S. 2000. Alkaloid uptake increases fitness in a hemiparasitic plant via reduced herbivory and increased pollination. *Am. Nat.* 156:92–99.

AYRES, M. P., CLAUSEN, T. P., MACLEAN, S. F., REDMAN, A. M., and REICHARDT, P. B. 1997. Diversity of structure and antiherbivore activity in condensed tannins. *Ecology* 78:1696–1712.

BEATTIE, A. J. 1985. The Evolutionary Ecology of Ant–Plant Mutualisms. Cambridge University Press, Cambridge.

BERNAYS, E. A., COOPER-DRIVER, G., and BILGENER, M. 1989. Herbivores and plant tannins. *Adv. Ecol. Res.* 19:263–302.

BIDAL, C. 1997. Impact d'évolution des défenses biotiques sur l'élaboration des défenses chimiques et mécaniques des plantes à fourmis. Unpublished DEA thesis. University of Montpellier.

BLATTNER, F. R., WEISING, K., BÄNFER, G., MASCHWITZ, U., and FIALA, B. 2001. Molecular analysis of phylogenetic relationships among myrmecophytic *Macaranga* species (Euphorbiaceae). *Mol. Phylo. Evol*. 19:331–334.

BRINKER, A. M. and SEIGLER, D. S. 1989. Methods for the detection and quantitative determination of cyanide in plant materials. *Phytochem. Bull.* 21:24–31.

BRYANT, J. P., CHAPIN, S. F., III, and KLEIN, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

COLEY, P. D. 1986. Costs and benefits of defense by tannins in a neotropical tree. *Oecologia* 70:238–241.

COLEY, P. D., BRYANT, J. P., and CHAPIN, F. S. 1985. Resource availability and plant antiherbivore defence. *Science* 230:895–899.

DAVIDSON, D. W. and MCKEY, D. 1993. The evolutionary ecology of symbiotic ant–plant relationships. *J. Hym. Res.* 2:13–83.

DAVIES, S. J. 1996. The comparative ecology of *Macaranga* (Euphorbiaceae). Thesis. Harvard University, Cambridge, Massachusetts.

DAVIES, S. J. 1999. New species of *Macaranga* (Euphorbiaceae) section Pachystemon from Borneo. *Kew Bull.* 54:147–154.

DAVIES, S. T. 1998. Photosynthesis of nine pioneer *Macaranga* species from Borneo in relation to life history. *Ecology* 79:2292–2308.

FIALA, B. 1996. Ants benefit pioneer trees: The genus *Macaranga* as an example of ant–plant associations in dipterocarp forest ecosystems, pp. 102–123, *in* A. Schulze and D. Schöne (eds.). Dipterocarp Forest Ecosystems: Structure, Function, Ecology and Sustainable Management. Samarinda, Singapore.

FIALA, B. and MASCHWITZ, U. 1990. Studies on the South East Asian ant–plant association *Crematogaster borneensis/Macaranga*: Adaptations of the ant-partner. *Insectes Soc.* 37:212–231.

FIALA, B. and MASCHWITZ, U. 1991. Extrafloral nectaries in the genus *Macaranga* (Euphorbiaceae) in Malaysia: Comparative studies of their possible significance as predispositions for myrmecophytism. *Biol. J. Linn. Soc*. 44:287–305.

FIALA, B. and MASCHWITZ, U. 1992a. Food bodies and their significance for obligate ant associations in the tree genus *Macaranga* (Euphorbiaceae). *Bot. J. Linn. Soc.* 110:61–75.

FIALA, B. and MASCHWITZ, U. 1992b. Domatia as most important adaptations in the evolution of myrmecophytes in the palaeotropical tree genus *Macaranga* (Euphorbiaceae). *Plant Syst. Evol.* 180:53–64.

FIALA, B., MASCHWITZ, U., THO, Y. P., and HELBIG, A. J. 1989. Studies on a South East Asian ant–plant association: Protection of *Macaranga* trees by *Crematogaster borneensis*. *Oecologia* 79:463–470.

FIALA, B., MASCHWITZ, U., and THO, Y. P. 1991. The association between *Macaranga* and ants in South East Asia, pp. 263–270, *in* C. Huxley and R. Cutler (eds.). Interaction Between Ants and Plants. Oxford University Press, Oxford.

FIALA, B., GRUNSKY, H., MASCHWITZ, U., and LINSENMAIR, K. E. 1994. Diversity of ant–plant interactions: Protective efficacy in *Macaranga* species with different degrees of ant association. *Oecologia* 97:186–192.

FIALA, B., JAKOB, A., MASCHWITZ, U., and LINSENMAIR, K. E. 1999. Diversity, evolutionary specialisation and geographic distribution of a mutualistic ant–plant complex: *Macaranga* and *Crematogaster* in South East Asia. *Biol. J. Linn. Soc.* 66:305–331.

FOLGARAIT, P. J. and DAVIDSON, D. W. 1994. Antiherbivore defense of myrmecophytic *Cecropia* under different light regimes. *Oikos* 71:305–320.

FOLGARAIT, P. J. and DAVIDSON, D. W. 1995. Myrmecophytic Cecropia: Antiherbivore defenses under different nutrient treatments. *Oecologia* 104:189–206.

GAUME, L. and MCKEY, D. 1999. An ant–plant mutualism and its host-specific parasite: Activity rhythms, young leaf patrolling, and effects on herbivores of two specialist plant-ants inhabiting the same myrmecophyte. *Oikos* 84:130–144.

GAUME, L., MCKEY, D., and ANSTETT, M.-C. 1997. Benefits conferred by "timid" ants: Active antiherbivore protection of the rainforest tree *Leonardoxa africana* by the minute ant *Petalomyrmex phylax*. *Oecologia* 112:209–216.

GERSHENZON, J. 1994. The cost of plant chemical defense against herbivores: A biochemical perspective, pp. 105–173, *in* E. A. Bernays (ed.). Insect–Plant Interactions, Vol. 5. CRC Press, Boca Raton, Florida.

HAGERMAN, A. E. and BUTLER, L. G. 1991. Tannins and lignins, pp. 355–388, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites, 2nd ed., Vol. I: The Chemical Participants, Academic press, San Diego, California.

HEIL, M. 1998. Quantitative Kosten-Nutzen-Analyse verschiedener Ameisen-Pflanzen-Assoziationen innerhalb der Gattung *Macaranga*. Wissenschaft & Technik Verlag, Berlin.

HEIL, M., FIALA, B., ZOTZ, G., MENKE, P., and MASCHWITZ, U. 1997. Food body production in *Macaranga triloba* (Euphorbiaceae): A plant investment in antiherbivore defence via symbiotic ant partners. *J. Ecol.* 85:847–861.

HEIL, M., FIALA, B., KAISER, W., and LINSENMAIR, K. E. 1998. Chemical contents of *Macaranga* food bodies: Adaptations to their role in ant attraction and nutrition. *Funct. Ecol.* 12:117–122.

HEIL, M., FIALA, B., BOLLER, T., and LINSENMAIR, K. E. 1999. Reduced chitinase activities in ant-plants of the genus *Macaranga*. *Naturwissenschaften* 86:146–149.

HEIL, M., STAEHELIN, C., and MCKEY, D. 2000. Low chitinase activity in *Acacia* myrmecophytes: A potential trade-off between biotic and chemical defenses? *Naturwissenschaften* 87:555–558.

HEIL, M., FIALA, B., MASCHWITZ, U., and LINSENMAIR, K. E. 2001. On benefits of indirect defense: Short- and long-term studies in antiherbivore protection via mutualistic ants. *Oecologia* 126:395–403.

HERMS, D. A. and MATTSON, W. J. 1992. The dilemma of plants: To grow or to defend. *Q. Rev. Biol.* 67:283–335.

HÖLLDOBLER, B. and WILSON, E. O. 1990. The Ants. Harvard University Press, Cambridge, Massachusetts.

HUXLEY, C. R. and CUTLER D. F. (eds.) 1991. Ant–Plant Interactions. Oxford University Press, Oxford.

ITIOKA, T., NOMURA, M., INUI, Y., ITINO, T., and INOUE, T. 2000. Difference in intensity of ant defense among three species of *Macaranga* myrmecophytes in a southeast Asian dipterocarp forest. *Biotropica* 32:318–326.

JANZEN, D. H. 1966. Coevolution of mutualism between ants and acacias in Central America. *Evolution* 20:249–275.

JANZEN, D. H. 1973. Dissolution of mutualism between *Cecropia* and *Azteca* ants. *Biotropica* 5:15–28.

LETOURNEAU, D. K. 1998. Ants, stem-borers, and fungal pathogens: Experimental tests of a fitness advantage in *Piper* plants. *Ecology* 79:593–603.

LIN, J. H. 1993. Studies on tannins of the bark of *M. tanarius* (L.) Muell. Et Arg. *J. Food Drug Anal.* 1:273–280.

LIN, J. H. 1994. Studies on tannins from the bark of *M. sinensis* (Baill.) Muell.-Arg. *J. Food Drug Anal.* 2:201–209.

LIN, J. H., NONAKA, G. I., and NISHIOKA, I. 1990a. Tannins and related compounds XCIV. Isolation and characterization of seven new hydrolizable tannins from the leaves of *Macaranga tanarius* (L.) Muell. Et Arg. *Chem. Pharm. Bull.* (*Tokyo*) 38:1218–1223.

LIN, J. H., ISHIMATSU, M., TANAKA, T., NONAKA, G. I., and NISHIOKA, I. 1990b. Tannins and related compounds XCIV. Structures of macarangins and macarinins. New hydrolyzable tannins possessing macaronyl and tergalloyl ester groups from the leaves of *M. sinensis* Baill. Muell.-Arg. *Chem. Pharm. Bull.* (*Tokyo*) 38:1844–1851.

MARQUIS, R. J. and BRAKER, H. E. 1994. Plant-herbivore interactions: Diversity, specificity and impact, pp. 263–281, *in* L. McDade, G. H. Hartshorn, H. Hespenheide and K. Bawa (eds.). University of Chicago Press, Chicago, Illinois.

NOMURA, M., ITIOKA, T., and ITINO, T. 2000. Variations in abiotic defense within myrmecophytic and nonmyrmecophytic species of *Macaranga* in a Bornean dipterocarp forest. *Ecol. Res.* 15:1–11.

REHR, S. S., FEENY, P. P., and JANZEN, D. H. 1973. Chemical defense in central American non-ant acacias. *J. Anim. Ecol.* 42:405–416.

RHOADES, D. F. 1979. Evolution of plant chemical defense against herbivores. pp. 4–53, *in* G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, San Diego, California.

RICKSON, F. R. 1980. Developmental anatomy and ultrastructure of the ant-food bodies (Beccarian bodies) of *M. triloba* and *M. hypoleuca* (Euphorbiaceae). *Am. J. Bot.* 67:285–292.

ROSENTHAL, G. A. and BERENBAUM, M. R. (eds.) 1991. Herbivores—Their Interactions with Secondary Plant Metabolites, Vol. 1: The Chemical Participants. Academic Press, New York.

SAGERS, C. L. and COLEY, P. D. 1995. Benefits and costs of defense in a neotropical shrub. *Ecology* 76:1835–1843.

SCHUPP, E. W. 1986. *Azteca* protection of *Cecropia* ant occupation benefits juvenile trees. *Oecologia* 70:379–385.

SEIGLER, D. S. and EBINGER, J. E. 1987. Cyanogenic glycosides in ant-acacias of Mexico and Central America. *South West Nat.* 32:499–503.

SIMMS, E. L. and RAUSHER, M. D. 1989. The evolution of resistance to herbivory in *Ipomoea purpurea*. II. Natural selection by insects and costs of resistance. *Evolution* 43:573–585.

SRIVASTAVA, R. P. and PROKSCH, P. 1991. Contact toxicity and feeding inhibitory activity of chromones from Asteraceae against *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Entomol. Gener.* 15:265–274.

TURNER, M. 1995. Foliar defences and habitat adversity of three woody plant communities in Singapore. *Funct. Ecol.* 9:279–284.

WHITMORE, T. C. 1969. First thoughts on species evolution in Malayan *Macaranga* (studies in *Macaranga* III). *Biol. J. Linn. Soc.* 1:223–231.

WHITMOTE, T. C. 1975. *Macaranga*, pp. 140–159, *in* H. K. Air-Shaw (ed.). The Euphorbiaceae of Borneo. *Kew Bulletin Additional. Series* 4. Kew Gardens, UK.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

GUSTATORY RESPONSIVENESS TO POLYCOSE IN FOUR SPECIES OF NONHUMAN PRIMATES

MATTHIAS LASKA,[1,*] STEFANIE KOHLMANN,[1]
HANS-PETER SCHEUBER,[2] LAURA TERESA HERNANDEZ SALAZAR,[3]
and ERNESTO RODRIGUEZ LUNA[3]

[1]*Department of Medical Psychology*
[2]*Department of Surgery*
University of Munich Medical School
D-80336 Munich, Germany
[3]*Instituto de Neuro-Etologia*
Universidad Veracruzana
C.P. 91000 Xalapa, Mexico

(Received October 24, 2000; accepted May 29, 2001)

Abstract—The taste responsiveness of six squirrel monkeys, five pigtail macaques, four olive baboons, and four spider monkeys to polycose, a starch-derived polysaccharide, was assessed in two-bottle preference tests of brief duration (2 min). In experiment 1, the monkeys were given the choice between tap water and defined concentrations of polycose dissolved in tap water. In experiment 2, the animals were given the choice between polycose and sucrose, fructose, glucose, lactose, and maltose presented in equimolar concentrations of 100 and 200 mM, respectively. The animals were found to prefer concentrations of polycose as low as 10 mM (pigtail macaques), 30 mM (olive baboons and spider monkeys), and 60 mM (squirrel monkeys) over tap water. Relative taste preferences were stable across the concentrations tested and indicate an order of relative effectiveness (sucrose > polycose ≥ maltose) in squirrel monkeys, spider monkeys, and olive baboons that is similar to the order of relative sweetness in humans. Pigtail macaques, however, displayed an order of relative effectiveness (maltose > polycose ≥ sucrose) that differs markedly from that found in the other primate species tested and is similar to relative taste preferences found in rodents such as rats. Both the high sensitivity of the pigtail macaques to polycose and their vivid predilection for this polysaccharide and its disaccharide constituent maltose suggest that *Macaca nemestrina*, unlike other primates, but like rodents, may have specialized taste receptors for starch.

Key Words—Gustatory preference thresholds, relative taste preferences, polycose.

*To whom correspondence should be addressed; e-mail: Laska@imp.med.uni-muenchen.de

INTRODUCTION

Carbohydrates, in the form of sugar and starch, represent a major source of metabolic energy for a great number of animal species. With the exception of some fruits, plants usually contain much more starch than sugar (Hizukuri, 1996). However, whereas taste perception of sugars has been the subject of considerable research at both the behavioral and the electrophysiological level in a variety of species (Spector, 2000), studies on the taste of starch have been restricted to the rat. One possible reason for this bias may be the fact that sugars have a highly attractive taste both for humans and many other species, whereas starch has a rather bland taste for humans and has been assumed to be tasteless and, thus, unattractive to other animals as well (Kare, 1971; Pfaffmann, 1977).

Comparative studies of sugar taste sensitivity and preference have revealed marked differences among species. With regard to sensitivity, for example, preference thresholds for sucrose may differ by more than two orders of magnitude, even between closely related species belonging to the same order of mammals, such as the spider monkey and the slow loris (Laska et al., 1996; Glaser, 1986). While many species display a strong preference for the taste of sugars, others, such as cats and chickens, are indifferent to soluble carbohydrates (Kare, 1971; Beauchamp et al., 1977). Species have also been found to differ in their relative preference for individual sugars. Among species that prefer sucrose, some, such as rats, spiny mice, and gerbils, show a preference for maltose, while others, such as dogs, armadillos, or cows, are either indifferent or averse to maltose (Kare, 1971; Feigin et al., 1987).

In contrast to the wealth of knowledge about differences among species regarding their responsiveness to sugars, little is known as to whether species also differ in their sensitivity and preference for starch and other polysaccharides.

In a series of landmark studies, Sclafani and coworkers have shown that rats are strongly attracted to starch and starch-derived polysaccharides such as polycose (Sclafani and Clyne, 1987). Further, they showed that rats have a markedly lower taste preference threshold for polycose than for sugars such as sucrose (Sclafani and Nissenbaum, 1987), and that, at least at concentrations up to 300 mM, they clearly prefer this polysaccharide to sucrose and other mono- or disaccharides (Sclafani and Mann, 1987). These results, along with other findings, led the authors to conclude that rats have two types of carbohydrate taste receptors, one for polysaccharides and one for sucrose, which produce qualitatively distinct gustatory sensations (Nissenbaum and Sclafani, 1987). Recent electrophysiological studies lend support to this idea, as they have demonstrated the neural activity profile of the rat evoked by polycose and other starch-derived polysaccharides to be markedly different from those of other taste stimuli termed sweet by humans (Giza et al., 1991; Sako et al., 1994).

The presumed function of polysaccharide taste receptors is to facilitate the identification of starch-rich foods (Sclafani, 1991). This latter supposition is supported by findings that showed other rodents, such as gerbils, hamsters, and spiny mice, which also feed on grains and other parts of plants containing high amounts of starch, display similar preferences for polysaccharides as rats (Feigin et al., 1987; Rehnberg et al., 1996).

Given the paucity of data on taste sensitivity and preference for polysaccharides in species other than rodents, we decided to test the gustatory responsiveness of four species of nonhuman primates to polycose. This highly water-soluble mixture of glucose chains with α-1,4 linkages (2% glucose, 7% maltose, 55% maltooligosaccharides of 3–10 glucose units, and 36% maltopolysaccharides of >10 glucose units) is produced from an acid enzyme hydrolysis of corn starch and, apart from its frequent use as a taste stimulus in studies of gustatory performance in rodents, it is also used as a dietary supplement in humans.

The four primate species employed here, squirrel monkeys, spider monkeys, olive baboons, and pigtail macaques, are known to differ—at least to some degree—in their dietary habits (Clutton-Brock and Harvey, 1977; Caldecott, 1986; Chapman, 1987; Ross, 1992), allowing us to address the question of whether possible differences in taste responsiveness to polysaccharides in nonrodent mammals may reflect an evolutionary adaptation to dietary specialization.

Thus, the aims of the present study are threefold: (1), to assess whether different species of nonhuman primates are attracted to polycose; (2), to determine taste preference thresholds for this substance as a first and conservative approximation of gustatory sensitivity; and (3), to assess relative preferences for this polysaccharide in direct comparison with five food-associated mono- and disaccharides.

The possibility of including two New World primate species and two Old World primate species in this study allowed us to address additionally the question of whether the degree of phylogenetic relatedness rather than dietary specialization may affect the taste responsiveness of nonhuman primates to polysaccharides.

METHODS AND MATERIALS

Animals. Testing was carried out with six male adult squirrel monkeys (*Saimiri sciureus*), two male and three female adult pigtail macaques (*Macaca nemestrina*), two male and two female adult olive baboons (*Papio hamadryas anubis*), and one male and three female adult spider monkeys (*Ateles geoffroyi*). Animals of all four species were housed as social groups in enclosures with adjacent single cages that could be closed by sliding doors to allow temporary separation of animals for individual testing [for details of maintenance see Laska (1996, 2000) and Laska et al. (1996, 1999a,b)]. Animals were fed commercial monkey chow, fresh fruit,

and vegetables *ad libitum* but were deprived of water overnight before testing on the following morning. The amount of food offered daily to the animals was such that leftovers were still present on the floor the next morning and, thus, it was unlikely that ravenous appetite affected the animals' ingestive behavior in the tests.

Procedure. Gustatory responsiveness to polycose (reagent grade, Ross Products, Columbus, Ohio) was assessed by using a two-bottle preference test of short duration (Richter and Campbell, 1940). Twice each day, approximately 1 and 2 hr before feeding, the animals were separated and allowed 2 min to drink from a pair of simultaneously presented graduated cylinders with metal drinking spouts.

In experiment 1, monkeys were given a choice between tap water and defined concentrations of polycose dissolved in tap water. Testing started at a concentration of 200 mM and proceeded in the following steps (100, 50, 20, 10 mM, etc.) until the animals failed to show a significant preference. Subsequently, intermediate concentrations were tested in order to determine the preference threshold value more exactly. To keep up the animals' motivation and willingness to cooperate, testing did not follow a strict descending staircase procedure but followed a pseudorandomized scheme in which trials with high and, thus, presumably readily perceptible and attractive concentrations of polycose were alternated with low and presumably less attractive concentrations.

In experiment 2, monkeys were given a choice between equimolar concentrations of polycose and sucrose, fructose, glucose, maltose, and lactose (reagent grade, Merck, Darmstadt, Germany). In order to assess whether preferences are stable at different concentration levels, two test series were performed at 100 and 200 mM, respectively.

In both experiments, each pair of stimuli was presented 10 times, and the position of the stimuli was randomized in order to counterbalance possible position preferences. All animals had served in previous studies using the same method (Laska, 1994, 1996, 1997, 1999, 2000; Laska et al., 1996, 1998, 1999a,b, 2000). They were trained to enter the single cages voluntarily and were completely accustomed to the procedure.

The experiments reported here comply with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 86-23, revised 1985) and also with current German and Mexican laws.

Data Analysis. For each animal, the amount of liquid consumed from each bottle was recorded, summed for the 10 test trials with a given stimulus combination, converted to percentages (relative to the total amount of liquid consumed from both bottles), and 66.7% (i.e., 2/3 of the total amount of liquid consumed) was taken as the criterion of preference. This rather conservative criterion was chosen for reasons of comparability of data, as the same criterion had been used in previous studies using the same method with the same primate species (Laska, 1994, 1996, 1997, 1999, 2000; Laska et al., 1996, 1998, 1999a,b, 2000), and in order to avoid misinterpretation of data due to a too-liberal criterion.

Additionally, two-tailed binomial tests (Siegel and Castellan, 1988) were performed, and an animal was only regarded as significantly preferring one of the two stimuli if it reached the criterion of 66.7% and consumed more from the bottle containing the preferred stimulus in at least 8 of 10 trials (binomial test, $P < 0.05$).

Preliminary analysis of the data indicated that there were no reliable differences in choice behavior and liquid consumption between the males and females of a species nor between the first and the second presentation of the day. Intraindividual variability in the amount of liquid consumed across the 10 test trials with a given stimulus combination was low and averaged less than 20%. Thus, a theoretically possible bias in the overall preference score due to excessive drinking in aberrant trials did not occur. Therefore, the data for the males and females of a species obtained in the 10 test trials were combined and are reported as group means and standard deviations.

Comparisons of the rank order of preference for the five saccharides tested across species and concentrations, respectively, were made by calculating Spearman rank-order correlation coefficients (r_s), which were tested for significance by computing z scores (Siegel and Castellan, 1988).

RESULTS

Experiment 1. Figure 1 shows the taste preference thresholds for polycose to be 10 mM in the pigtail macaques, 30 mM in the spider monkeys and the olive baboons, and 60 mM in the squirrel monkeys. All animals of a given species significantly discriminated these concentrations from tap water, and in some cases single individuals even scored slightly lower preference threshold values. All animals, however, failed to show a significant preference for the lowest concentrations presented, suggesting that the preference for higher concentrations was indeed based on the chemical nature of the stimulus. In most cases, interindividual variability of scores was low for both sub- and suprathreshold concentrations tested (cf. SDs in Figure 1).

Across-species comparisons of the degree of preference for polycose displayed by the four primate species showed that at concentrations of 50, 100, and 200 mM, respectively, the pigtail macaques were more attracted to the polysaccharide than the other species.

Experiment 2. Figure 2 shows the mean group preferences of the four primate species given a choice between polycose and maltose, sucrose, fructose, glucose, and lactose, presented at equimolar concentrations of 100 mM.

Squirrel monkeys significantly preferred sucrose and fructose over polycose and showed a trend to prefer polycose over maltose, glucose, and lactose that fell short of statistical significance. Spider monkeys significantly preferred sucrose and fructose over polycose, polycose over glucose and lactose, and showed a

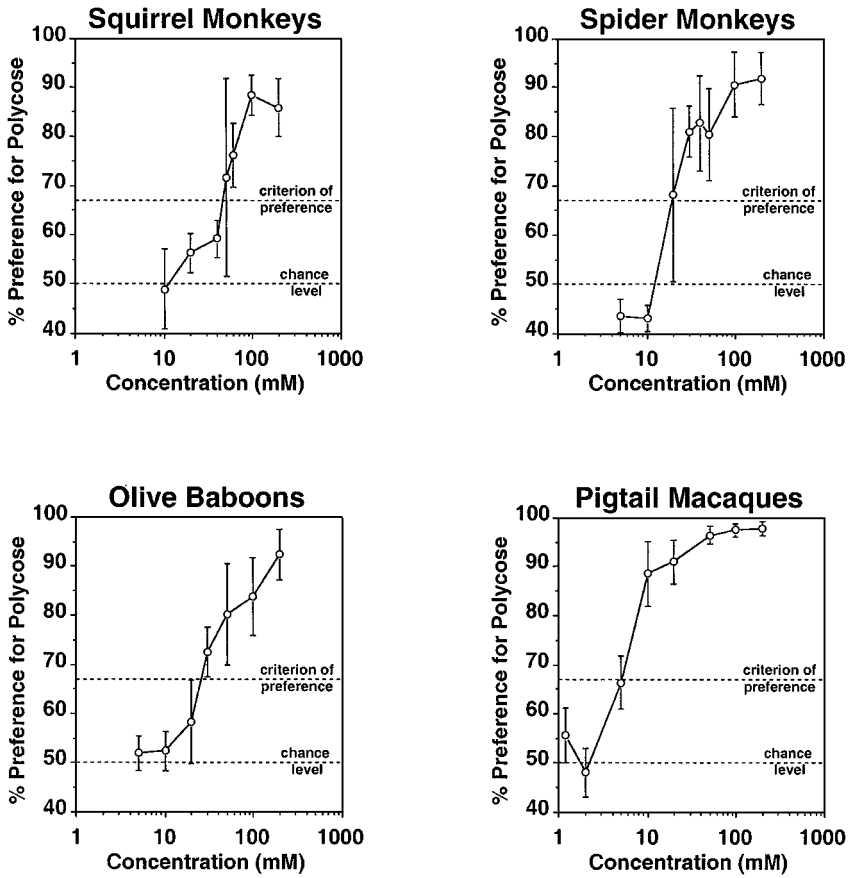


FIG. 1. Taste responsiveness of six squirrel monkeys, five pigtail macaques, four spider monkeys, and four olive baboons to aqueous solutions of polycose tested against tap water. Each data point represents the mean value (±SD) of 10 test trials of 2 min per animal.

nonsignificant trend to prefer polycose over maltose. Olive baboons significantly preferred sucrose over polycose; polycose over maltose, glucose, and lactose; and showed a nonsignificant trend to prefer polycose over fructose. Pigtail macaques significantly preferred maltose over polycose; polycose over fructose, glucose, and maltose; and showed a trend to prefer polycose over sucrose that fell short of statistical significance.

Interindividual variability within a given species was remarkably low, as can be inferred from the small SDs, and with only few exceptions all animals of a species either reached the criterion of preference (>66.7% of total consumption, plus binomial test, $P < 0.05$) in a given task or all failed to do so.

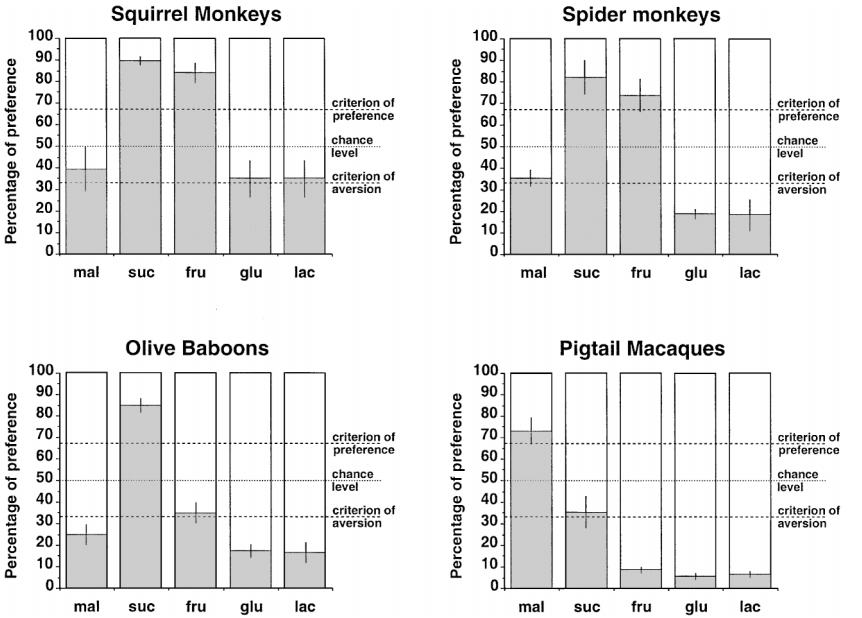


FIG. 2. Relative taste preferences of six squirrel monkeys, five pigtail macaques, four spider monkeys, and four olive baboons given a choice between aqueous solutions of polycose and sucrose, fructose, glucose, maltose, and lactose presented at equimolar concentrations of 100 mM. The shaded portion of a bar indicates the preference for a given saccharide and the white portion of a bar indicates the preference for polycose. Each bar represents the mean value (±SD) of 10 test trials of 2 min per animal.

Across-species comparisons of the patterns of preferences displayed by the four primate species showed that the preference rankings of squirrel monkeys, spider monkeys, and olive baboons for the five mono- and disaccharides tested against polycose were the same (sucrose > fructose > maltose > glucose > lactose) and, thus, significantly correlate with each other (Spearman, $P < 0.05$ for all pairs). The preference ranking of pigtail macaques for the same sugars tested against polycose (maltose > sucrose > fructose > lactose > glucose) was markedly different from that of the other three species (Spearman, $P > 0.05$ for all pairs).

Figure 3 shows the mean group preferences of the four primate species given a choice between equimolar concentrations (200 mM) of polycose and maltose, sucrose, fructose, glucose and lactose.

Squirrel monkeys significantly preferred sucrose and fructose over polycose and failed to show any preference in the combinations of polycose versus maltose, glucose, and lactose. Spider monkeys significantly preferred sucrose over polycose, polycose over glucose and lactose, and showed a nonsignificant trend to prefer fructose and maltose over polycose. Olive baboons significantly preferred sucrose

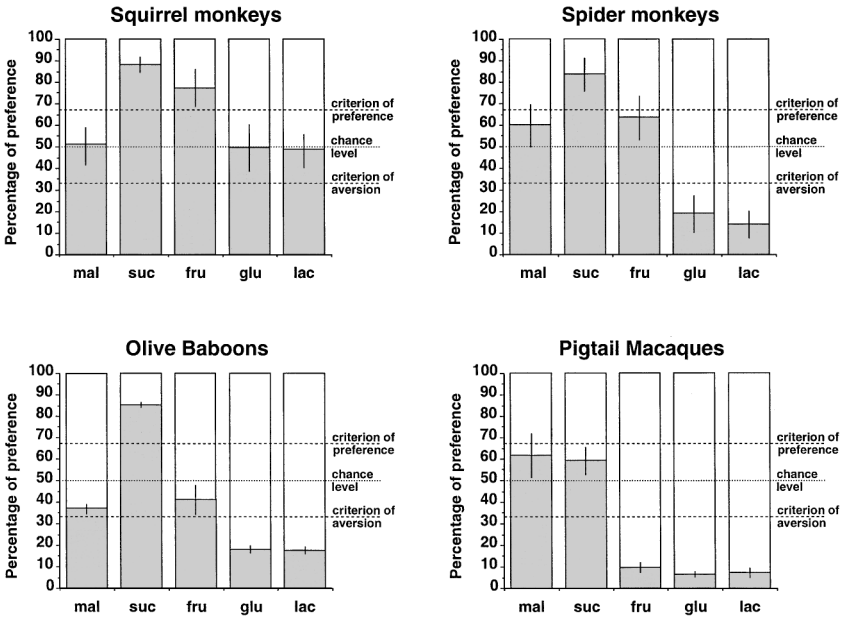


FIG. 3. Relative taste preferences of six squirrel monkeys, five pigtail macaques, four spider monkeys, and four olive baboons given a choice between aqueous solutions of polycose and sucrose, fructose, glucose, maltose, and lactose presented at equimolar concentrations of 200 mM. The shaded portion of a bar indicates the preference for a given saccharide and the white portion of a bar indicates the preference for polycose. Each bar represents the mean value (±SD) of 10 test trials of 2 min per animal.

over polycose, polycose over glucose and lactose, and showed a nonsignificant trend to prefer polycose over maltose and fructose. Pigtail macaques significantly preferred polycose over fructose, glucose, and maltose and showed a trend to prefer maltose and sucrose over polycose that fell short of statistical significance.

Interindividual variability within a given species was remarkably low as can be inferred from the small SDs, and, with only few exceptions, all animals of a species either reached the criterion of preference in a given task or all failed to do so.

Across-species comparisons of the patterns of preferences displayed by the four primate species revealed that the preference rankings of squirrel monkeys, spider monkeys, and olive baboons for the five mono- and disaccharides tested against polycose were the same (sucrose > fructose > maltose > glucose > lactose) and, thus, significantly correlate with each other (Spearman, $P < 0.05$ for all pairs), whereas the preference ranking of the pigtail macaques for the same sugars tested against polycose (maltose > sucrose > fructose > lactose > glucose) was markedly different from that of the other three species (Spearman, $P > 0.05$ for all pairs).

Within-species comparisons of the patterns of preference across the two concentrations tested revealed that the preference rankings of all four primate species for the five mono- and disaccharides tested against polycose remained the same at 100 mM and 200 mM and thus correlate significantly with each other (Spearman, $P < 0.05$ for all four pairs).

There was, however, a general trend (16 of 20 cases) for the preferences to be less pronounced with the higher concentration tested (200 mM) compared to the lower concentration (100 mM).

DISCUSSION

Table 1 compares the taste preference threshold values for polycose obtained in the present study with those for maltose and sucrose obtained with the same method in the four primate species tested here, with those of other mammalian species obtained by using a similar method, and with detection or recognition threshold values obtained for humans, with psychophysical procedures. A comparison of the preference threshold values for polycose shows *Macaca nemestrina* to be the most sensitive nonhuman primate species tested so far. Remarkably, their preference threshold value for this polysaccharide is even lower than the recognition threshold value of humans (Hettinger et al., 1996) in which, to the best of our

TABLE 1. TASTE PREFERENCE THRESHOLDS FOR POLYCOSE, MALTOSE, AND SUCROSE IN FOUR PRIMATE SPECIES TESTED AND IN OTHER MAMMALIAN SPECIES

Species	Threshold (mM)		
	Polycose	Maltose	Sucrose
Saimiri sciureus[1,2a]	60	90	10
Ateles geoffroyi[1,3]	30	20	3
Papio hamadryas anubis[1,4]	30	20	10
Macaca nemestrina[1,5]	10	10	10
Rattus norvegicus[6]	0.1	2.5	2.6
Rattus norvegicus[7]	<1	5	5
Meriones unguiculatus[7]	5	>10	5
Mesocricetus auratus[7]	<1	>10	>10
Acomys cahirinus[7]	5	10	>10
Homo sapiens[8,9]	30	38	10

[a1]Present study, [2]Laska (1996), [3]Laska et al. (1996), [4]Laska et al. (1999), [5]Laska (2000), [6]Sclafani and Nissenbaum (1987), [7]Feigin et al. (1987), [8]Hettinger et al. (1996), study established a recognition threshold for polycose, [9]ASTM (1973) study established detection thresholds rather than preference thresholds for maltose and sucrose.

knowledge, no proper detection threshold value has been determined. More importantly, pigtail macaques are the only primate species tested so far whose preference threshold value for polycose and maltose is as low as that for sucrose. Thus, they differ from the other primates tested that all, including humans, display lower thresholds for sucrose compared to polycose and its disaccharide constituent maltose. The pattern of preference threshold values displayed by the pigtail macaques for these three saccharides is similar to that reported in rodents such as rats, hamsters, gerbils, and spiny mice, which all have been shown to be at least as sensitive to polycose and maltose as to sucrose (Sclafani and Nissenbaum, 1987; Feigin et al., 1987).

The reliability of the preference thresholds established here is supported by one of the few earlier studies that assessed taste responses of nonhuman primates to polycose. Using 24-hr solution versus water tests, Sunderland and Sclafani (1987) reported that squirrel monkeys prefer polycose over water at concentrations of 100 and 200 mM, but not at 10 and 50 mM. Bonnet macaques prefer this polysaccharide over water at all four concentrations.

Although all rodent species tested so far show lower preference threshold values for polycose than the pigtail macaques and, thus, all primates tested here, the similarity in the across-substance patterns of sensitivity between *Macaca nemestrina* and rats, hamsters, gerbils, and spiny mice (cf. Table 1) suggests that this primate species, and perhaps also other members of the genus *Macaca*, may have specialized taste receptors for starch and starch-derived polysaccharides that other primates are thought to lack (Sclafani, 1991).

This supposition is supported by our findings in the second experiment. Table 2 compares the relative taste preferences for suprathreshold concentrations of polycose, maltose, and sucrose in the four primate species tested here with those of other mammalian species obtained with the same or a similar method and with ratings on relative sweetness obtained in humans with psychophysical procedures.

Squirrel monkeys, spider monkeys, and olive baboons clearly preferred sucrose over equimolar concentrations of polycose and maltose and, thus, show a pattern of relative preferences that is similar to the ranking of relative sweetness of these three saccharides found in humans (Feigin et al., 1987). Pigtail macaques, in contrast, were at least as attracted to the polysaccharide and its disaccharide constituent as to sucrose. Their pattern of relative preferences is similar to that found in rats. Using solution versus solution tests of brief duration, a method almost identical to the one employed here, Sclafani and Mann (1987) showed that, at concentrations of 30, 100, and 300 mM, *Rattus norvegicus* prefers polycose and maltose over sucrose and that this ranking of relative preference is reversed only when presenting the saccharides at equimolar concentrations of 500 mM.

The reliability of the relative preferences reported here is supported by earlier studies that assessed relative taste preferences for food-associated mono- and disaccharides in squirrel monkeys (Laska, 1997), spider monkeys (Laska et al.,

TABLE 2. RELATIVE TASTE PREFERENCES FOR POLYCOSE, MALTOSE, AND SUCROSE IN FOUR PRIMATE SPECIES TESTED AND IN OTHER MAMMALIAN SPECIES

Species and concentration (mM)	Rank order of preference
Saimiri sciureus[1a]	
100	sucrose > polycose ≥ maltose
200	sucrose > polycose = maltose
Ateles geoffroyi[1]	
100	sucrose > polycose ≥ maltose
200	sucrose ≥ maltose ≥ polycose
Papio hamadryas anubis[1]	
100	sucrose > polycose > maltose
200	sucrose > polycose ≥ maltose
Macaca nemestrina[1]	
100	maltose > polycose ≥ sucrose
200	maltose = polycose = sucrose
Rattus norvegicus[2]	
30	polycose > maltose > sucrose
100	polycose ≥ maltose ≥ sucrose
300	polycose ≥ sucrose ≥ maltose
500	sucrose > maltose ≥ polycose
Saimiri sciureus[3]	
100	sucrose > maltose = polycose
Macaca radiata[3]	
100	polycose = maltose = sucrose
Homo sapiens[4]	
100	sucrose > maltose = polycose
200	sucrose > maltose > polycose

[a1]Present study, [2]Sclafani and Mann (1987), [3]Sunderland and Sclafani (1988), [4]Feigin et al. (1987) study established relative sweetness rather than preference.

1998), and pigtail macaques (Laska, 2000). Using the same method as in the present study, i.e., solution versus solution tests of brief duration, *Saimiri sciureus* and *Ateles geoffroyi* preferred sucrose over maltose at equimolar concentrations of 50, 100, 200, and 400 mM, respectively, whereas *Macaca nemestrina* preferred maltose over sucrose at all four concentrations.

In line with these findings, Sunderland and Sclafani (1987) reported that squirrel monkeys showed a significantly higher degree of preference for sucrose than for polycose or maltose in 24-hr solution versus water tests using concentrations of 10, 50, 100, and 200 mM, respectively, whereas bonnet macaques displayed preferences for maltose and polycose that were at least as strong as those for sucrose.

The question arises as to possible reasons why the pigtail macaques differ from the other primates tested in their responsiveness to polycose. Old World

primates and New World primates have been shown to differ in their ability to perceive substances such as aspartame and thaumatin, which both taste sweet to humans, suggesting that phylogenetic relatedness might account for differences or correspondences in taste perception among species (Glaser, 1993). However, in the present study, olive baboons, an Old World primate species like the pigtail macaques, showed the same pattern of relative preferences for polycose as squirrel monkeys and spider monkeys, two New World primate species. Thus, it seems unlikely that membership in one of these taxa is responsible for the observed differences among species.

Differences in dietary habits have been shown repeatedly to provide plausible explanations for differences in taste performance among species (Kare, 1971; Pfaffmann, 1977). Among New World primates, for example, the degree of frugivory has been found to correlate positively with sensitivity to food-associated mono- and disaccharides (Laska, 1996). This seems to make sense in terms of optimal foraging theory, as frugivorous species rely on soluble carbohydrates to meet their energy requirements more than nonfrugivores (Laska et al., 1996).

Sclafani (1991) proposed that the high sensitivity and preference for polycose and other starch-derived polysaccharides observed in the rat may also be explained by dietary habits. Anatomical features such as teeth and gut morphology suggest that members of the genus *Rattus* are primarily granivorous, i.e., they feed on a starch-rich diet, lending support to this hypothesis. Although considerable information on the dietary habits of the four primate species employed in the present study are at hand (Clutton-Brock and Harvey, 1977; Caldecott, 1986; Chapman, 1987; Ross, 1992), no definitive conclusions can be drawn at to whether differences in feeding specializations may account for the observed differences in polycose responsiveness, because unfortunately, none of the studies reported the proportion of starch-rich plants in the diet of any species. Furthermore, pigtail macaques do not seem to differ markedly in the proportion of fruits and seeds in their diet (72% of total intake) from olive baboons (63%) and spider monkeys (80%), but only from squirrel monkeys (26%) (Clutton-Brock and Harvey, 1977). Using the proportion of animal matter in the diet as an indicator of the maximally possible proportion of starch-containing plants also shows the squirrel monkey (72%) to be markedly different from the other three species (1–10%), (Clutton-Brock and Harvey, 1977), but nevertheless the squirrel monkeys showed the same pattern of relative preference for polycose as the spider monkeys and the olive baboons. Thus, the ultimate reason for the observed differences in polycose responsiveness between pigtail macaques and olive baboons, spider monkeys, squirrel monkeys, and humans remains elusive. One can speculate, however, as to the possible proximate reason.

In addition to behavioral studies measuring voluntary intake of sapid solutions, two lines of evidence lend further support to the idea that rats have specialized taste receptors for starch and stach-derived polysaccharides. Firstly,

studies using conditioned taste aversion paradigms showed that rats display little cross-generalization between polycose and sucrose, but some degree of cross-generalization between polycose and its disaccharide constituent maltose (Nissenbaum and Sclafani, 1987; Sako et al., 1994). Similar results were obtained using the same method in the hamster, another rodent species that cross-generalized between glycogen and maltose, but not between glycogen and sucrose (Rehnberg et al., 1996). A more recent study showed cross-generalization between sucrose and polycose in the same rodent species, suggesting that the two substances share common perceptual taste characteristics (Formaker et al., 1998). Secondly, electrophysiological studies have shown that the rat's chorda tympani responses to sugars were strongly suppressed by gurmarin, an antisweet peptide, whereas this substance had essentially no effect on polycose responses (Sako et al., 1994). Application of $KHCO_3$ onto the rat's tongue enhanced chorda tympani responses to sugars, but not to polycose (Sako et al., 1994). In line with these findings, single unit activity in the nucleus tractus solitarius of the rat in response to polycose was markedly different from that in response to substances representing the traditional four basic taste qualities (Giza et al., 1991).

To the best of our knowledge, studies on conditioned taste aversion using polycose in nonhuman primates have not been performed, but findings from electrophysiological studies in cynomolgus and rhesus macaques lend some support to the idea that members of the genus *Macaca* might have specialized taste receptors for starch and other polysaccharides. Plata-Salaman et al. (1993) recorded the activity of single neurons in the gustatory cortex of *Macaca fascicularis* in response to 19 chemicals sweet to humans. They found that all 19 substances formed a coherent cluster in the taste space generated from the correlations among patterns of neural activity evoked by the stimuli, suggesting relative similarity of taste quality across substances. Whereas simple carbohydrates such as glucose, fructose, sucrose, and maltose formed the center of this cluster, polycose was somewhat distant from these sugars, indicating some degree of difference in taste quality.

The results of the present study demonstrate that all four species of nonhuman primates employed here are clearly attracted to aqueous solutions of polycose. Further, we showed that pigtail macaques have lower taste preference thresholds for this starch-derived polysaccharide compared to squirrel monkeys, spider monkeys, and olive baboons and that their pattern of relative preference (maltose $>$ polycose $\geq$ sucrose) differs from that of the other three primate species (sucrose $>$ polcose $\geq$ maltose). These findings suggest that *Macaca nemestrina*, unlike other primates, but similar to rodents, may have specialized taste receptors for starch.

Acknowledgments—We thank Andreas Russwurm and Alejandra Arango Cessa for help in collecting data, and the Deutsche Forschungsgemeinschaft (La 635/10-1), the Volkswagen Foundation (I/75 354), and Patronato Pro-Universidad Veracruzana for financial support. L.T.H.S. was awarded a grant by Conacyt Mexico (no.124786).

REFERENCES

ASTM, 1973. Compilation of odor and taste threshold values data. Data Series DS 48. American Society for Testing and Materials, Philadelphia, Pennsylvania.

BEAUCHAMP, G. K., MALLER, O., and ROGERS, J. G. 1977. Flavor preferences in cats (*Felis catus* and *Panthera* sp.). *J. Comp. Physiol. Psychol*. 91:1118–1127.

CALDECOTT, J. O. 1986. An Ecological and Behavioural Study of the Pig-Tailed Macaque. Contributions to Primatology, Vol. 21. Karger, Basel.

CHAPMAN, C. A. 1987. Flexibility in the diets of three species of Costa Rican primates. *Folia Primatol*. 49:90–105.

CLUTTON-BROCK, T. H. and HARVEY, P. H. 1977. Species differences in feeding and ranging behaviour in primates, pp. 557–584, *in* T. H. Clutton-Brock (ed.). Primate Ecology. Academic Press, New York.

FEIGIN, M. B., SCLAFANI, A., and SUNDAY, S. R. 1987. Species differences in polysaccharide and sugar taste preferences. *Neurosci. Biobehav. Rev*. 11:231–240.

FORMAKER, B. K., Kearns, C. E., and FRANK, M. E. 1998. The taste of polycose in hamsters. *Chem. Senses* 23:675–682.

GIZA, B. K., SCOTT, T. R., SCLAFANI, A., and ANTONUCCI, R. F. 1991. Polysaccharides as taste stimuli: Their effect in the nucleus tractus solitarius of the rat. *Brain Res*. 555:1–9.

GLASER, D. 1993. The effects of sweeteners in primates, pp. 353–363, *in* M. Mathlouti, J. A. Kanters, and G. G. Birch (eds.). Sweet-Taste Chemoreception. Elsevier, London.

GLASER, D. 1986. Geschmacksforschung bei Primaten. Vjschr. *Naturforsch. Ges. Zurich* 131:92–110.

HETTINGER, T. P., FRANK, M. E., and MYERS, W. E. 1996. Are the tastes of polycose and monosodium glutamate unique? *Chem. Senses* 21:341–347.

HIZUKURI, S. 1996. Starch: Analytical aspects, pp. 347–429, *in* A. C. Eliasson (ed.). Carbohydrates in Food. Marcel Dekker, New York.

KARE, M. 1971. Comparative study of taste, pp. 278–292, *in* L. M. Beidler (ed.). Handbook of Sensory Physiology, Vol. IV, Part 2. Springer, Berlin.

LASKA, M. 1994. Taste difference thresholds for sucrose in squirrel monkeys (*Saimiri sciureus*). *Folia Primatol*. 63:144–148.

LASKA, M. 1996. Taste preference thresholds for food-associated sugars in the squirrel monkey, *Saimiri sciureus*. *Primates* 37:93–97.

LASKA, M. 1997. Taste preferences for five food-associated sugars in the squirrel monkey (*Saimiri sciureus*). *J. Chem. Ecol*. 23:659–672.

LASKA, M. 1999. Taste responsiveness to food-associated acids in the squirrel monkey (*Saimiri sciureus*). *J. Chem. Ecol*. 25:1623–1632.

LASKA, M. 2000. Gustatory responsiveness to food-associated sugars and acids in pigtail macaques *Macaca nemestrina*. *Physiol. Behav*. 70:495–504.

LASKA, M., CARRERA SANCHEZ, E., RODRIGUEZ RIVERA, J. A., and RODRIGUEZ LUNA, E. 1996. Gustatory thresholds for food-associated sugars in the spider monkey (*Ateles geoffroyi*). *Am. J. Primatol*. 39:189–193.

LASKA, M., CARRERA SANCHEZ, E., and RODRIGUEZ LUNA, E. 1998. Relative taste preferences for food-associated sugars in the spider monkey (*Ateles geoffroyi*). *Primates* 39:91–96.

LASKA, M., SCHEUBER, H.-P., CARRERA SANCHEZ, E., and RODRIGUEZ LUNA, E. 1999a. Taste difference thresholds for sucrose in two species of nonhuman primates. *Am. J. Primatol*. 48:153–160.

LASKA, M., SCHÜLL, E., and SCHEUBER, H.-P. 1999b. Taste preference thresholds for food-associated sugars in baboons, *Papio hamadryas anubis*. *Int. J. Primatol*. 20:25–34.

LASKA, M., HERNANDEZ SALAZAR, L. T., RODRIGUEZ LUNA, E., and HUDSON, R. 2000. Gustatory responsiveness to food-associated acids in the spider monkey (*Ateles geoffroyi*). *Primates* 41:175–183.

NISSENBAUM, J. W. and SCLAFANI, A. 1987. Qualitative differences in polysaccharide and sugar tastes in the rat: A two-carbohydrate taste model. *Neurosci. Biobehav. Rev.* 11:187–196.

PFAFFMANN, C. 1977. Biological and behavioral substrates of the sweet tooth, pp. 3–24, *in* J. M. Weiffenbach (ed.). Taste and Development. The Genesis of Sweet Preference. US Government Printing Office, Washington, DC.

PLATA-SALAMAN, C. R., SCOTT, T. R., and SMITH-SWINTOSKY, V. L. 1993. Gustatory neural coding in the monkey cortex: The quality of sweetness. *J. Neurophysiol*. 69:482–493.

REHNBERG, B. G., MACKINNON, B. I., HETTINGER, T. P., and FRANK, M. E. 1996. Analysis of polysaccharide taste in hamsters: Behavioral and neural studies. *Physiol. Behav*. 59:505–516.

RICHTER, C. P. and CAMPBELL, K. H. 1940. Taste thresholds and taste preferences of rats for five common sugars. *J. Nutr.* 20:31–46.

ROSS, C. 1992. Basal metabolic rate, body weight and and diet in primates: An evaluation of the evidence. *Folia Primatol*. 58:7–23.

SAKO, N., SHIMURA, T., KOMURE, M., MOCHIZUKI, R., MATSUO, R., and YAMAMOTO, T. 1994. Differences in taste responses to polycose and common sugars in the rat as revealed by behavioral and electrophysiologcal studies. *Physiol. Behav*. 56:741–745.

SCLAFANI, A. 1991. Starch and sugar tastes in rodents: An update. *Brain Res. Bull*. 27:383–386.

SCLAFANI, A. and CLYNE, A. E. 1987. Hedonic response of rats to polysaccharide and sugar solutions. *Neurosci. Biobehav. Rev*. 11:173–180.

SCLAFANI, A. and MANN, S. 1987. Carbohydrate taste preferences in rats: Glucose, sucrose, maltose, fructose and polycose compared. *Physiol. Behav*. 40:563–568.

SCLAFANI, A. and NISSENBAUM, J. W. 1987. Taste preference thresholds for polycose, maltose, and sucrose in rats. *Neurosci. Biobehav. Rev*. 11:181–185.

SIEGEL, S. and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill, New York.

SPECTOR, A. C. 2000. Linking gustatory neurobiology to behavior in vertebrates. *Neurosci. Biobehav. Rev*. 24:391–416.

SUNDERLAND, G. and SCLAFANI, A. 1988. Taste preferences of squirrel monkeys and bonnet macaques for polycose, maltose and sucrose. *Physiol. Behav*. 43:685–690.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

Spodoptera exigua OVIPOSITION AND LARVAL FEEDING PREFERENCES FOR PIGWEED, *Amaranthus hybridus*, OVER SQUARING COTTON, *Gossypium hirsutum*, AND A COMPARISON OF FREE AMINO ACIDS IN EACH HOST PLANT

ALLAN T. SHOWLER

Kika de la Garza Subtropical Agricultural Research Center, USDA-ARS
2413 E. Highway 83, Bldg. 201
Weslaco, Texas 78596

(Received March 28, 2001; accepted June 14, 2001)

Abstract—The beet armyworm, *Spodoptera exigua* (Hübner), can utilize a number of different host plants for oviposition and larval development, but some host plants are preferred over others. This study, using cage choice tests and olfactometer assays, demonstrates the beet armyworm's preference for pigweed, *Amaranthus hybridus* L., over cotton, *Gossypium hirsutum* L. Cage and olfactometer choice assays indicated that olfaction plays an important role in host selection by ovipositing females. First instars exhibited no feeding preference, but the more mobile third instars showed a significant feeding preference for excised pigweed leaves. The higher quantities and more diverse accumulations of free amino acids in pigweed might, in conjunction with other physiochemical and mechanical attractant and deterrent factors in the two-plant species system, play roles in the tendency of the beet armyworm to select pigweed over cotton and in providing a superior array of easily absorbed amino acids as compared to cotton.

Key Words—*Gossypium hirsutum*, cotton, *Amaranthus*, pigweed, beet armyworm, *Spodoptera exigua*, host plant selection, oviposition, free amino acids.

INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner), is known to feed on more than 50 plant species from over 10 families worldwide (Wilson, 1932; Smits et al., 1987). It originated in southern Asia and was introduced to the United States in Oregon in 1876 and again in California in 1882 (Wilson, 1932). It has since become

0098-0331/01/1000-2013$19.50/0

a pest of many crops in the United States and Mexico. In cotton, the beet armyworm is deleterious especially when pesticide applications disrupt beneficial arthropod populations (Eveleens et al., 1973; Raulston et al., 1997). However, Ali and Gaylor (1992) determined that beet armyworm larvae took longer to develop, had lower pupal weight, and required a greater number of stadia when raised on cotton, *Gossypium hirsutum* L., as compared to artificial diet or pigweed, *Amaranthus hybridus* L., a preferred wild host (Taylor, 1931). Similarly, *Amaranthus* spp. and *Portulaca oleracea* L. were found to be the most suitable hosts for *Spodoptera latifascia* (Walker) based on larval and pupal weights, survival, and development times in contrast to host plants such as maize, *Zea mays* (L.), sorghum, *Sorghum bicolor* (L.), *Ixophorus unisetus* (Presl.) Schlect., *Ipomoea* sp., and *Melampodium divaricatum* (Rich. ex. Pers.) Dc. (Portillo et al., 1998). *Spodoptera latifascia* was unable to utilize sorghum and maize sufficiently to sustain growth and development, but no evidence of decreased oviposition preference for these crop hosts was observed (Portillo, 1994). Despite heavy damage to sorghum and maize caused by *S. latifascia*, these crop plants are relatively unsuitable hosts for larval development (Portillo et al., 1998).

It is known that cotton leaves contain chemicals, including volatile terpenoids, that attract and/or deter herbivorous arthropods (Harborne, 1988). Gossypol has been shown to reduce beet armyworm larval weights, increase the length of the egg to adult development interval, and cause mortality (Bottger and Patana, 1966). Hairs, trichomes, nectaries, other deterrents and attractants (Schuster and Maxwell, 1974; Reynolds et al., 1975; Hassan et al., 1990; Ring et al., 1993), and various ecological factors (Berdegué et al., 1998) can also influence preference for one plant or another.

Host plant free amino acid accumulations have been associated with higher numbers of nematodes (Showler et al., 1990) and arthropods (Jayaraj and Seshadri, 1967; Blua et al., 1994). Gravid beet armyworms appear to prefer to oviposit on drought-stressed cotton (Ruberson et al., 1994; Ruberson, 1996), and it is known that water-deficit stress in many plants results in elevated levels of some free amino acids (Labanauskas et al., 1981; Shen et al., 1989; Gzik, 1996). Feeding behavior of tobacco budworm, *Heliothis virescens* (F.), corn earworm, *Heliothis armigera* (Hübner), and *Spodoptera littoralis* (Boisdval) adults was correlated with the electrophysiological response of sensilla on the proboscis. Responses of the sensilla to amino acids and sugar were correlated to feeding behavior of each of these species (Blaney and Simmonds, 1988).

This study compares the oviposition and feeding preference of beet armyworm adults and larvae for pigweed or early squaring cotton. The free amino acid analyses of both plant species might indicate that, in addition to volatiles and other chemical and mechanical defenses, free amino acid accumulations might play a role in host plant selection and contribute to the nutritional value of the preferred host plant species being compared.

METHODS AND MATERIALS

Oviposition Preference. Beet armyworm larvae were obtained from a laboratory colony at the Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas, and reared on a soybean-wheat germ diet (Shaver and Raulston, 1971). The insects were maintained in environmental chambers at 30°C, 85% relative humidity, and a 13L : 11D photoperiod. Pupae were sexed and placed in individual cups until adult emergence. On the day of emergence, females were paired individually with single males in 0.5-liter cardboard containers. The next day, females were placed alone in a container lined with wax paper as oviposition substrate. Each container was monitored daily for the appearance of the first egg mass, which signified the presence of a gravid female moth. Different females were used for each replication.

Gravid beet armyworm females were provided a choice of cotton or pigweed for oviposition in 1.8- × 1.8- × 1.1-m cages. Since maximum egg-laying occurs during the first and second nights of ovipositional activity (Fye and McAda, 1972; Tisdale and Sappington, 2000), only females that had initiated ovipositional activity on the previous night were used in the oviposition choice assays. Four 7.5-liter pots of cotton plants (three per pot) at early squaring (diameter 3–5 mm), and four pots of pigweed plants (three per pot) at heading stage were randomly placed within each cage. Four gravid beet armyworm females were placed in each cage and allowed to oviposit for 48 hr, then each plant was inspected for egg masses. The assay was replicated 30 times during May–July 2000.

In order to determine whether female beet armyworms select the host plants by olfaction, a 78- × 32- × 38-cm introduction chamber with screen ventilation on three sides and Plexiglas on the front was connected to two separate 90- × 40- × 40-cm plywood choice chambers (each with a wire mesh back door) using two 25-cm-diameter × 35-cm-long white plastic tubes; one tube connected one choice chamber to the Plexiglas front panel of the introduction chamber, and the other tube connected from a second choice chamber to the front panel of the introduction chamber. An electric fan (20 cm diam.) was placed 1.5 m behind each choice chamber so that airflow through the tubes was about 0.4 m/sec. The introduction chambers were positioned at the open entrance of a 4.9-m-wide workshop door so that volatiles blown through the chambers would dissipate into the atmosphere (pilot tests using colored smoke demonstrated air flow). A 7.5-liter pot containing three pigweed plants at the heading stage was placed at random in one of the choice chambers and a 7.5-liter pot containing three cotton plants at the early squaring stage was placed in the other choice chamber. The plants were not tall enough to be seen through the plastic connecting pipes. Five gravid female beet armyworm moths were placed in the introduction chamber. After 48 hr, the host plants in the choice chambers and the walls of the choice chambers were examined for egg clusters. This experiment was replicated 30 times.

Feeding Preference of Larvae. Ten beet armyworm first instars were placed in covered, ventilated Petri dishes containing one cotton leaf of intermediate age and one pigweed leaf of similar size (17.40 ± 0.13 and 17.14 ± 0.15 cm^2, respectively, $N = 40$, $P = 0.203$) each with the petiole inserted through the cap of a 0.6-ml plastic vial of water to maintain leaf turgor. The two leaves were placed 2 cm apart, and the larvae were introduced 2 cm away from either leaf. This arrangement was replicated 100 times. After being stored for 24 hr at ambient room temperature ($\approx$23.9°C, 13L : 11D photoperiod), larval positions relative to the leaves were recorded.

In a separate Petri dish assay, one third instar was placed equidistant from a pigweed leaf and a cotton leaf. Larval position relative to the leaves was recorded after 1, 4, 6, and 24 hr. Each of the 30 replications was comprised of 10 Petri dishes.

Analysis of Free Amino Acids. Fully extended leaves among the topmost six leaves on the plant were taken from six different cotton plants at the squaring stage, and leaves from six different pigweed plants at head stage were collected. These 12 samples were immediately used to obtain 1 g leaf material per sample and completely homogenized with 10 ml 0.1 N HCl using a Virtishear homogenizer (Virtis, Gardiner, New York). At least 5 g homogenate from each sample was placed in separate 10-ml tubes and centrifuged at 10,000 rpm for 30 min. Samples were stored at −80°C.

One ml of supernatant from each sample was passed through a 0.5-μm filter fitted to a 5-ml plastic syringe. Samples were placed in the autosampler of an Agilent (Agilent Technologies, Atlanta, Georgia) reversed-phase high-performance liquid chromatograph (HPLC) with a binary pump delivering solvent A [1.36 g sodium acetate trihydrate + 500 ml purified HPLC grade water + 150 μl triethylamine (TEA) + sufficient acetic acid to bring pH to 7.2 ± 0.05] and solvent B [1.36 g sodium acetate trihydrate + 100 ml purified HPLC grade water (acetic acid added to this mixture to bring pH to 7.2 ± 0.05) + 200 ml acetonitrile + 200 ml methanol] at 0.3 and 0.45 ml/min on a concave gradient through a C_{18} fluorescence column. Absorbances at 262 and 338 nm were monitored on a variable wavelength detector for 40 min/sample. The autosampler measured and mixed a total of 6 μl sodium borate buffer (0.4 N, pH 10.2 in water), 1 μl 9-fluorenylmethylchloroformate (FMOC), and 1 μl orthophthalaldehyde (OPA) derivatizing agents, and 2 μl of sample; then 2 μl was injected for chromatographic separation of free amino acids. Identification and quantification of derivatized free amino acids was achieved by calibrating with a standard mixture of amino acids. Peak integration accuracy was enhanced by manual establishment of peak baselines.

For normally distributed data, statistical differences were determined using the paired t test, and for nonparametric data, Yates' corrected χ^2 test was used (Analytical Software, 1998). All experiments were conducted between March and July 2000.

RESULTS

Oviposition Preference. In the cage assay, beet armyworm females oviposited 4.69 times more egg clusters on pigweed than on cotton (Table 1). On pigweed, the mean number of eggs was 4.55 times higher than that deposited on cotton, and the number of eggs per cluster was 1.12 times higher on pigweed than on cotton. In the olfactometer assay, 3.27 times more egg clusters were found on pigweed than on cotton (Table 1). Pigweed had 3.44 times more eggs compared to cotton, and the number of eggs per cluster was 1.03 times higher on pigweed than on cotton. There were 2.05 times more egg clusters deposited on the choice chamber walls and the plant pots in the cotton chambers (1.23 ± 0.21) than in the pigweed chambers (0.60 ± 0.14; $\chi^2 = 2.74$; $df = 29$, $P = 0.098$)

Feeding Preference of Larvae. First-instar beet armyworms showed no preference for excised cotton (4.68 ± 0.24 larvae/leaf) or pigweed (4.29 ± 0.23 larvae/leaf) leaves ($\chi^2 = 0.76$, $df = 99$, $P = 0.38$). Once first instars found either of the hosts, they stayed on them. However, third instars displayed a preference for pigweed over cotton ($\chi^2_{24\text{hr}} = 44.8$, $df = 29$, $P < 0.0001$) at each of the four time intervals (Figure 1). After 24 hr, pigweed leaves that had been chosen were usually chewed to the midribs and some larvae consumed the petioles inside of the plastic water capsules rather than moving over to untouched cotton leaves. Those larvae that fed on the cotton leaves consumed a visually estimated 25% or less than the pigweed leaf area eaten.

TABLE 1. EGGS, CLUSTERS, AND EGGS/CLUSTER OVIPOSITED BY BEET ARMYWORMS ON PIGWEED AND COTTON: CAGE AND OLFACTOMETER CHOICE ASSAYS

Experiment and host	Eggs[a]	Clusters[a]	Eggs/cluster[b]
Cage			
Cotton	115.2 ± 36.0^c	2.2 ± 0.6^c	48.7 ± 5.2^c
Pigweed	524.5 ± 33.3^c	10.2 ± 0.8^c	54.4 ± 2.6^c
χ^2	4375.2	85.5	
t			0.49
P	<0.0001	<0.0001	0.63
Olfactometer			
Cotton	137.0 ± 14.6^c	2.9 ± 0.3^c	48.5 ± 2.0^c
Pigweed	471.9 ± 53.8^c	9.5 ± 1.1^c	49.9 ± 1.3^c
χ^2	2989.0	55.2	
t			0.51
P	<0.0001	<0.0001	0.61

[a]Means for cotton and pigweed ($N = 30$) compared within each assay using χ^2.
[b]Means in cage and olfactometer assays for cotton ($N = 17$ and 27, respectively) and pigweed ($N = 30$ of each plant). Compared within each assay using t test.
[c]Mean ± SE.

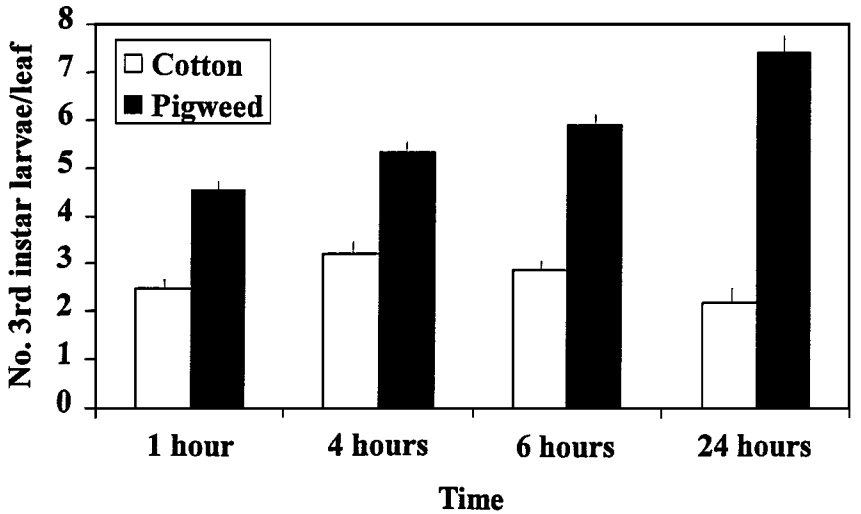


FIG. 1. Numbers of third instar beet armyworms (±SE) (one larva used per dish, 10 dishes per replication) on pigweed and cotton leaves over time in excised leaf choice assay ($N = 30$).

Analysis of Free Amino Acids. Free alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, threonine, and valine were detected in either or both cotton and pigweed (Table 2). Free cysteine, lysine, methionine, and tyrosine were not found in any of the samples. Other free amino acids, such as tryptophan, could not be detected. Twelve of the 17 detectable free amino acids were found in pigweed. Ten to 12 of the detectable free amino acids were found in cotton; the variation occurred because, in some cotton plants, free histidine and free phenylalanine were absent.

Pigweed leaves contained 1.86 times ($t = 3.91$, $df = 5$, $P = 0.0002$) the quantity of total free amino acids as compared to cotton. The greater accumulation of total free amino acids in pigweed over cotton largely resulted from higher concentrations of arginine (5.28 times, $t = 7.19$, $df = 5$, $P = 0.0008$), glutamic acid (1.85 times, $t = 3.53$, $df = 5$, $P = 0.0168$), glycine (4.03 times, $t = 2.73$, $df = 5$, $P = 0.0415$), histidine (98.03 times, $t = 8.12$, $df = 5$, $P = 0.0005$), leucine (absent in cotton, $t = 3.94$, $df = 5$, $P = 0.0104$), proline (2.27 times, $t = 2.76$, $df = 5$, $P = 0.0396$), and serine (2.25 times, $t = 2.39$, $df = 5$, $P = 0.0621$). In cotton, four free amino acids occurred in significantly higher quantities than in pigweed: alanine (absent in pigweed, $t = 11.11$, $df = 5$, $P = 0.0001$), isoleucine (2.88 times; $t = 3.79$; $df = 5$; $P = 0.0128$), phenylalanine (2.65 times, $t = 9.45$, $df = 5$, $P = 0.0002$), and threonine (3.07 times, $t = 5.16$, $df = 5$, $P = 0.0086$).

Amino acids that are indispensable to insects are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and

TABLE 2. FREE AMINO ACID (FAA) CONCENTRATIONS IN PIGWEED AND COTTON LEAVES

FAA	Concentration (pmoles/μl extract, mean $\pm$ SE)		P^a
	Pigweed	Cotton	
Alanine	0	1029.2 $\pm$ 3.7	0.0001
Arginine	4898.0 $\pm$ 500.2	927.02 $\pm$ 71.14	0.0008
Aspartic acid	1741.1 $\pm$ 137.6	2343.4 $\pm$ 278.4	0.1279
Glutamic acid	4819.0 $\pm$ 391.6	2603.2 $\pm$ 348.2	0.0168
Glycine	196.5 $\pm$ 53.9	48.8 $\pm$ 3.8	0.0415
Histidine	3808.4 $\pm$ 463.5	38.8 $\pm$ 1.5	0.0005
Isoleucine	316.1 $\pm$ 49.9	909.4 $\pm$ 109.6	0.0128
Leucine	54.6 $\pm$ 13.8	0	0.0104
Phenylalanine	121.7 $\pm$ 9.8	322.7 $\pm$14.2	0.0002
Proline	49.7 $\pm$ 5.4	21.9 $\pm$ 8.0	0.0396
Serine	1272.6 $\pm$ 195.0	566.6 $\pm$ 138.4	0.0621
Threonine	164.1 $\pm$ 32.8	503.4 $\pm$ 38.8	0.0036
Valine	109.0 $\pm$ 23.1	116.9 $\pm$ 27.9	0.7670
Total free essential amino acids	9471.8 $\pm$ 980.5	2818.2 $\pm$ 184.5	0.0020
Total FAAs	17551.0 $\pm$ 1675.5	9431.3 $\pm$ 576.0	0.0112

[a] Level of significance shown in right column determine by paired t test; $N = 6$.

valine, although there are variations with regard to different insect species (Chapman, 1971). The HPLC was able to detect all of the free essential amino acids except tryptophan. Of the nine detectable free essential amino acids, cotton had up to six (arginine, histidine, isoleucine, phenylalanine, threonine, and valine), and pigweed had seven (arginine, histidine, isoleucine, leucine, phenylalanine, threonine, and valine). In addition to having one more free essential amino acid than cotton, pigweed had 3.36 times higher concentration of total free essential amino acids ($t = 5.92$; $df = 5$; $P = 0.0020$) than cotton (Figure 2). In cotton, free essential amino acids constituted 29.88% of the total amount of free amino acids; in pigweed free essential amino acids comprised 53.97% of the total quantity of detectable free amino acids. Three of cotton's six free essential amino acids were significantly more abundant in cotton than in pigweed: isoleucine, phenylalanine, and threonine.

DISCUSSION

Whatever the interplay of mechanisms or cues that govern beet armyworm oviposition preference for one host plant or another, the extent to which the beet armyworm propagates itself is in the balance. Many insects prefer to oviposit on

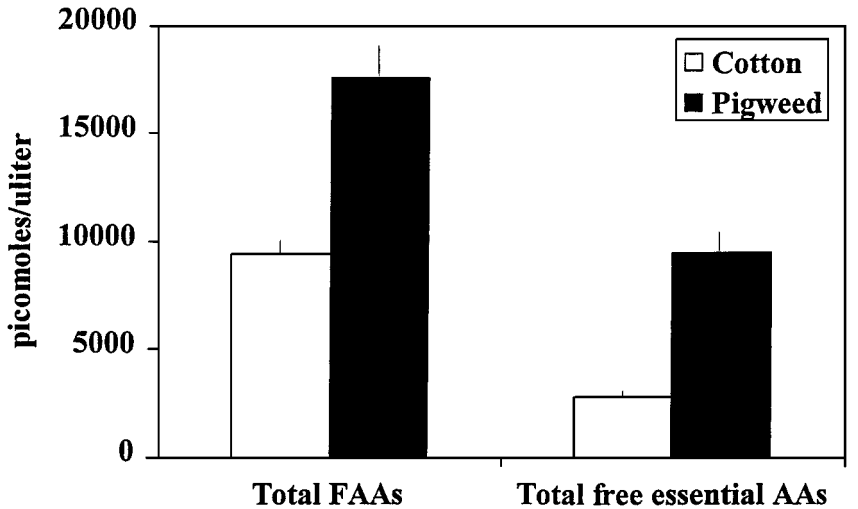


FIG. 2. Picomoles (±SE) of total free amino acids in pigweed and cotton contrasted to quantities of free essential amino acids ($N = 6$). Cotton and Pigweed had significantly different ($p \leq 0.05$) quantities of total free amino acids, and total free essential amino acids.

certain plant species because those hosts enhance larval development and survival by providing a suitable diet (Singer, 1972, 1983), although there are exceptions (Wiklund, 1974; Courtney, 1981). Berdegué et al. (1998) demonstrated that beet armyworm choice of oviposition host was not molded by the nutritional quality of the two common host plants studied, *Chenopodium murale* L. and *Apium graveolens* L. Thompson (1988) described four ecologically based hypotheses that suggest selection pressures acting alone or together help explain host plant preferences, in addition to nutrition and attractive and deterrent plant volatiles.

In the time hypothesis, which seems to best explain the result of the beet armyworm's choice between *C. murale* and *A. graveolens* (Berdegué et al., 1998), females may oviposit on unsuitable novel hosts without the benefit of evolutionary time to select away from detrimental behavior. Examples of this involve introduced insects with novel plants or the introduction of exotic plants where the insects are indigenous (Straatman, 1962; Chew, 1977; Rodman and Chew, 1980; Legg et al., 1986). The patch dynamic hypothesis predicts that eggs will be laid mostly on the plant host that they encounter more frequently, but this fails to explain examples of strong preference for scattered weed species in large monocultures (Koike et al., 1996). In the enemy-free-space hypothesis, herbivores might prefer nutritionally inferior host plants if they offer superior protection from natural enemies (Kester and Barbosa, 1991; Fox and Eisenbach, 1992; Benrey and Denno, 1997). Further,

volatiles emitted by cotton leaves damaged by feeding larvae can alert biological control agents to their presence (Elzen et al., 1984; Tumlinson et al., 1992). The parasite/grazer hypothesis suggests that herbivores that can develop on more than one host are less likely to exhibit strong host plant oviposition preferences than are herbivores that can develop on only one host plant (Thompson, 1988). Although the beet armyworm adult and larval choice of pigweed over cotton could involve one or more of these explanations, they do not identify the cues that the insect responds to in making the choice.

Cotton leaves and squares emit volatile terpenoids and other compounds (Thompson et al., 1971), the composition of which can change in response to various stimuli (Heath and Manukin, 1992; Surburg et al., 1993). Loughrin et al. (1995) found that 23 volatiles are emitted from cotton leaves by damaged beet armyworm larvae (terpenes and lipoxygenase-hydroperoxidase lyase-derived volatiles predominated). Although there were no apparent differences in volatile emission among different varieties of undamaged cotton, average volatile emission from damaged leaves of a naturalized variety was about 7 times higher than from damaged leaves of commercial varieties. In choice tests, however, the larvae preferred feeding on leaves of the commercial cultivars. Huang and Renwick (1992) showed that the oviposition preference for host plants of *Pieris rapae* L. and *P. oleracea* Harris were explained in most cases by the presence of stimulants and deterrents in plant extracts. The two *Pieris* species apparently evolved differential sensitivities to the chemical stimuli that trigger or deter oviposition. The balance of positively and negatively interpreted sensory signals evoked by plant chemicals appears to have played an important role in acceptance or rejection of a plant by both *Pieris* species. Such intermingling of cues, however, makes definitive identification of chemical causal agents challenging. Attraction of tobacco budworm (Tingle and Mitchell, 1992), pink bollworm, *Pectinophora gossypiella* (Saunders) (Wiesenborn and Baker, 1990), and the boll weevil, *Anthonomus grandis* Boheman (Geuldner et al., 1970; Chang et al., 1985; Dickens, 1990) to cotton blossom volatiles has been demonstrated, and green leaf volatiles linalool, myrcene, and benzaldehyde were shown to elicit responses in the beet armyworm (Dickens et al., 1993). Cotton plants with gossypol, however, were less preferred as hosts to many herbivorous arthropods (Bottger et al., 1964; Maxwell et al., 1965; Lukefahr and Martin, 1966). Hedin et al. (1991) showed that female tobacco budworm moths oviposited in the terminals of the cotton plant; the hatched larvae migrated to the terminal area and then to small squares on which they fed, finally burrowing into the anthers where they developed. Calyx crowns of resistant lines are high in terpenoid aldehydes, including gossypol. The cage and olfactometer assays in this study suggest that volatiles emitted from cotton and pigweed play roles in the tendency for beet armyworms to oviposit on pigweed, which lacks gossypol. That the numbers of egg clusters deposited on cotton choice chamber walls and plant pots were double those on the pigweed choice chambers further suggests that, although not

statistically significant ($P = 0.098$), cotton may be less preferred than pigweed once the moths entered the choice chambers.

Yoshida and Parella (1992) concluded that factors other than total nitrogen and leaf moisture may influence the nutritional ecology of *S. exigua* in the case of various chrysanthemum varieties. As little as 0.1% concentration of gossypol in the diet reduced weights of larval beet armyworm, boll weevil, cotton bollworm, *H. zea* (Boddie), cabbage looper *Trichoplusia ni* (Hübner), and salt-marsh caterpillar *Estigmene acrea* (Drury) and increased the number of days required for development from eggs to adults (Bottger and Patana, 1966). Gossypol, generally accepted as being an element of cotton's resistance to some pests (Lukefahr and Martin, 1966), was found to be present in equal quantities in both susceptible and resistant lines, while the hemigossypolone and heliocides H_1 and H_2 were greatly increased in resistant lines (Hedin et al., 1991).

In the case of the common cutworm, *S. litura* F., larvae fed less as the quality of their diet declined. The longer development time, involving extra stadia, permitted the larvae to consume as much as those raised on a better diet (Itoyama et al., 1999). This accounts for the conclusion of Ali and Gaylor (1992) that cotton is a relatively poor host plant for the beet armyworm. In this experiment, first-instar beet armyworms fed on the plant, cotton or pigweed, that they first encountered regardless of nutritional value, or the presence of deleterious chemicals or any mechanical defenses (e.g., trichomes and hairs). The limited mobility of first-instar beet armyworms makes them highly dependent on the female parent's ability to select the most nutritious host. The more mobile third-instar beet armyworm (Smits et al., 1987), however, exhibited a significant tendency to feed on pigweed even after 1 hr had elapsed following their release. The mechanism for making the selection, however, could not be determined in this study. Many of the larvae moved about the Petri dish and over both leaves before settling onto one or the other to feed. However, the third instars did tend to choose the plant species with the higher amounts of more diverse free amino acids. Third-instar beet armyworms can move from one plant to another, but it is likely that the need for mobility arises primarily when the larvae defoliate leaves and must move on to find other leaves on the same plant. In the case of first- and third- instar beet armyworms, survival and vitality of progeny probably depend heavily on host selection by the gravid female.The results of Itoyama et al. (1999) also agree with the observation that beet armyworm third instars consumed less cotton leaf area than pigweed leaf area during 48 hr intervals. Nevertheless, similar to the findings of Portillo (1994) using *S. latifascia*, the beet armyworm will oviposit on cotton and can inflict economic damage.

In this two-plant system, it appears that gravid beet armyworms tend to negatively interpret the blend of volatiles emanating from cotton leaves and, thus, apparently orient mostly toward pigweed, although it might also be that the insect

orients toward pigweed because it is a superior food source. Cavin and Rodriguez (1988) found that β-carbolene alkaloids are found worldwide among many plant families. Chronic dietary exposure tests of harman, harmine, and other simple β-carbolene alkaloids for activity against beet armyworm revealed potent antifeedant and possible toxic effects. It seems that in this study, pigweed did not repel or deter oviposition and larval feeding, and, in agreement with Ali and Gaylor (1992), larval development did not appear to be impeded by nutritional deficiencies or toxins. Blaney and Simmonds (1988) correlated feeding behavior of tobacco budworm, corn earworm, and *S. littoralis* adults with the electrophysiological response on sensilla on the proboscis. Assuming that beet armyworms can detect amino acids and sugars, that such detection has a role in determination of host plants for oviposition, and that foliar free amino acid content is detected by the sensilla, the greater levels of important unbound amino acids in pigweed help to explain the oviposition preference for pigweed. Al-Zubaidi and Capinera (1984) demonstrated that beet armyworm larval development time and mortality were decreased and egg production was increased by nitrogen application to host plants, including pigweed. Foliage became more digestible and efficiency of conversion of ingested and digested food increased as nitrogen levels increased. Nitrogen application to plants has been shown to result in increased levels of free amino acids (Magalhaes et al., 1995).

Insect physiologists have shown that individual amino acids differ greatly in their effect on insect performance; subsets of amino acids are the mechanistic basis of the correlations between total plant nitrogen and insect performance. Synthetic diets mimicking amino acid distribution in anthers were found to be successful for tobacco budworm larval growth and development (Hedin et al., 1991). At least to some extent, nutritional strength has been linked with insect host plant preference (Scribner and Slansky, 1981; Bernays and Chapman, 1994), and higher levels of readily available (unbound) nitrogen presumably improve insect development (Helms et al., 1971). Similarly, host plant increases in concentrations of certain free amino acids have been shown, in some cases, to be preferred by herbivorous insects. Studies show that plants stressed by moisture (Labanauskas et al., 1981; Shen et al., 1989; Gzik, 1996; Showler, in preparation) and diseases (Seitz and Hochster, 1964; Jensen, 1969; Lodh et al., 1971) accumulate significantly higher quantities of some free amino acids, especially proline. McQuate and Connor (1990) demonstrated that mildly water-deficit-stressed soybeans were preferred by larvae of Mexican bean beetle, *Epilachna varivestis* Mulsant. Beet armyworm eggs were reported as being most concentrated on stressed cotton plants in fields (Ruberson et al., 1994; Ruberson, 1996). Jayaraj and Seshadri (1967) found that nymphs of *Empoasca kerri* Pruthi were consistently more abundant on the leaves of pigeon pea, *Cajanus cajan* (L.) Millsp., infected with a mosaic virus than they were on healthy plants. The infected leaves

had a higher content of total nitrogen, peptides, and free amino acids than the healthy plants. Several species of aphids have been shown to benefit more from plants infected with viruses than from uninfected plants (Kennedy, 1951; Ajayi and Dewar, 1982; Blua et al., 1994), and other studies demonstrate altered free amino acid concentrations in virus-infected plants (Singh et al., 1974; Showler et al., 1990).

Insects absorb nitrogen through the gut primarily in the form of free amino acids or very small peptides (Brodbeck and Strong, 1987). Thus, the initial cost of proteolysis is saved if amino acids are ingested in this form. The distinction between free amino acids and those bound in proteins may be important for insects that are physiologically incapable of ingesting large peptides (e.g, aphids), but this issue is separate from the energy costs of proteolysis. In terms of optimizing insect development, it is probable that the balance between different amino acids is particularly important (Chapman, 1971). Vanderzant (1958), for example, found that although pink bollworm larvae could survive on a diet that included only the essential amino acids, growth was slow and adults were small. The inclusion of certain dispensable amino acids was necessary for optimum growth and development, and in some cases, amino acids such as glycine have been shown to be essential (Hinton et al., 1951; Davis, 1956). It is suggested that beet armyworm larvae obtain a less diverse source of readily available (unbound) essential amino acids on cotton (because cotton leaves lack free leucine and sometimes free histidine and free phenylalanine) than on pigweed. Those essential amino acids that are not available in the soluble form in pigweed (alanine, lysine, and methionine) and cotton (leucine, lysine, and methionine) can be acquired in varying extent through proteolysis of foliar proteins. Pink bollworm larvae raised on a diet in which one of the essential amino acids was omitted grew slowly and failed to survive beyond the second instar (Vanderzant, 1958). Increased glutamic acid caused growth stimulation in pink bollworm, and this could also be the case with the higher concentrations of glutamic acid in pigweed than in cotton. Vanderzant (1958) also determined that increased arginine and histidine was associated with larger pink bollworm larvae, so it is possible that the higher accumulation of free essential amino acids and total free amino acids could make pigweed more nutritious and attractive to the beet armyworm. The greater diversity and abundance of free essential amino acids in pigweed, in combination with other governing factors, might have a role in host selection, and corresponds to evidence that pigweed is a superior host for developing beet armyworm (Ali and Gaylor, 1992).

Acknowledgments—Thanks to Jaime Cavazos, Raúl Cantú, Francisco Garcia, Jesse de Anda, Robert Campos, Mohamed Osman, and Abraha Garza for assistance in the field and laboratory; to Dave Eckert for HPLC setup and calibration; and to Tong-Xian Liu, Thomas Reagan, Eileen Paine, and Tim Paine for critical reviews.

REFERENCES

AJAYI, O. and DEWAR, A. M. 1982. The effect of barley yellow dwarf virus on honeydew production by the cereal aphids, *Sitobion avenae* and *Metapolophium dirhodum*. *Ann. Appl. Biol.* 100:203–212.

ALI, A. and GAYLOR, M. J. 1992. Effects of temperature and larval diet on development of the beet armyworm (Lepidoptera: Noctuidae). *Environ. Entomol*. 4:780–786.

AL-ZUBAIDI, F. S. and CAPINERA, J. L. 1984. Utilization of food and nitrogen by the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), in relation to food type and dietary nitrogen levels. *Environ. Entomol*. 13:1604–1608.

ANALYTICAL SOFTWARE. 1998. Statistix for Windows. Analytical Software, Tallahassee, Florida.

BENREY, B. and DENNO, R. F. 1997. The slow-growth-high-mortality hypothesis: A test using the cabbage butterfly. *Ecology* 78:987–999.

BERDEGUÉ, M., REITZ, S. R., and TRUMBLE, J. T. 1998. Host plant selection and development in *Spodoptera exigua*: Do mother and offspring know best? *Entomol. Exp. Appl*. 89:57–64.

BERNAYS, E. A. and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York.

BLANEY, W. M. and SIMMONDS, M. S. J. 1988. Food selection in adult and larvae of three species of Lepidoptera: A behavioral and electrophysiological study. *Entomol. Exp. Appl*. 49:111–121.

BLUA, M. J., PERRING, T. M., and MADORE, M. A. 1994. Plant virus-induced changes in aphid population development and temporal fluctuations in plant nutrients. *J. Chem. Ecol*. 20:691–707.

BOTTGER, G. T. and PATANA, R. 1966. Growth, development, and survival of certain Lepidoptera fed gossypol in the diet. *J. Econ. Entomol*. 59:1166–1168.

BOTTGER, G. T., SHEEHAN, E. T., and LUKEFAHR, M. J. 1964. Relation of gossypol of cotton plants to insect resistance. *J. Econ. Entomol*. 57:283–285.

BRODBECK, B. and STRONG, D. 1987. Amino acid nutrition of herbivorous insects and stress to host plants, pp. 346–364, *in* P. Barbosa and J. C. Schultz (eds.). Insect Outbreaks. Academic Press, London.

CAVIN, J. C. and RODRIGUEZ, B. 1988. The influence of dietary β-carbolene alkaloids on growth rate, food consumption, and food utilization of larvae of *Spodoptera exigua* (Hübner). *J. Chem. Ecol*. 14:475–484.

CHANG, J. F., BENEDICT, J. H., and PAYNE, T. L. 1985. Trapping of cotton terpine volatiles and boll weevil pheromone, pp. 397–398, *in* Proceedings Beltwide Cotton Conference, National Cotton Council, Memphis, Tennessee.

CHAPMAN, R. F. 1971. The Insects Structure and Function. Elsevier, New York.

CHEW, F. S. 1977. Coevolution of pierid butterflies and their cruciferous foodplants. II. The distribution of eggs on potential foodplants. *Evolution* 31:568–579.

COURTNEY, S. P. 1981. Coevolution of pierid butterflies and their cruciferous host plants. III. *Anthocharis cardamines* (L.). Survival, development, and oviposition on different host plants. *Oecologia* 51:91–96.

DAVIS, G. R. F. 1956. Amino acid requirements of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) for pupation. *Can. J. Zool*. 34:82–85.

DICKENS, J. L. 1990. Orientation to host plant and conspecifics by the boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae) electrophysiological and behavioral correlations. *Symp. Biol. Hung*. 39:103–110.

DICKENS, J. L., VISSER, J. H., and VAN DER PERS, J. N. C. 1993. Detection and deactivation of pheromone and plant odor components by the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). *J. Insect Physiol*. 39:503–516.

ELZEN, G. W., WILLIAMS, J. J., and VINSON, S. B. 1984. Isolation and identification of cotton synomones mediating searching by parasitoid *Campoletis sonorensis*. *J. Chem. Ecol*. 10:1251–1264.

EVELEENS, K. G., VAN DEN BOSCH, R., and EHLER, L. 1973. Secondary outbreak induction of beet armyworm by experimental insecticide applications in cotton in California. *Environ. Entomol*. 2:497–503.

FOX, L. R. and EISENBACH, J. 1992. Contrary choices: Possible explanation of enemy-free space by herbivorous insects in cultivated vs. wild crucifers. *Oecologia* 89:574–579.

FYE, R. E. and MCADA, W. C. 1972. Laboratory studies on the development, longevity, and fecundity of six lepidopterous pests of cotton in Arizona. USDA Technical Bulletin. No. 1454.

GUELDNER, R. C., THOMPSON, A. C., HARDEE, D. D., and HEDIN, P. A. 1970. Constituents of the cotton bud. XIX. Attractancy to the boll weevil of the terpenoids and related plant constituents. *J. Econ. Entomol*. 63:1819–1920.

GZIK, A. 1996. Accumulation of proline and pattern of alpha-amino acids in sugar beet plants in response to osmotic, water, and salt stress. *Environ. Exp. Bot*. 36:29–38.

HARBORNE, J. B. 1988. Introduction to Ecological Biochemistry, 3rd ed. Academic Press, New York.

HASSAN, S. T. S., WILSON, L. T., and BLOOD, P. R. B. 1990. Oviposition by *Heliothis armigera* and *H. punctigera* (Lepidoptera: Noctuidae) on okra leaf and smooth-leaf cotton. *Environ. Entomol*. 19:710–716.

HEATH, R. R. and MANUKIN, A. 1992. Development and evaluation of systems to collect volatile semiochemicals from insects and plants using a charcoal-infused medium for air purification. *J. Chem. Ecol*. 18:1209–1226.

HEDIN, P. A., PARROTT, W. L., and JENKINS, J. W. 1991. Effects of cotton plant allelochemicals and nutrients on behavior and development of tobacco budworm. *J. Chem. Ecol*. 17:1107–1121.

HELMS, J. A., COBB, F. W. JR., and WHITNEY, H. S. 1971. Effect of infection by *Verticicladiella wagenerii* on the physiology of *Pinus ponderosa*. *Phytopathol*. 61:920–925.

HINTON, T., NOYES, D. T., and ELLIS, J. 1951. Amino acids and growth factors in a chemically defined medium for *Drosophila*. *Physiol. Zool*. 34:335–353.

HUANG, X. and RENWICK, J. A. A. 1992. Differential selection of host plants by two *Pieris* species: The role of oviposition stimulants and deterrents. *Entomol. Exp. Appl*. 68:59–69.

ITOYAMA, K., KAWAHIRO, Y., MURATA, M., and TOJO, S. 1999. Fluctuations of some characteristics in the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae) reared under different diets. *Appl. Entomol. Zool*. 34:315–321.

JAYARAJ, S. and SESHADRI, A. R. 1967. Preference of the leafhopper *Empoasca kerri* Pruthi (Homoptera: Jassidae) to pigeon pea [*Cajanus cajan* (L.) Milsp.] plants infected with sterility mosaic virus. *Curr. Sci*. 36:353–355.

JENSEN, S. G. 1969. Composition and metabolism of barley leaves infected with barley yellow dwarf virus. *Phytopathology* 59:1694–1699.

KENNEDY, J. S. 1951. Benefits to aphids from feeding on galled and virus-infected leaves. *Nature* 168:825–826.

KESTER, K. M. and BARBOSA, P. 1991. Behavioral and ecological constraints imposed by plants on insect parasitoids: Implications for biological control. *Biol. Control* 1:94–106.

KOIKE, S. T., SCHULBACH, K. F., and Chaney, W. E. 1996. Celery production in California. University of California Division of Agriculture, and Natural Resources, report 7220.

LABANAUSKAS, C. K., STOLZY, L. H., and HANDY, M. F. 1981. Protein and free amino acids in field-grown cowpea seeds as affected by water stress at various growth stages. *Plant Soil* 63:355–368.

LEGG, D. E., SCHENK, T. C., and CHANG, H. C. 1986. European corn borer (Lepidoptera: Pyralidae) oviposition preference and survival on sunflower and corn. *Environ. Entomol*. 15:631–634.

LODH, S. B., RAVEL, P., and SINGH, S. 1971. Studies on nitrogen metabolism in tomato (*Lycopersicon esculentum* Mill.) infested with tomato leaf curl virus. *Indian J. Exp. Biol*. 9:281–283.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1995. Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol*. 21:1217–1227.

LUKEFAHR, M. J. and MARTIN, D. F. 1966. Cotton-plant pigments as a source of resistance in the bollworm and tobacco budworm. *J. Econ. Entomol*. 59:176–179.

MAGALHAES, J. R., HUBER, D. M., and TSAI, C. Y. 1995. Influence of the form of nitrogen on ammonium, amino acids and N-assimilating enzyme activity in maize genotypes. *J. Plant Nutr*. 18:747–763.

MAXWELL, F. G., LA FEVER, H. N., and JENKINS, J. N. 1965. Blister beetles on glandless cotton. *J. Econ. Entomol*. 58:792–793.

MCQUATE, G. T. and CONNOR, E. F. 1990. Insect responses to plant water deficits. I. Effect of water deficits in soybean plants on the feeding preference of Mexican bean beetle larvae. *Ecol. Entomol*. 15:419–431.

PORTILLO, H. E. 1994. Feeding performance and oviposition preference of *Spodoptera latifascia*, *S. frugiperda*, and *Metaponpheumata rogenhoferi* (Lepidoptera: Noctuidae) on sorghum, maize, and noncrop vegetation. PhD dissertation. Mississippi State University, Starkville, Mississippi.

PORTILLO, H. E., PITRE, H. N., MECKENSTOCK, D. H., and ANDREWS, K. L. 1998. Performance of a lepidopteran pest complex (Langosta) (Lepidoptera: Noctuidae) on sorghum, maize, and noncrop vegetation in Honduras. *Environ. Entomol*. 27:70–79.

RAULSTON, J. R., SPURGEON, D. W., and ZAMORA, O. 1997. Spatial and temporal patterns of beet armyworm trap captures in northeastern Mexico, pp. 1229–1232, *in* Proceedings, Beltwide Cotton Conference, National Cotton Council, Memphis, Tennessee.

REYNOLDS, H. T., ADKISSON, P. L., and SMITH, R. F. 1975. Cotton pest management, pp. 379–443, *in* R. L. Metchalf and W. H. Luckman (eds.). Introduction to Insect Pest Management. John Wiley & Sons, New York.

RING, D. R., BENEDICT, J. H., WALMSLEY, M. L., and TREACY, M. F. 1993. Cotton yield response to cotton fleahopper (Hemiptera: Miridae) infestations on the Lower Gulf Coast of Texas. *J. Econ. Entomol*. 86:1811–1819.

RODMAN, J. and CHEW, F. S. 1980. Phytochemical correlates of herbivory in a community of native and naturalized cruciferae. *Biochem. Syst Ecol*. 8:43– 50.

RUBERSON, J. R. 1996. Environmental conditions and biological control of the beet armyworm, pp. 116–118, *in* Proceedings, Beltwide Cotton Conference, National Cotton Council, Memphis, Tennessee.

RUBERSON, J. R., HERZOG, G. A., LAMBERT, W. R., and LEWIS, W. J. 1994. Management of the beet armyworm: Integration of control approaches, pp. 857-858, *in* Proceeding, Beltwide Cotton Conference, National Cotton Council, Memphis, Tennessee.

SCHUSTER, M. F. and MAXWELL, F. G. 1974. The impact of nectariless cotton on plant bugs, boll worms, and beneficial insects, pp. 86–87, *in* Proceedings, Beltwide Cotton Conference, National Cotton Council, Memphis, Tennessee.

SCRIBNER, J. M. and SLANSKY, F., JR. 1981. The nutritional ecology of insects. *Annu. Rev. Entomol*. 26:183–211.

SEITZ, E. W. and HOCHSTER, R. M. 1964. Free proline in normal and in grown gall tumor tissue of tomato and tobacco. *Life Sci*. 3:1033–1037.

SHAVER, T. N. and RAULSTON, J. R. 1971. A soybean-wheat germ diet for rearing the tobacco budworm. *Ann. Entomol. Soc. Am*. 64:1077–1079.

SHEN, L., FOSTER, J. G., and ORCUTT, D. M. 1989. Composition and distribution of free amino acids in flatpea (*Lathyrus sylvestris* L.) as influenced by water deficit and plant age. *J. Exp. Bot*. 40: 71–79.

SHOWLER, A. T., REAGAN, T. E., and SHAO, K. P. 1990. Nematode interactions with weeds and sugarcane mosaic virus in Louisiana sugarcane. *J. Nematol*. 22:31–38.

SINGER, M. C. 1972. Complex components of habitat suitability within a butterfly colony. *Science* 179:75–77.

SINGER, M. C. 1983. Determinants of multiple host use by a phytophagous insect population. *Evolution* 37:389–403.

SINGH, S., VERMA, V. S., GOSSWAMI, B. K., and PADMA, R. 1974. Influence of pigweed mosaic virus on free amino acids of pigweed (*Amaranthus viridis* L.). *Bio. Plant* 16:76–77.

SMITS, P. H., VAN VELDEN, M. C., VAN DEVRIE, M., and VLAK, J. M. 1987. Feeding and dispersion of *Spodoptera exigua* larvae and its relevance for control with a nuclear polyhedrosis virus. *Entomol. Exp. Appl*. 43:67–72.

STRAATMAN, R. 1962. Notes on certain Lepidoptera ovipositing on plants which are toxic to their larvae. *J. Lepid. Soc*. 16:99–103.

SURBURG, H., GUENTERT, M., and HARDER, H. 1993. Volatile compounds from flowers. Analytical and olfactory aspects, pp. 168–186, *in* R. Teranishi, R.G. Buttery, and H. Sugisawa (eds.). Bioactive Volatile Compounds from Plants. American Chemical Society Symposium Series 585, American Chemical Society, Washington, D.C.

TAYLOR, J. S. 1931. Notes on the biology and *Laphygma exempta* Walk., and *L. exigua* Hübn.(Lepidoptera: Noctuidae). *Bull. Entomol. Res*. 22:209–210.

THOMPSON, J. N. 1988. Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. *Entomol. Exp. Appl*. 47:3–14.

THOMPSON, A. C., BAKER, D. N., GUELDNER, R. C., and HEDIN, P. A. 1971. Identification and quantitative analysis of the volatile substances emitted by maturing cotton in the field. *Plant Physiol*. 48:50–52.

TINGLE, F. C. and MITCHELL, E. R. 1992. Attraction of *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) to volatiles from extracts of cotton flowers. *J. Chem. Ecol*. 18:907–914.

TISDALE, R. A. and SAPPINGTON, T. W. 2000. Beet armyworm fecundity and oviposition behavior, pp. 1209–1213, *in* Proceedings, Beltwide Cotton Conference, National Cotton Council, Memphis, Tennessee.

TUMLINSON, J. H., TURLINGS, T. C. J., and LEWIS, W. J. 1992. The semiochemical complexes that mediate insect parasitoid foraging. *Agric. Zool. Rev*. 5:221–252.

VANDERZANT, E. S. 1958. The amino acid requirements of the pink bollworm. *J. Econ. Entomol*. 51:309–311.

WIESENBORN, W. D. and BAKER, T. C. 1990. Upwind flight to cotton flowers by *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *Environ. Entomol*. 19:490–493.

WIKLUND, C. 1974. Oviposition preferences in *Papilio machaon* in relation to the host plants of the larvae. *Entomol. Exp. Appl*. 17:189–198.

WILSON, J. W. 1932. Notes on the biology of *Laphrygma exigua* Hübner. *Fla. Entomol*. 16:33–39.

YOSHIDA, H. A. and PARELLA, M. P. 1992. Development and use of selected chrysanthemum cultivars by *Spodoptera exigua* (Lepidoptera: Noctuidae). *J. Econ. Entomol*. 85:2377–2382.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

CHEMICAL RECOGNITION OF PARTNER PLANT SPECIES BY FOUNDRESS ANT QUEENS IN *Macaranga–Crematogaster* MYRMECOPHYTISM

YOKO INUI,[1] TAKAO ITIOKA,[2,*] KAORI MURASE,[2] RYOHEI YAMAOKA,[3] and TAKAO ITINO[4]

[1]*Center for Ecological Research*
Kyoto University
Ôtsu 520-2113, Japan

[2]*Graduate School of Bioagricultural Sciences*
Nagoya University
Chikusa-ku, Nagoya 464-8601, Japan

[3]*Department of Applied Biology*
Kyoto Institute of Technology
Sakyô-ku, Kyoto 606-0962, Japan

[4]*Faculty of Agriculture*
Kagawa University
Miki-chô, Kita-gun, Kagawa 761-0795, Japan

(Received October 25, 2000; accepted June 3, 2001)

Abstract—The partnership in the *Crematogaster–Macaranga* ant–plant interaction is highly species-specific. Because a mutualistic relationship on a *Macaranga* plant starts with colonization by a foundress queen of a partner *Crematogaster* species, we hypothesized that the foundress queens select their partner plant species by chemical recognition. We tested this hypothesis with four sympatric *Macaranga* species and their *Crematogaster* plant-ant species. We demonstrated that foundress *Crematogaster* queens can recognize their partner *Macaranga* species by contact with the surface of the seedlings, that they can recognize compounds from the stem surface of seedlings of their partner plant species, and that the gas chromatographic profiles are characteristic of the plant species. These findings support the hypothesis that foundress queens of the *Crematogaster* plant-ant species select their partner *Macaranga* species by recognizing nonvolatile chemical characteristics of the stem surfaces of seedlings.

Key Words—partner-plant selection, partner-plant recognition, ant–plant interactions, mutualism, chemical recognition, myrmecophyte, ant dispersal, insect–plant coevolution.

*To whom correspondence should be addressed; e-mail: B51552B@nucc.cc.nagoya-u.ac.jp

0098-0331/01/1000-2029$19.50/0

INTRODUCTION

Myrmecophytism, a type of ant–plant interaction, is one of the most conspicuous examples of the coevolution of mutualism between an animal and a plant (Huxley and Cutler, 1991; Davidson and McKey, 1993). In the mutualistic interactions, the ants (plant-ants) protect their host plants from herbivores and clinging vines, while the host plants (myrmecophytes) provide nest sites and, in some cases, food for the ants (Janzen, 1966; Buckley, 1982; Davidson and McKey, 1993). Myrmecophytism is often obligate: plant-ants and myrmecophytes cannot survive or have difficulty in surviving without each other. Many characteristics of both plant-ants and myrmecophytes are highly specialized and adapted for the mutualistic interaction (Davidson and McKey, 1993).

Macaranga (Euphorbiaceae) is a tree genus including more than 25 species of myrmecophytes, found predominantly in the tropical areas of South East Asia (Whitmore, 1969; Fiala and Maschwitz, 1991, 1992a). Most myrmecophytes have obligate mutualistic relationships with the species-specific specialist ant (plant-ant) species of the genera *Crematogaster* and *Camponotus*. The plants harbor partner plant-ants in their hollow stems and produce food bodies that the plant-ants harvest as their main food (Fiala et al., 1989; Fiala and Maschwitz, 1991, 1992b). In many obligate myrmecophytic species, the mutualistic and symbiotic interactions begin with the foundress ant queens colonizing the plant partners at a very early stage of plant growth. The queens produce ant workers who patrol the host trees and exclude herbivores (Fiala et al., 1989). Both host plants and plant-ants are dependent on each other for survival (Fiala and Maschwitz, 1990; Fiala et al., 1999).

In some cases, many myrmecophytic species have evolved within a particular genus, such as *Acacia* (Leguminosae), *Cecropia* (Cecropiaceae), and *Macaranga* (McKey and Davidson, 1993). The *Macaranga–Crematogaster* system, however, is the only case where the partnership between plant and ant species is highly species-specific in a wide variety of myrmecophytic species within a single genus (Fiala et al., 1999). One species of *Macaranga* has mutualistic relationships mainly with only one or two species of *Crematogaster* plant-ants, and one ant species interacts with a narrow range of *Macaranga* species, in some cases just one or two species (Fiala et al., 1999). Although several species of obligate myrmecophytic *Macaranga* species often coexist in similar habitats in a locality (Davies et al., 1998; Itioka et al., 2000), the species-specificity is well maintained (Itino et al., unpublished data). What factors affect the species-specific partnership in *Macaranga–Crematogaster* myrmecophytism? The first objective of this study was to test whether foundress queens of *Crematogaster* plant-ants can distinguish between *Macaranga* species.

It is well known that ants use various semiochemicals, such as pheromones and cuticular hydrocarbons, for communication in their socialized colonies and

for recognition of nestmates, trophobionts, and prey organisms (Traniello, 1980; Jessen and Maschwitz, 1986; Bonavita et al., 1987; Hölldobler and Carlin, 1987). We also know that closely related but different plant species have different nonvolatile chemicals on their surfaces. Thus, it is likely, in the *Macaranga*–*Crematogaster* system, that foundress queens may recognize their host plant species chemically. The second objective of this study was to examine whether chemical cues, characteristic of each *Macaranga* species, are used for host plant recognition by *Crematogaster* foundress queens.

To examine these two objectives, we conducted three experiments on four obligate myrmecophytic species of *Macaranga* and their specific *Crematogaster* plant-ants: (1) preference experiments to observe how foundress queens of each ant species respond to seedlings presented experimentally; (2) chemical analyses of extracted from the stem surfaces of *Macaranga* seedlings; and (3) preference experiments using esters extracted from the stem surfaces of *Macaranga* seedlings to test whether foundress queens use these as chemical cues for host plant recognition.

METHODS AND MATERIALS

Study Site. All experimental plants and ants were collected in lowland mixed dipterocarp forest in Lambir Hills National Park, Sarawak, Malaysia (4°20′N, 113°50′E, altitude 55–70 m). The details of the study site were described by Watson (1985), Inoue and Hamid (1994) and Kato et al. (1995). At least 20 species, including at least 11 myrmecophytic species, of *Macaranga* occur at the study site (Nagamasu and Momose, 1997).

Life History. We investigated four *Macaranga* species: *M. winkleri*, *M. trachyphylla*, *M. beccariana*, and *M. hypoleuca*, which are all abundant at the study site. Although the preferred light and soil conditions differ widely among the four species (Davies et al., 1998), it is not uncommon to find seedlings and mature trees of multiple species sharing a single microhabitat, such as a tree gap or a riverbank (Itioka, personal observation). All the experiments, except for chemical analysis, were conducted during September and October 1998 and March 1999.

The four species are all obligate myrmecophytes that harbor specific plant-ant species of the *Crematogaster* genus. The details of the basic biology of the mutualistic interactions between typical obligate myrmecophytes of *Macaranga* and their *Crematogaster* plant-ants have been described by Fiala et al. (1989) and Fiala and Maschwitz (1990, 1991, 1992a,b). The *Macaranga* plants produce pearly secreta, called food bodies, on the backs of young leaves or stipules. These food bodies, which the symbiont ants utilize as their main food resource, are rich in carbohydrates, sugars, and lipids (Rickson, 1980; Fiala and Maschwitz, 1992b; Heil et al., 1997). In return, the symbiont ants protect their host plants from

phytophagous insects and clinging vines (Fiala et al., 1989). When they reach 10–30 cm in height, the hollow stems of *Macaranga* seedlings swell and then form domatia between the nodes. At this stage, foundress ant queens settle on the seedlings, although the average heights of seedlings that ant queens begin to colonize depends on species. A foundress makes a hole in the wall of a domatium, enters the domatium, closes the hole, and starts to make her new colony. Thereafter, the mutualism begins.

Each of the four *Macaranga* species associates with a unique plant-ant species at the study site. The *Crematogaster* ant species associated with the four *Macaranga* species have not yet been described, although they have been distinguished by analysis of mtDNA (Itino et al., unpublished data). At the study site, the ant species inhabiting *M. beccariana* and *M. hypoleuca* are almost identical to *Crematogaster decamera*. Those inhabiting *M. trachyphylla* and *M. winkleri* are a species closely related to *C. borneensis* and an undescribed non-*decamera Crematogaster* species, respectively (Seiki Yamane, personal communication; see Itioka et al., 2000 for further details). Although the two species resembling *C. decamera* are morphologically indistinguishable, they can be clearly distinguished from the species inhabiting *M. trachyphylla* and *M. winkleri* by the morphology of queens and workers.

Host Plant Recognition by Ant Queen (Preference Experiments). To examine whether foundress ant queens recognize and differentiate among different congeneric plant species, we offered seedlings of each of the four *Macaranga* species to foundress ants of each *Crematogaster* plant-ant species. Seedlings that were less than 30 cm in height and not yet colonized by ants were collected at random from several tree gaps and riverbank areas within the study site. The seedlings were potted in a shade house at a distance from the forest ridge. We used cloth shades to match the light levels in the shade house to those of a typical forest gap. We allowed the seedlings to grow until they bore one to three domatia and used them for the preference experiments described below. Nulliparous ant queens were collected from domatia of intact and 10- to 20-cm-high seedlings of each *Macaranga* species in the field, and kept in a plastic bag for one day before they were used. We considered a queen with no offspring in the domatia to be nulliparous. We omitted queens that were in domatia with lignified entrance holes, which indicated that a long time had elapsed after the queens' settlement. We checked the species of the ant queens morphologically. When we found nonspecific plant-ant queens in the collection, which was rare, we omitted them. Single ant queens were placed on the youngest well-developed leaf of a potted seedling and observed to see whether they entered the stem of the offered seedling within an hour or within a day. Each individual queen was used in the experiments only once or twice; each queen was placed once on a seedling of the same species from which she was collected and once on a seedling of one of the other three species. The order of the two trials was randomized and the interval between the trials was an hour. The experimental seedlings were used only once for the experiments.

Chemical Analyses of Surface Extracts of Macaranga *Seedlings.* To investigate the differences in chemical characteristics that might be used as cues for host recognition by the foundress ant queens, we obtained extracts from the stem surfaces of seedlings of each species. Seedlings were collected and cultivated in the shade house as above, then the hollow stems were cut out. The stem part that covered two or three consecutive nodes (domatia) was sampled from each seedling. Each stem sample was soaked in ethyl acetate for 30 min. The extracts from each stem sample were analyzed separately by gas chromatography (GC) and used as samples in the bioassays (see below). We sampled stem pieces from at least seven seedlings of each species.

Each ethyl acetate extract was evaporated completely and then dissolved in 40 μl hexane. We added 100 ng docosane as an internal standard and then analyzed 2 μl of each sample by GC in a GC-14A gas chromatograph (Shimadzu Inc.) equipped with a flame ionization detector and a DB-1 glass capillary column (30 m $\times$ 0.25 mm ID; 0.25 μm film thickness; J&W Scientific Inc.). Helium was used as the carrier gas. The oven temperature was programmed as follows: 80°C for 1 min, increasing by 20°C/min to 320°C and held there for 10 min.

Bioassays Offering Extracts of Macaranga *Seedling to Ant Queens.* To examine whether the chemicals extracted from *Macaranga* seedlings can be used as chemical cues for host plant recognition by ant queens, we offered the extracts from the stem surfaces to foundress queens. Ethyl acetate extracts of each *Macaranga* species were applied to tube-shaped filter papers (60 mm $\times$ 5 mm ID) and allowed to dry completely. The quantity of extract was equivalent to the extract from the stem part of a node. About 1 hr after the application of the extract, single ant queens were placed on the center of a glass tray (90 mm ID $\times$ 20 mm) with the extract-impregnated paper tube, and their behavior was observed for 30 min. Talc was applied to the wall of the glass tray so that the ant queen inside could not climb up the wall. When the ant queen climbed the paper tube and touched it with her antennae, we regarded the behavior as showing interest and recorded the time during which she showed such behavior and stayed on the paper tube. A paper tube treated with pure ethyl acetate was used as a control treatment. Experimental ants were prepared and collected in the same way as in the preference experiments, and the trial schedule was also the same. Each ant queen was used twice: she was offered a paper tube containing the extract of her host species once and one containing the extract of one other species or a control once, in random order.

RESULTS

Host Recognition by Ant Queen. We defined a partner *Macaranga* species for a particular experimental ant queen as the *Macaranga* species of the seedling from which the queen was derived. The frequencies with which ant queens of the

TABLE 1. COMPARISON OF ANT QUEEN PREFERENCES TO SEEDLINGS OF DIFFERENT *Macaranga* SPECIES[a]

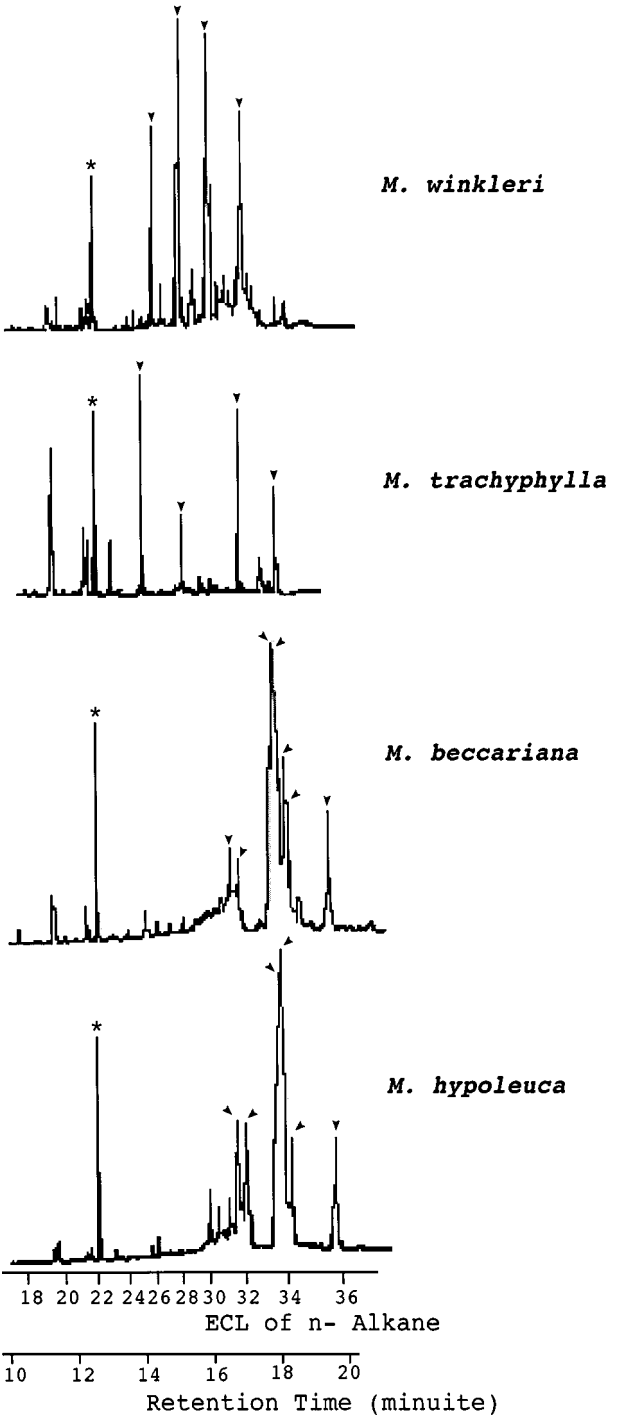
Plant species of offered seedlings	Ant species (plant species that ant queens associate with)			
	M. winkleri	*M. trachyphylla*	*M. beccariana*	*M. hypoleuca*
M. winkleri	77.8 (18)	15.3 (13)	42.9 (7)	40.0 (5)
M. trachyphylla	0 (8)	71.0 (31)	50.0 (4)	60.0 (5)
M. beccariana	0 (7)	13.3 (15)	91.3 (23)	81.3 (25)
M. hypoleuca	0 (7)	0 (4)	77.8 (18)	84.0 (25)

[a] Percentage of ant queens that entered the offered seedlings of each species; the number of experimental ant queens is given in parenthesis.

three species that associate with *M. winkleri*, *M. trachyphylla*, and *M. beccariana* entered the stem domatia of the partner *Macaranga* species were higher than those at which queens entered the stem domatia of the other two nonpartner *Macaranga* species (Table 1; Fischer's exact test, $\chi^2 = 20.263$, $P < 0.0001$ for queens from *M. winkleri* and $\chi^2 = 19.177$, $P < 0.0001$ for queens from *M. trachyphylla*, and $\chi^2 = 8.694$, $P = 0.0069$ for queens from *M. beccariana*). The queens derived from *M. winkleri* rejected all seedlings of the three nonpartner *Macaranga* species, including *M. hypoleuca*. Some individual ant queens derived from *M. trachyphylla* and *M. beccariana*, however, entered the seedling domatia of nonpartner *Macaranga* species, although they showed a significant preference for their original partner species as shown above. Ant queens from *M. trachyphylla* did not enter the seedlings of *M. hypoleuca* at all, whereas queens from *M. beccariana* entered them frequently (77.8%, 14/18 queens). For queens derived from *M. beccariana*, there was no difference between the frequency of entrance into *M. beccariana* seedlings and into *M. hypoleuca* seedlings (Fischer's exact test, $\chi^2 = 1.479$, $P = 0.377$). The frequency with which ant queens derived from *M. hypoleuca* entered the seedling domatia of *M. hypoleuca* was not higher than the frequency with which they entered the domatia of the three nonpartner species (Fischer's exact test, $\chi^2 = 1.545$, $P = 0.324$). The difference was smallest in the case of *M. beccariana*; ant queens derived from *M. hypoleuca* entered the seedling domatia of *M. hypoleuca* and *M. beccariana* with almost equal frequency.

Chemical Analyses of Extracts of Surfaces of Macaranga *Seedling.* Figure 1 shows gas chromatograms of the extracts from the seedling stem surfaces of the

FIG. 1. Gas chromatograms of ethyl acetate extracts from the surface of the four *Macaranga* species. The chromatograms presented are those that contain the least "noisy" peaks of all the individual seedlings of each species. Arrows mark the main peaks that are found in the samples from each seedling of that species in notable amounts. Asterisks indicate the peaks of the internal standard, docosane C_{22} (100 ng).


M. winkleri
M. trachyphylla
M. beccariana
M. hypoleuca
18 20 22 24 26 28 30 32 34 36
ECL of n- Alkane
10 12 14 16 18 20
Retention Time (minuite)

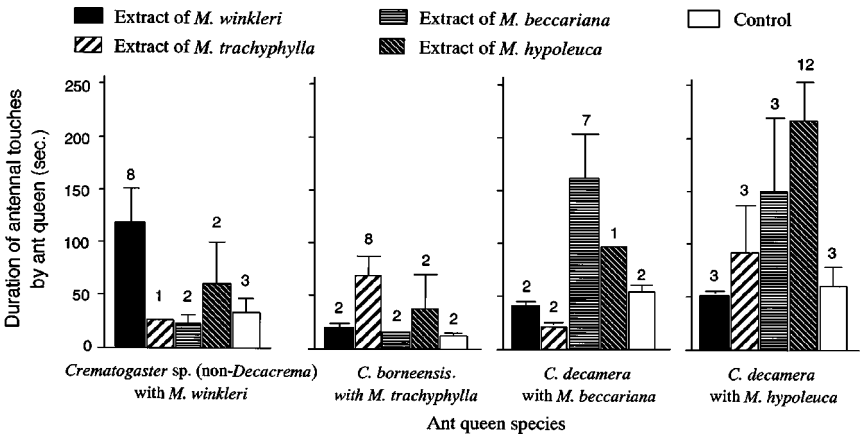


FIG. 2. The ant queen preferences among the extracts of the four *Macaranga* species. The preference is expressed by the mean duration of antennal touches by the ant queen of the given paper tube. Vertical lines on the bars indicate standard errors. Sample sizes are indicated above the bars.

four *Macaranga* species. All seedlings of a species had the same main GC peaks without exception. The four main GC peaks of *M. winkleri* were not found in any gas chromatograms of the other three species, and similarly the four main peaks in the gas chromatograms of *M. trachyphylla* were not found in the other three species. *M. hypoleuca* and *M. beccariana* showed seven and six main GC peaks, respectively; six were the same for these two species and were not found in the other two species. Moreover, the relative proportions of the six peaks (peak heights) were similar in *M. hypoleuca* and *M. beccariana*.

Bioassays Offering Extracts of Macaranga *Seedlings to Ant Queens*. More than 90% of bioassayed ant queens stayed longer on the paper tubes treated with the extracts of their partner species than on those treated with the nonvolatile extracts of their nonpartner species or controls (Figure 2; *t* test: $df = 14$, $t = 5.791$, $P < 0.0001$ for ants from *M. winkleri*; $df = 14$, $t = 2.162$, $P = 0.0484$ for ants from *M. trachyphylla*; $df = 12$, $t = 6.315$, $P < 0.0001$ for ants from *M. beccariana*; $df = 22$, $t = 6.963$, $P < 0.0001$ for ants from *M. hypoleuca*).

DISCUSSION

Recent work on the specificity in the partnership between *Macaranga* myrmecophytes and *Crematogaster* plant-ants (Fiala et al., 1999; Itino et al., unpublished data) has verified that the *Macaranga–Crematogaster* myrmecophytism is highly species-specific. To clarify the mechanisms of this species specificity, it

is necessary to investigate the colonizing process of the partner myrmecophytes by foundress queens, since a mutualistic relationship (symbiosis) on a particular *Macaranga* plant starts with colonizing or settling of a seedling by a foundress queen. The recognition of the partner plants by foundress queens is a crucial phase in the process.

The results of the preference experiments, in which foundress queens were artificially placed on seedlings, suggest that foundress queens of some *Crematogaster* species can recognize their partner *Macaranga* species by contact with the plant surface at the colonizing phase. Foundress queens derived from *M. winkleri* and *M. trachyphylla* seedlings showed clear acceptance of their original partner *Macaranga* species but rejected nonpartner species. Simultaneously, however, the results suggest that the recognition of or preference for the original partner myrmecophyte is not complete in some cases, especially with foundress queens derived from *M. beccariana* and *M. hypoleuca* seedlings. These foundress queens more frequently accepted the nonoriginating *Macaranga* myrmecophytes as nest sites than did the ant queens derived from *M. winkleri* and *M. trachyphylla*. Moreover, foundress queens from *M. beccariana* and *M. hypoleuca* seemed almost equally to accept *M. beccariana* and *M. hypoleuca*. The ability to distinguish between *M. beccariana* and *M. hypoleuca* by contact with seedlings seems to be weaker in the foundress queens derived from either of these two species than in the other two ant species. Thus, the ants vary in the intensity of their preferences for or in their ability to recognize particular partner species of *Macaranga* by contact with the plant surface.

There are two possible reasons for the differences among the ant species and among the plant species in the results of the preference experiments: the phylogenetic relationships between the *Macaranga* species and the strength of the mutualistic relationships. Because *M. beccariana* and *M. hypoleuca* are considered to be phylogenetically closely related species (S. J. Davies, personal communication), the queens derived from these two species tend to have a weaker preference for a particular one. The characteristics that ant queens use to discriminate among partner plants may be similar in the two plant species. In fact, as mentioned below, the characteristics of nonvolatile chemicals on the stem surfaces of these two plant species are almost identical. On the other hand, *M. winkleri* is phylogenetically more remote from the other three species, which may be why ant queens derived from *M. winkleri* are the most persistent in their preference for their original partner species.

The strength of the mutualistic relationship or interdependency may be an important factor in the persistent preference by foundress queens for a particular partner species. It is reasonable to infer that ant species that are most dependent on partner myrmecophytes have been selected for their superior ability to distinguish their own partner plant species because they have a particularly strong preference for the partner species. Among *M. winkleri*, *M. trachyphylla*, and *M. beccariana*,

the myrmecophytism—the intensity of the mutual dependency between plant and ant—is highest on *M. winkleri* and next highest on *M. trachyphylla* (Itioka et al., 2000; Nomura et al., 2000). Although the intensity of the interdependency between *M. hypoleuca* and its plant-ant has not yet been investigated, it seems to be the weakest in these four *Macaranga* species. Because the microhabitat of *M. hypoleuca* is the most shady and, therefore, its growth is slowest, the food supply provided for its plant-ants appears to be lower than that of the others (Murase, personal observation), and the plant-ant is observed to be the least aggressiveness (Itioka, unpublished data).

Together with the clear results of the preference experiments, the results of the bioassay offering extracts of *Macaranga* seedlings to ant queens suggest that foundress queens recognize their own specific partner-plant species from the chemical characteristics of the plant surface and that they use compounds of low volatity on the stem surfaces of *Macaranga* seedlings in the recognition. These findings are supported by the differences among the gas chromatographs of the surface extracts. The fact that the GC profiles of *M. winkleri* and *M. trachyphylla* are distinct from those of the other species is consistent with the finding that foundress queens derived from these two species much less frequently failed to recognize their own original partner species. Further, the similarity of the chemical patterns of *M. beccariana* and *M. hypoleuca* is consistent with the finding that these two species are equally accepted as partner plants by foundress queens derived from either of them. It may be difficult for these two ant species to distinguish their own partner species from a closely related species. Thus, the extent to which the chemical patterns differ correlates with the ability of foundress queens to distinguish the partner plant in the preference experiment.

Our results demonstrate that high-boiling compounds on the stem surface play an important role as semiochemicals in the recognition of the partner-plant species by plant-ant foundress queens; however, this does not exclude other mechanisms from being involved in the process of selection and recognition of the partner plant species. Differences in microhabitat; in the ant's reproductive, dispersal, and colonization seasons (or the plant's receptive season for ant colonization); characteristics of volatile chemicals of seedlings; morphology, coloration, and physical features of seedling surfaces, etc., might all be used by foundress queens. These factors should be investigated in the future. However, differences in microhabitat are unlikely to be involved in the selection of the partner species by foundress queens in our system, because, except for *M. hypoleuca*, the spatial distribution of seedlings of the *Macaranga* species studied overlapped markedly at the microhabitat level (unpublished data). This is different from the habitat segregation in the myrmecophytism between *Cecropia* plants and their plant-ants reported by Yu and Davidson (1997). In the case of *M. hypoleuca*, differences in microhabitat, coloration of foliage, and leaf surface texture might be

cues in selection and recognition by plant-ants, because only *M. hypoleuca* favors a shady habitat, such as the forest floor, and has whitish, lustrous and waxy leaves.

Acknowledgments—We thank Hua Seng Lee and Abang A. Hamid of the Forest Department of Sarawak, Tamiji Inoue of the Center for Ecological Research, Kyoto University, and Kazuhiko Ogino of the University of Shiga Prefecture for providing the opportunity to carry out this study. We also thank Seiki Yamane of Kagoshima University for valuable comments on ant identification, and Takayuki Ohgushi for helpful comments on the early version of the manuscript. This work was partly supported by grants from the Japan Ministry of Education, Science and Culture for International Scientific Research (10041163) and for Creative Basic Research (09NP1501).

REFERENCES

BONAVITA, C. A., CLÉMENT, J. L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in ant *Camponotus vagus* Scop. *J. Entomol. Sci*. 22:1–10.

BUCKLEY, R. C. 1982. Ant-Plant Interactions in Australia. Dr. W. Junk Publishers, The Hague, The Netherlands.

DAVIDSON, D. W. and MCKEY, D. 1993. The evolutionary ecology of symbiotic ant–plant relationships. *J. Hym. Res*. 2:13–83.

DAVIES, S. J., PALMIOTTO, P. A., ASHTON, P. S., LEE, H. S., and LAFRANKIE, J. V. 1998. Comparative ecology of 11 sympatric species of *Macaranga* in Borneo: Tree distribution in relation to horizontal and vertical resource heterogeneity. *J. Ecol*. 86:662–673.

FIALA, B. and MASCHWITZ, U. 1990. Studies on the South East Asian ant-plant association *C. borneeensis–Macaranga*: Adaptations of the ant-partner. *Insectes Soc*. 37:212–231.

FIALA, B. and MASCHWITZ, U. 1991. Extrafloral nectaries in genus *Macaranga* (Euphorbiaceae) in Malaysia: Comparative studies of their possible significance as predispositions for myrmecophytism. *Biol. J. Linn. Soc*. 44:287–305.

FIALA, B. and MASCHWITZ, U. 1992a. Domatia as most important adaptations in the evolution of myrmecophytes in the paleotropical tree genus *Macaranga* (Euphrobiaceae). *Plant Syst. Evol*. 180:53–64.

FIALA, B. and MASCHWITZ, U. 1992b. Food bodies and their significance for obligate ant-association in the tree genus *Macaranga* (Euphorbiaceae). *Bot. J. Linn. Soc*. 110:61–75.

FIALA, B., MASCHWITZ, U., THO, Y. P., and HELBIG, A. J. 1989. Studies of a South East Asian ant-plant association: Protection of *Macaranga* trees by *Crematogaster borneensis*. *Oecologia* 79:463–470.

FIALA, B., JACOB, A., MASCHWITZ, U., and LINSENMAIR, E. 1999. Diversity, evolutionary specialization and geographic distribution of a mutualistic ant-plant complex: *Macaranga* and *Crematogaster* in South East Asia. *Biol. J. Linn. Soc*. 66:305–331.

HEIL, M., FIALA, B., LINSENMAIR, K. E., ZOTZ, G., MENKE, P., and MASCHWITZ, U. 1997. Food body production in *Macaranga triloba* (Euphorbiaceae): A plant investment in antiherbivore defence via symbiotic ant partners. *J. Ecol*. 85:847–861.

HÖLLDOBLER, B. and CARLIN, N. F. 1987. Anatomy and specificity in the chemical communication signals of social insects. *J. Comp. Physiol. A*. 161:567–581.

HUXLEY, C. R. and CUTLER, D. F. 1991. Ant–Plant Interactions, Oxford University Press, New York.

INOUE, T. and HAMID, A. A. 1994. Plant Reproductive System and Animal Seasonal Dynamics (Long-Term Study of Dipterocarp Forests in Sarawak). Center for Ecological Research, Kyoto University, Otsu, Japan.

ITIOKA, T., NOMURA, M., INUI, Y., ITINO, T., and INOUE, T. 2000. Difference in intensity of ant defense among three species of *Macaranga* myrmecophytes in a Southeast Asian dipterocarp forest. *Biotropica* 32:318–326.

JANZEN, D. H. 1966. Coevolution of mutualism between ants and acacias in Central America. *Evolution* 20:249–275.

JESSEN, K. and MASCHWITZ, U. 1986. Orientation and recruitment behavior in the ponerine ant *Pachycondyla tesserinoda* (Emery): Laying of individual-specific trails during tandem running. *Behav. Ecol. Sciobiol*. 19:151–155.

KATO, M., INOUE, T., HAMID, A. A., NAGAMASU, T., MERDEK, M. B., NONA, A. R., ITINO, T., YAMANE, S. K., and YUMOTO, T. 1995. Seasonality and vertical structure of light-attracted insect communities in a dipterocarp forest in Sarawak. *Res. Pop. Ecol*. 37:59–79.

MCKEY, D. and DAVIDSON, D. W. 1993. Ant-plant symbioses in Africa and the neotropics: History, biogeography and diversity, pp. 568–606, *in* P. Goldblatt (ed.). Biological Relationships between Africa and South America. Yale University Press, New Haven, Connecticut.

NAGAMASU, H. and MOMOSE, K. 1997. Flora of Lambir Hills National Park, Sarawak, with special reference to the Canopy Biology Plot, pp. 20–67, *in* T. Inoue and A. A. Hamid (eds.). General Flowering of Tropical Rainforests in Sarawak. Center for Ecological Research, Kyoto University, Otsu, Japan.

NOMURA, M., ITIOKA, T., and ITINO, T. 2000. Variations in abiotic defense within myrmecophytic and non-myrmecophytic species of *Macaranga* in a Bornean dipterocarp forest. *Ecol. Res*. 15:1–11.

RICKSON, F. R. 1980. Developmental anatomy and ultrastructure of the ant-food bodies of *Macaranga triloba* and *M. hypoleuca*. *Am. J. Bot*. 67:285–292.

TRANIELLO, J. F. A. 1980. Colony specificity in the trail pheromone of an ant. *Naturwissenschaften* 67:361–362.

WATSON, H. 1985. Lambir Hills National Park: Resource Inventory with Management Recommendations. National Parks and Wildlife Office, Forest Department, Kuching, Sarawak, Malaysia.

WHITMORE, T. C. 1969. First thoughts on species evolution in Malayan *Macaranga* (Studies in *Macaranga* III). *Biol. J. Linn. Soc*. 1:223–231.

YU, D. W. and DAVIDSON, D. W. 1997. Experimental studies of species-specificity in *Cecropia*–ant relationships. *Ecol. Monogr*. 67:273–294.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

IDENTIFICATION, SYNTHESIS, AND FIELD EVALUATION OF THE SEX PHEROMONE OF THE CITRUS FRUIT BORER *Ecdytolopha aurantiana*

WALTER S. LEAL,[1,2,]* J. MAURÍCIO S. BENTO,[3] YASUHIRO MURATA,[4] MIIKIO ONO,[4] JOSÉ R. P. PARRA,[3] and EVALDO F. VILELA[5]

[1]*Department of Entomology*
University of California
Davis, California 95616

[2]*National Institute of Sericultural and Entomological Science*
1-2 Ohwashi, Tsukuba, 305-8634 Japan

[3]*Departamento de Entomologia*
Fitopatologia e Zoologia Agrícola, ESALQ-USP
13418-900, Piracicaba-SP, Brazil

[4]*Fuji Flavor Co. Ltd.*
3-5-8 Midorigaoka
Hamura-city, Tokyo 190-11, Japan

[5]*Departamento de Biologia Animal*
UFV, 36570-000, Viçosa-MG, Brazil

(Received March 10, 2001; accepted May 23, 2001)

Abstract— The sex pheromone of the citrus fruit borer *Ecdytolopha aurantiana* has been identified by gas chromatography coupled to an electroantennographic detector (GC-EAD). The electron impact mass spectral (EI-MS) fragmentation of the major EAD-active peak gave identifying features for a monounsaturated acetate. Further analyses by chemical ionization mass spectrometry (CI-MS), vapor-phase infrared spectroscopy (GC-IR), along with chemical derivatization (DMDS reaction), led to full characterization of the major component as (*E*)-8-dodecenyl acetate (*E*8–12 : Ac). The second constituent was identified as the related alcohol, (*E*)-8-dodecenol (*E*8–12 : OH). The two compounds were indistinguishable from the authentic synthetic standards in chemical and EAD analyses. Samples of the two compounds were obtained by a facile synthesis utilizing lithium chemistry. Field tests showed that captures in traps baited with a mixture of *E*8–12 : Ac and *E*8–12 : OH at 100 : 1 and 10 : 1 ratios were not significantly different from the catches in traps having two virgin females. Dosage tests showed better performance of traps baited with 1 mg than those with 0.1 mg of the pheromone blend, either in 100 : 1 or 10 : 1 ratio.

*To whom correspondence should be addressed; e-mail: wsleal@ucdavis.edu

0098-0331/01/1000-2041$19.50/0

Key Words—Lepidoptera, Tortricidae, *Ecdytolopha aurantiana*, sex pheromone, (*E*)-8-dodecenol, (*E*)-8-dodecenyl acetate, GC-EAD.

INTRODUCTION

The citrus fruit borer *Ecdytolopha aurantiana* Lima (Lepidoptera: Tortricidae) is an economically important pest in Latin America, in particular, in Brazil, where it causes crop losses to orange growers estimated at US$ 50 million/yr (Anonymous, 2000). Recent studies on the sexual behavior and field ecology of *E. aurantiana* on citrus trees suggest that a female-released sex pheromone may be useful for monitoring populations of the citrus fruit borer (Bento et al., 2001). A synthetic pheromone system would allow growers to minimize the number of insecticide sprays, which, without a reliable monitoring system, becomes an economically and environmentally costly way to prevent crop damage. This prompted us to undertake the identification, synthesis, and field evaluation of the citrus fruit borer pheromone.

METHODS AND MATERIALS

Analytical Procedures. Low-resolution electron impact mass spectrometry (EI-MS) was carried out with a HP 6890 gas chromatograph (GC; Agilent, formerly Hewlett-Packard, Palo Alto, California) linked to a mass selective detector (MSD 5973; Agilent). Chromatographic resolution was done on a HP-5MS column (30 m $\times$ 0.25 mm; 0.25 μm) that was operated at 70°C for 1 min, increased to 270°C at a rate of 10°C/min, and finally held at this temperature for 10 min. Chemical ionization (CI) was performed on a HP 5890 II Plus GC connected to a Mass Engine 5989B (Agilent). The reactant gas was 5% ammonia diluted in helium. Samples were separated on a HP-5MS column (30 m $\times$ 0.25 mm; 0.25 μm). The oven temperature was set at 60°C for 1 min, increased to 200°C at a rate of 5°C/min, increased again to 270°C at 10°C/min, and finally held at this temperature for 10 min. Vapor-phase Fourier transform infrared (FTIR) spectra were recorded with a HP 6890 GC coupled to a FTIR system equipped with a light pipe interface, FTS-40A, GC/C32 (Bio-Rad, Cambridge, Massachusetts). The GC was equipped with a HP-5 column (30 m $\times$ 0.32 mm; 0.25 μm) operated at 70°C for 1 min, increased to 250°C at a rate of 10°C/min, and finally held at this temperature for 10 min. The light pipe and the transfer line were set at 230°C and 250°C, respectively. In all GCs, helium was used as carrier gas at 1 ml/min, except for the GC-FTIR, for which the flow rate was 2 ml/min. GC with electroantennographic detection (EAD) was performed according to the method of Struble and Arn (1984). A HP 5890 II Plus GC was modified to have the effluent from the capillary column split into EAD and flame ionization detector (EAD/FID, 3 : 1).

E. aurantiana antennae were placed in the previously described acrylic stage (Leal et al., 1992). The stage was set inside the glass transfer line (2 cm away from the GC outlet) and connected with gold wires to an amplifier (gain 5) and filtered through a passive filter (cutoff frequency 0.12 Hz). The signal was fed into an A/D 35900E interface (Agilent). FID and EAD signals were acquired with a 3365 Series II Chemstation (Agilent). To obtain EAD responses for synthetic (*Z*)- and (*E*)-8-dodecenyl acetate (10 ng of each), six to eight samples of the two isomers were injected successively within 5–6 min in split mode.

Pheromone Extraction and Isolation. For extraction, hexane (Fisher, Pesticide Residue Grade) was distilled in an all-glass distillation apparatus. The last segments of the abdomen (segments VIII and IX) of 400 three-day-old virgin females were extracted with distilled hexane (4 ml). Extractions were done between 6:00 and 8:00 PM, coinciding with the mating time of *E. aurantiana*, as previously observed in a greenhouse (Bento et al., 2001). The crude extract was subjected to flash column chromatography on a Pasteur pipet filled with silica gel (Wakogel C-200; Wako, Tokyo, Japan) and eluted successively with solutions of ether in hexane (0, 1, 3, 5, 10, 15, 20, 50, and 100%). All fractions were concentrated to ca. 1 virgin female-equivalent per microliter.

Derivatization. An aliquot of the ether–hexane (5 : 95) fraction (50 μl) was concentrated in a 1-ml V-vial (Wheaton, Millville, New Jersey) by a slow stream of argon. The residual material was dissolved in distilled dimethyl disulfide (DMDS, 80 μl), and a small crystal of iodine (ca. 200 μg) was added. A magnetic spin vane was introduced, and the vial was sealed with a Teflon-lined cap. The reaction mixture was stirred at 60°C overnight. After cooling to room temperature, hexane (100 μl) was added and the reaction was quenched and washed with sodium thiosulfate (5%; 50 μl). The organic phase was dried over anhydrous sodium sulfate, concentrated to 50 μl, and analyzed by GC-MS.

Syntheses. For identification purposes, commercially available (*Z*)- and (*E*)-8-dodecenyl acetate (IPO-DLO, Wageningen, The Netherlands) were used. (*Z*)- and (*E*)-Dodecenol were obtained by alkaline hydrolysis (KOH/MeOH) of the corresponding acetates. For field experiments, samples of (*E*)-8-dodecenyl acetate **1** and (*E*)-8-dodecenol **2** were prepared utilizing lithium chemistry (Figure 1). A solution

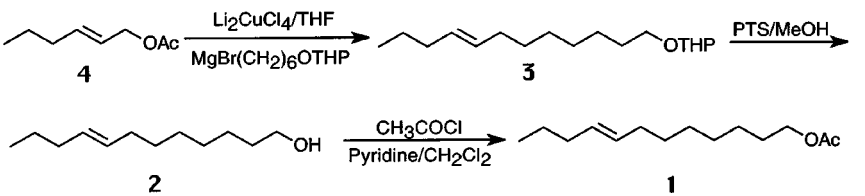


FIG. 1. Scheme for the preparation of (*E*)-8-dodecenol (**2**) and (*E*)-8-dodecenyl acetate (**1**) for the field evaluations.

of (*E*)-2-hexenyl acetate **4** (24 g, 90 mmol) and Li_2CuCl_4 (0.31 g, 1.44 mmol) in THF (14.4 ml) was added to 100 ml of a solution of $MgBr(CH_2)_6OTHP$ (26 g, 90 mmol) in THF at 10° (Figure 1). After stirring for 3 hr, the reaction mixture was poured into dilute HCl and extracted with ether. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum to give **3**. The crude product was stirred with *p*-toluenesulfonic acid monohydrate (PTS; 0.86 g, 4.5 mmol) in methanol (MeOH; 100 ml). After stirring for 2 hr, K_2CO_3 (1.3 g, 9 mmol) was added and stirring was continued for 2 hr. The reaction mixture was filtered, concentrated, and distilled to give **2** (6.6 g, 36 mmol; bp 107°/1.5 mm Hg) in overall 40% yield. EI-MS data: 55(100%), 41(55), 42(10), 43(14), 53(10), 54(39), 56(15), 57(13), 66(8), 67(98), 68(55), 69(35), 70(8), 71(6), 79(9), 81(90), 82(84), 83(20), 95(57), 96(45), 97(9), 109(24), 110(18), 123(9), 124(6), 138(5), 166(8). IR ν_{max} (cm^{-1}): 2960 (s), 2925 (s), 2860 (s), 1740 (s), 1675 (w), 1240 (s), 965 (s).

To a solution of **2** (1.84 g, 10 mmol) and pyridine (1.19 g, 15 mmol) in CH_2Cl_2 (30 ml) at 10°, CH_3COCl (1.02g, 13 mmol) was added. After stirring for 2 hr, the reaction mixture was poured into water and extracted with ether. The ether layer was washed with brine, dried over anhydrous sodium sulfate, concentrated, and distilled to give **1** (2.21 g, 9.8 mmol; 98% yield; bp 104°/1.5 mm Hg). EI-MS data: 82(100%), 41(39), 43(63), 54(33), 55(70), 56(10), 61(8), 67(90), 68(45), 69(25), 81(87), 83(20), 95(58), 96(63), 97(10), 109(29), 110(26), 123(13), 124(9), 137(7), 138(6), 166(29). IR ν_{max} (cm^{-1}): 3640 (s), 2962 (s), 2926 (s), 2862 (s), 1675 (w), 1050 (s), 965 (s). The sample was devoid of the *Z*-isomer as determined by chemical derivatization with *m*-chloroperbenzoic acid (Attygale, 1998). Under the above GC conditions, the epoxide derived from *E*8–12 : Ac appeared at 13.95 min, and no trace peak was detected at 14.18 min, the retention time for the epoxide derived from *Z*8–12 : Ac.

Field Experiments. Tests were done in commercial citrus groves in São Paulo, Brazil. The ratio of the two components (1 mg) was tested at the Fazenda São João, in Altair, which has 13-year-old, 3.5-m-high trees (variety: Valencia) planted in a 5-×8-m fashion. On the other hand, two dosages (0.1 and 1 mg) and two ratios (10 : 1 and 100 : 1) of the pheromone components were tested at the Fazenda Entre Rios, in Gavião Peixoto, which has 5-year-old, 3-m-high trees (variety: Pera Rio) planted in a 7- ×3.5-m arrangement. The test compounds were incorporated in a Fuji Flavor slow-releasing device made of ES fiber (Chisso Co. Ltd, Tokyo, Japan) and covered with polyethylene film. The pheromone dispensers were loaded into Sticky Delta traps (Fuji Flavor Co. Ltd. Tokyo, Japan), which were suspended at 3 or 3.5 m. The treatments were replicated four times over three consecutive days in a completely randomized block design and with an intertrap distance of 40 m. All treatments, including 2-day-old virgin females, were renewed daily. To stabilize the variance, capture data were transformed to log $(x + 1)$, in particular, because of the occurrence of zero catches in the unbaited traps (Snedecor and Cochran,

1989). The figures were drawn with the original data (before transformation). The means of the transformed data were tested for significance by ANOVA with JMP Software, Version 2 (SAS Institute, Cary, North Carolina). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

The response-guided strategy for the isolation of the citrus fruit borer sex pheromone, i.e., fractionating crude extract and monitoring the biological activity of each fraction with a bioassay, was unrewarding. Although observations of the typical behavioral responses of moth to pheromones, namely, wing-fanning, precopulatory behavior, and attraction, led us to the active fractions, activity was observed in multiple fractions for which GC-MS analyses showed many peaks, including homologous compounds. For example, the ether–hexane (5 : 95) fraction contained various monounsaturated acetates. It is unlikely these homologous compounds can be clearly separated by further purification by silica gel column so as to allow unambiguous identification of the pheromone components. On the other hand, gas chromatography–electroantennographic detection (GC-EAD), with the male *E. aurantiana* as the sensing element, allowed rapid identification of the active compounds. Analyses of the crude extract showed two EAD-active peaks, which appeared at 10.9 and 12.4 min (Figure 2). The results were consistent when GC-EAD was repeated either with the same or different antennae ($N = 16$). The

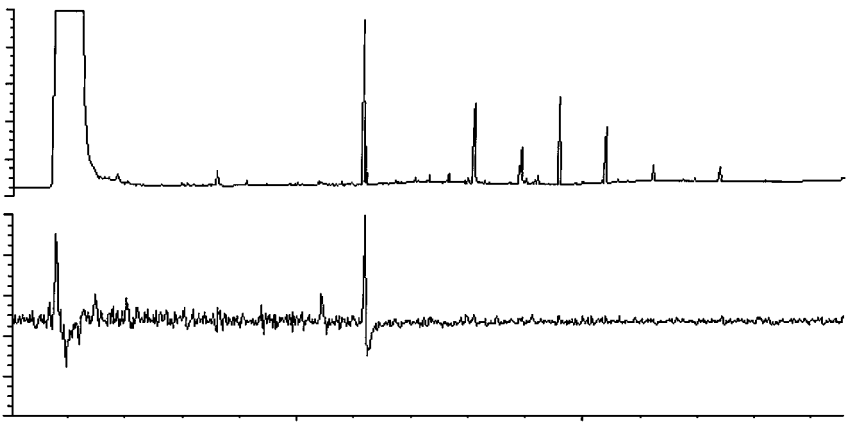


FIG. 2. Responses from flame ionization detector (FID, top) and electroantennographic detection (EAD, bottom; sensing element: male *E. aurantiana* antenna) to a hexane extract from abdominal tips of females (ca. 5 female-equivalents).

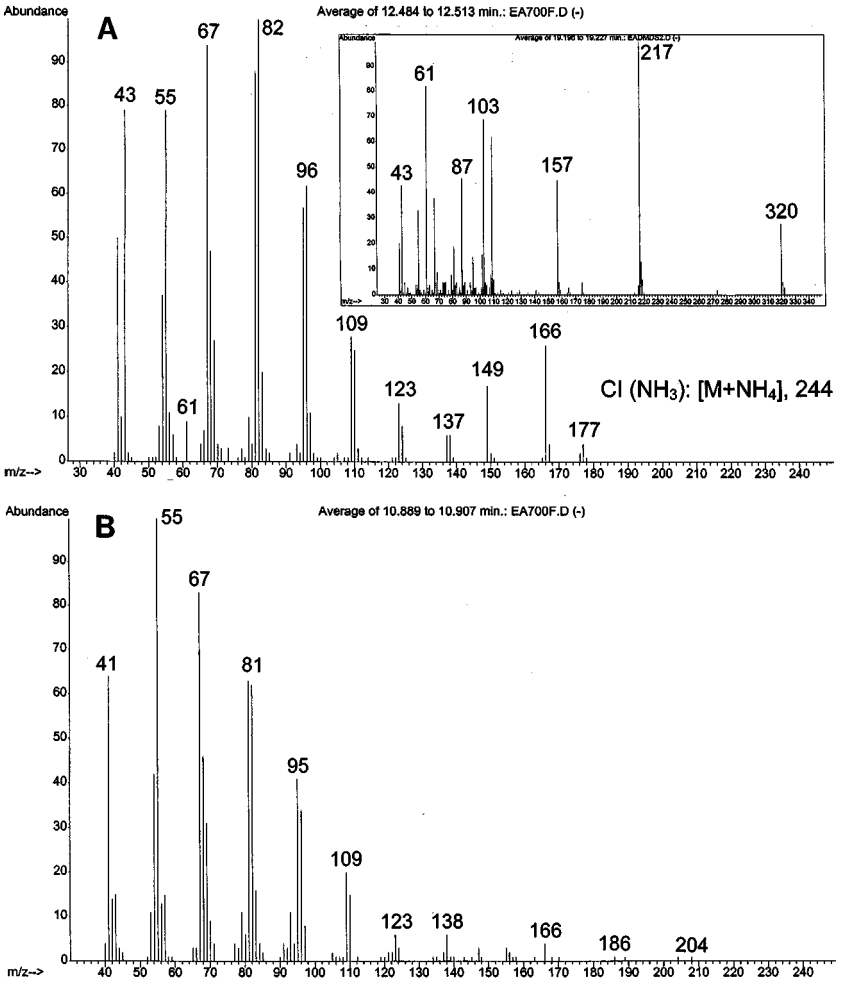


FIG. 3. Mass spectral data from the EAD-active peaks (Figure 2) at 12.4 min (A) and 10.9 min (B). Inset: MS of the major EAD-active peak after derizativation (DMDS adduct).

two EAD-active peaks were also detected in the fraction separated by silica gel column, the major compound being detected in the 3, 5, and 10% fractions and the minor compound in the 15 and 20% fractions. These are the biologically active fractions (above), but attraction was found only when two fractions (one of each group) were tested together.

The fragmentation pattern of the major EAD peak (Figure 3A) suggested that it was an acetate, as evidenced by the diagnostic peaks at *m/z* 61 from elimination

of the alkyl moiety and transfer of two hydrogen atoms to the fragment containing the oxygen atom. The quasimolecular ion (M^+-60) at *m/z* 166 indicated that the compound was a monounsaturated acetate with a molecular weight of 226. Chemical ionization mass spectrometry (CI-MS), with ammonia as the reactant gas, gave a base peak at *m/z* 244, which confirmed the molecular weight of 226. These findings suggested that the major EAD-active compound was dodecenyl acetate, in agreement with a library (Wiley) search, in which the best fit was 8-dodecenyl acetate. GC-MS analysis of the DMDS adducts showed the molecular weight at *m/z* 320 (Figure 3A, inset), an increment of *m/z* 94, suggesting the incorporation of two SCH_3 in one unsaturation in the parent compound. In addition, the base peak at *m/z* 217 and the peak at *m/z* 103 indicated that the unsaturation was located at position 8. Therefore, the compound was confirmed to be 8-dodecenyl acetate. The stereochemistry of the pheromone was determined by vapor-phase IR (Figure 4), which showed a typical *trans* band at 971 cm^{-1} (out of plane CH bending) and the lack of the =CH stretching (*cis* band) at ca. 3010 cm^{-1} (Leal, 1998). In conclusion, the major constituent was unambiguously identified as 8-(*E*)-dodecenyl acetate (**1**). In GC, GC-MS, GC-FTIR, and GC-EAD analyses, synthetic 8-(*E*)-dodecenyl acetate was indistinguishable from the natural product. Moreover, male antennae responded more strongly to the *E* than the *Z* isomer in EAD measurements (Figure 5), and the *Z* isomer was not detected in the gland extracts.

Although the FID peak of the EAD-active compound at 10.9 min (Figure 2) was very small, based on the structure of the major peak and the MS data of the minor component (Figure 3B), the second component was suggested to be the

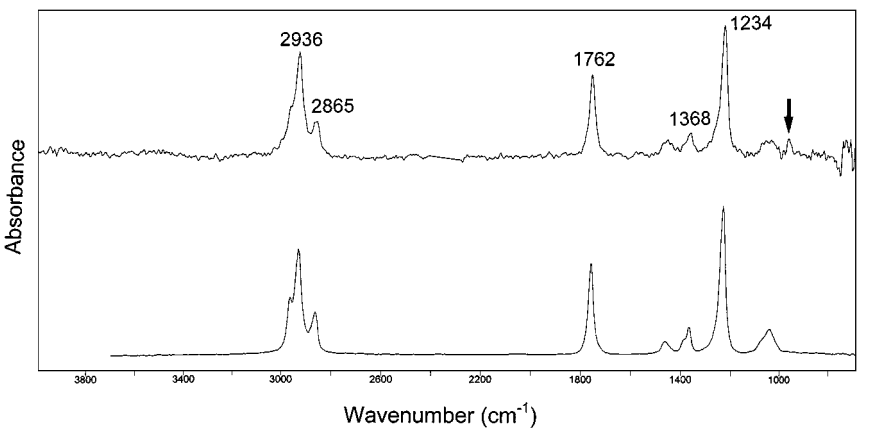


FIG. 4. Vapor-phase IR data generated with 10 female-equivalents of the major EAD-active peak (top). A library spectrum of a saturated homolog is given as reference (bottom). Arrow shows a characteristic *trans* band at 971 cm^{-1}.

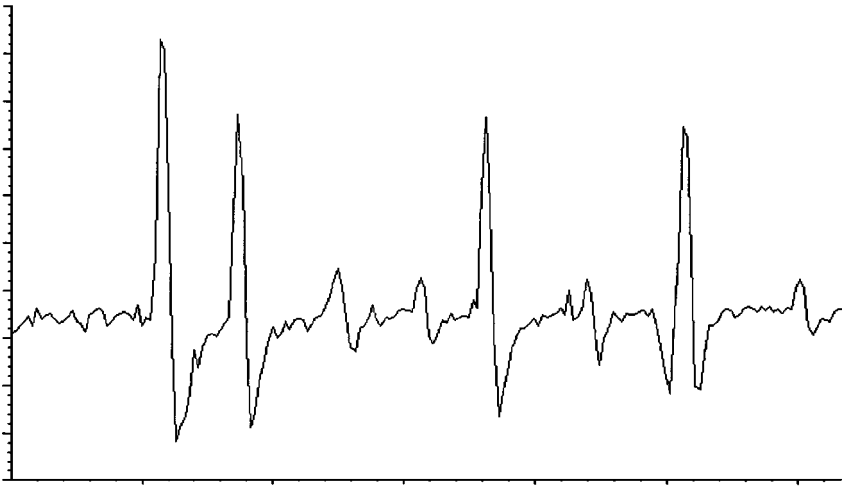


FIG. 5. Responses of a male *E. aurantiana* antenna to multiple injections of (*E*)- and (*Z*)-8-dodecenyl acetate introduced through a GC in split mode (50 : 1; ca. 0.2 ng/injection).

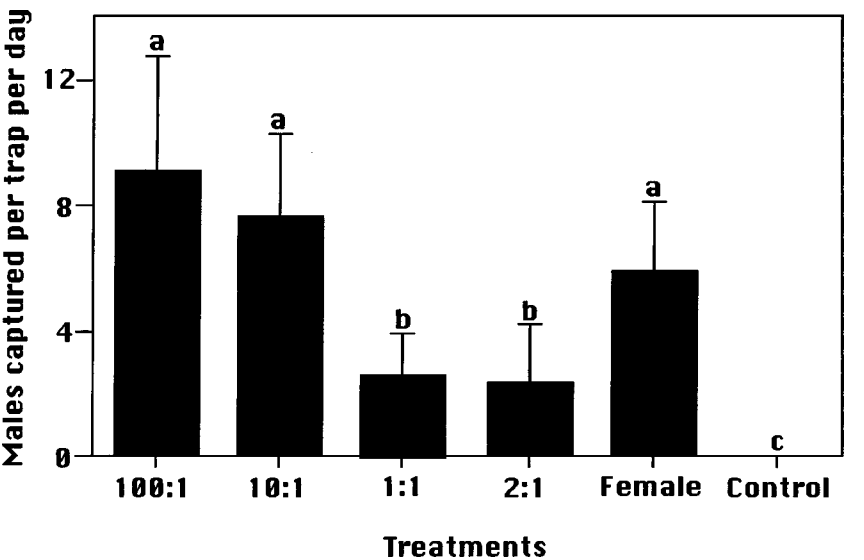


FIG. 6. Captures of *E. aurantiana* in sticky Delta traps baited with synthetic mixtures of the two pheromone components, (*E*)-8-dodecenyl acetate and (*E*)-8-dodecenol (1 mg of pheromone per trap).

related alcohol (**2**). A synthetic sample of the alcohol gave the same retention time as the minor peak, was EAD-active, and showed a similar MS profile. The MS of the synthetic compound devoid of impurities lacks some of the minor fragments, in particular, the ones at *m/z* 186, 189, 204, and 208, which are probably derived from an overlapping peak. Preliminary field experiments showed no significant catches of *E. aurantiana* in traps baited with only one of the two compounds, i.e., either (*E*)-8-dodecenol or (*E*)-8-dodecenyl acetate. However, captures in traps baited with the binary mixture were higher than control traps (Figure 6), regardless of the ratio of the two compounds. Captures in traps baited with high rates of *E*8–12 : Ac/*E*8–12 : OH (100 : 1 and 10 : 1) were not significantly different from the catches in traps baited with two virgin females. Performance of the traps with *E*8–12 : Ac/*E*8–12 : OH at 1 : 1 or 2 : 1 ratios were lower than the female traps, but better than the unbaited traps (control). Interestingly, there was no difference between the captures in traps having a 100 : 1 or 10 : 1 ratio of the two pheromone components. This is suggested to be due to the large number of treatments or because of the amount of pheromone (1 mg per device). In a separate set of experiments, the two ratios (100 : 1 and 10 : 1) were tested at different dosages. That traps baited with 1 mg of the pheromone blends captured more than those with

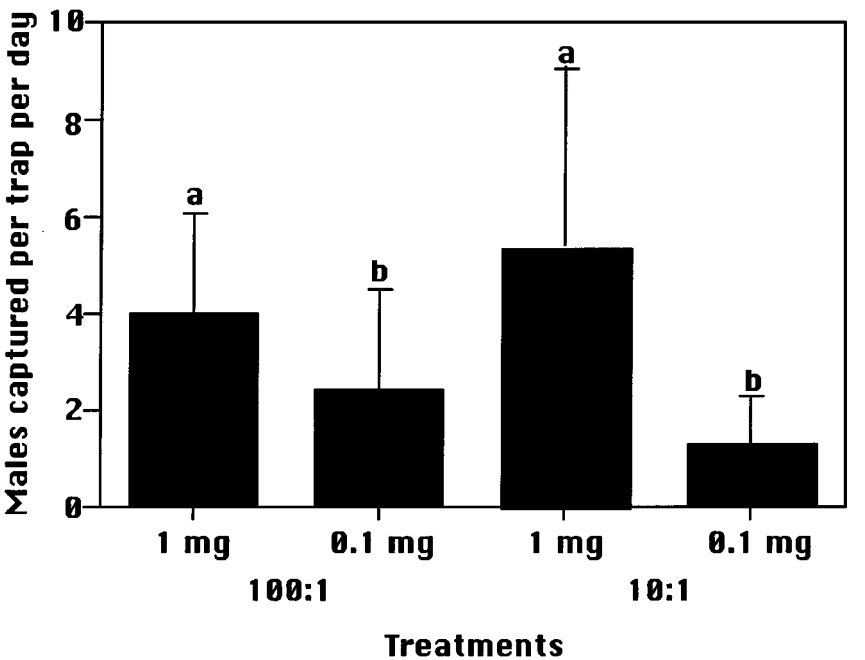


FIG. 7. Catches of *E. aurantiana* in sticky Delta traps with synthetic mixtures of the two pheromone components at two dosages and two different ratios.

0.1 mg (Figure 7) indicated that there was no saturation at the 1-mg level. In fact, the release rate of pheromone dispenser is very low as compared to the rubber septum. Furthermore, the catches in traps baited with 0.1 mg of the pheromone at the 100 : 1 and 10 : 1 ratios were not significantly different. It may be possible that the optimum ratio can be determined only in experiments under controlled conditions, such as wind tunnels. However, the optimal ratio for practical application in monitoring the insect population lies between 100 : 1 and 10 : 1 (*E*8–12 : Ac/*E*8–12 : OH). Although the scanty amount of the minor component prevented accurate analytical measurement of the ratio of the two compounds in the pheromone gland, it was closer to 100 : 1 than to 10 : 1.

Tortricid moths have been favorites of pheromone research. Sex pheromones have been characterized in over 60 species, and attractants of another 300 have been determined in field tests (Arn, 1991). The known female sex pheromones are all composed of primary aliphatic alcohols and their corresponding acetates and aldehydes. In general, they have 12 or 14 carbon atoms in the primary chain and one or two double bonds. (*E*)-8-Dodecenyl acetate and (*E*)-8-dodecenol have been identified as pheromone components of various moth species (Arn et al., 1998) and binary mixtures of **1** and **2** have been identified at least in two species, i.e., a sex pheromone from *Hedya chionosema* (Roelofs and Brown, 1982) and a sex attractant for *Strophedra nitidana* (Ando et al., 1977).

Acknowledgments—We appreciate the helpful discussion with members of our laboratory. We thank Rosa Molina, Peter Kasten, Jr., and Neide M. G. Zério (ESALQ/USP) for assistance in rearing insects.

REFERENCES

ANDO, T., YOSHIDA, S., TATSUKI, S., and TAKAHASHI, N. 1977. Sex attractants for male Lepidoptera. *Agric. Biol. Chem.* 41:1485–1492.

ANONYMOUS. 2000. Tecnologia contra o bicho-furão. *Revi. Fundecitrus* 96:8–10.

ARN, H. 1991. Sex pheromones, pp. 187–207, *in* L. P. S. Van der Geest and H. H. Evenhuis (eds.). World Crop Pests, 5. Tortricid Pests: Their Biology, Natural Enemies and Control. Elsevier, Tokyo.

ARN, H., TOTH, M., and PRIESNER, E. 1998. http://www.nysaes.cornell.edu/pheronet/

ATTYGALLE, A. B. 1998. Microchemical techniques, pp. 207–294, *in* J. G. Millar and K. F. Haynes (eds.). Methods in Chemical Ecology: Chemical Methods, Vol. 1. Kluwer Academic Press, New York.

BENTO, J. M. S., PARRA, J. R. P., VILELA, E. F., WALDER, J. M., and LEAL, W. S. 2001. Sexual behavior and diel activity of citrus fruit borer *Ecdytolopha aurantiana* (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 27:2053-2065.

LEAL, W. S. 1998. Infrared and ultraviolet techniques, pp. 185–206, *in* J. G. Millar and K. F. Haynes (eds.). Methods in Chemical Ecology: Chemical Methods, Vol. 1. Kluwer Academic Press, New York.

LEAL, W. S., HASEGAWA, M., MOCHIZUKI, F., and YASUDA, T. 1992. Behavioral and electrophysiological evidence of sex pheromone(s) in *Anomala schonfeldti* Ohaus (Coleoptera: Scarabeidae). *Appl. Entomol. Zool.* 27:592–594.

ROELOFS, W. L. and BROWN, R. L. 1982. Pheromones and evolutionary relatioships of Tortricidae. *Annu. Rev. Ecol. System.* 13:395–422.

SNEDECOR, G. W. and COCHRAN, W. G. 1989. Statistical Methods, 8th ed. Iowa University Press, Ames, Iowa.

STRUBLE, D. L. and ARN, H. 1984. Combined gas chromatography and electroantennogram recording of insect olfactory responses, pp. 161–178, *in* H. E. Hummel and T. A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

SEXUAL BEHAVIOR AND DIEL ACTIVITY OF CITRUS FRUIT BORER *Ecdytolopha aurantiana*

J. MAURÍCIO S. BENTO,[1] JOSÉ R. P. PARRA,[1] EVALDO F. VILELA,[2] JULIO M. WALDER,[3] and WALTER S. LEAL[4,*]

[1]*Departamento de Entomologia*
Fitopatologia e Zoologia Agrícola, ESALQ-USP
13418-900, Piracicaba-SP, Brazil

[2]*Departamento de Biologia Animal, UFV*
36570-000, Viçosa-MG, Brazil

[3]*CENA*
13418-900, Piracicaba-SP, Brazil

[4]*Department of Entomology*
University of California
Davis, California 95616

(Received March 10, 2001; accepted May 23, 2001)

Abstract—Males and virgin females of the citrus fruit borer *Ecdytolopha aurantiana* Lima, displayed two flight peaks during a 24-hr period, one at dawn and the other at dusk in an orange grove near Gavião Peixoto, São Paulo, Brazil. During the day, when temperatures were highest and relative humidity lowest, most individuals rested on leaves in the lower and middle crown. Moths rapidly moved higher in the crown after sunset, and many were observed flying above the tree canopy. This behavior was mainly associated with mating. Males and virgin females marked with fluorescent powder of different colors were observed in the dark with the aid of a black light. Mating was observed only in the upper crown of citrus trees from 6:00 to 9:00 PM, with a peak (64%) between 7:00 and 8:00 PM. Males of *E. aurantiana* were captured in traps baited either with virgin females or female extracts, suggesting the use of a long-range sex pheromone. At close distance (1–2 cm), males and females displayed a short-range communication behavior, with males exposing hairpencils and vibrating their wings. Females were frequently stimulated to contact the body of a male before copulation. The mean duration of copulation was 1 hr 40 min.

Key Words—Sex pheromone, *Ecdytolopha aurantiana*, attraction, mating behavior, hairpencils, diel periodicity.

*To whom correspondence should be addressed; e-mail: wsleal@ucdavis.edu

0098-0331/01/1000-2053$19.50/0

INTRODUCTION

The citrus fruit borer *Ecdytolopha aurantiana* Lima (Lepidoptera: Tortricidae) is a multivoltine species of brown and gray coloration (Lima, 1927; Schultz, 1939, White, 1993). *E. aurantiana* occurs in most neotropical areas, including Brazil (Prates and Pinto, 1988, 1991), Argentina (Schultz, 1939), Costa Rica and Trinidad-Tobago (Pickles, 1936). The larvae of *E. aurantiana* feed on and destroy citrus fruit, guava, *Psidium guajava* L. (Lima, 1927); banana, *Musa acuminata* Colla; coconut, *Cocos nucifera* L. (Meyrick, 1931); cherimolia, *Annona cherimolia* Mill. (Schultz, 1939); litchi, *Lichi chinensis* Sonn. (Lima, 1945); macadamia, *Macadamia integrifolia* Maiden and Betche (White, 1993), and sugar apple (fruta-do-conde), *Annona squamosa* L. (Nakano and Soares, 1995).

E. aurantiana was first observed to cause damage to citrus trees in the State of São Paulo, southeast Brazil, in 1915 (Lima, 1927). By the mid-1980s, this pest had been found in 54 municipalities in the State of São Paulo (Prates and Pinto, 1995) and in 10 other Brazilian States, with consequent reduced citrus production in these regions (Prates and Pinto, 1988, 1991). Yield losses of up to 50% in infested areas were estimated to occur in the State of São Paulo (Garcia et al., 1998), and the latest crop losses throughout the country are estimated at US$ 50 million pr/yr (Anonymous, 2000).

E. aurantiana is a difficult pest to control. Females usually deposit only one egg per fruit, and lay 150–200 eggs during their life (Garcia, 1999). After hatching, larvae pierce the peel and penetrate inside the fruit, where they feed on the pulp (Fonseca, 1934). Once larvae are inside the fruit, their control is impossible, and the fruit becomes unfit for consumption.

The major difficulty in the management of *E. aurantiana* is the lack of an accurate method for monitoring populations. Different sampling methods based on counts of eggs and first-instar larvae on the fruits are costly and inaccurate (Garcia, 1999). A system for adult detection by means of pheromone-based trapping of males would be an alternative method to anticipate the occurrence of this pest, and to control it in a more effective and economic manner, but the mating behavior of *E. aurantiana* has not been previously studied. Our objective was to report the sexual behavior and daily activity pattern of males and virgin females of *E. aurantiana* on citrus trees in an attempt to set up a rational program for the control of this pest.

METHODS AND MATERIALS

Insects. Adults of *E. aurantiana* were maintained in the laboratory on an artificial diet developed by Garcia and Parra (1999) at $27 \pm 2^{\circ}$C, $65 \pm 10\%$ relative humidity, and a 14D:10L photoregime. After emergence, the adults were sexed by the method of Garcia (1999) and transferred to wood cages ($25 \times 25 \times 25$ cm)

covered with nylon mesh. Males and virgin females were kept in different cages, with 200–400 individuals per cage. The production of adults in the laboratory varied during the experiment, ranging from 200 to 400 pairs a day. A small portion of insects (ca. 10–20%) was used for continuous rearing in the laboratory.

Greenhouse Experiments. Experiment 1 was conducted to determine the time of day, duration, and insect age at first mating. For each experiment, 10 pairs of *E. aurantiana* were selected soon after emergence and individually placed into transparent plastic cups (200 ml) inverted on Petri dishes (12 cm in diameter). The insects were maintained in a greenhouse at $25 \pm 2^{\circ}$C; $65 \pm 10\%$ relative humidity, and under natural light. Each Petri dish was lined with a filter paper of the same size, which was moistened daily with distilled water to keep the humidity high inside the plastic cup.

Preliminary assays showed that adults of *E. aurantiana* remained at rest during most of the day and the night, especially from 8:00 AM to 4:00 PM and from 10:00 PM to 5:00 AM. Thus, we observed pairs daily between 4:00 and 10:00 PM for seven days, with behavior recorded at 1-min intervals. To study mating behavior at night, we used a torchlight dimmed by three red sheets of cellophane paper and kept 1 m away from the subject. This experiment was replicated four times (i.e., 10 pairs with four replicates each; $N = 40$) in a completely randomized block design. Data (age at first mating and mating time) were transformed to percentage, and the differences between means were tested for significance by analysis of variance and compared by the Tukey test at 5% level. Tests were conducted on alternate days from November 5, 1998, to January 26, 1999.

Field Cage Experiments. Experiment 2 was designed to analyze the distribution and locomotion of males and virgin females of *E. aurantiana* during the day, as well as to determine the site of mating on the citrus trees. A $6 \times 25 \times 3.5$ m ($150\ m^2$) nylon field cage was constructed, covering six orange trees (6 years old) of the Pêra variety, 2.5–3 m high, in a commercial orange grove located on the campus of Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Piracicaba-SP, Brazil. The field cage was constructed in such a manner that the trees occupied its central line. Spacing between trees in the line was 4 m. Weeds present in the area covered by the field cage during the experiment were not eliminated.

Diel Distribution and Activity of Adult Moths. After emergence, males and females were sexed by the method of Garcia (1999) and maintained in cages, as previously described. At 2 days of age, males and females were marked with yellow and orange fluorescent powder, respectively. Insects were marked with ink diluted in ethyl alcohol, sprayed with a nasal spray, and dried with an electric fan at low speed (Southwood, 1995). Immediately after marking, the moths were released in the field cage at about 10:00 PM. On the following day, starting at 4:00 AM, observations were made for a period of 30 min at 1-hr intervals for 24 hr. During observations, the location and number of males and virgin females of *E. aurantiana* were recorded for: citrus trees, weeds, and soil and nylon netting.

When on citrus trees, moths were recorded on: leaves, fruit, and branches and trunk. The corresponding height on the tree was also considered: lower crown (0.5–1 m), middle crown (1.1–1.8 m), and upper crown (1.8–3 m). The sites of moth copulation and resting during day were marked with white plastic tape of ca. 20 cm. At night, the marked moths were visualized with the aid of a hand-held black light (model B-160 Spectroline). Movement of the moths was calculated as the difference between the individuals that moved from one place to another in relation to the previous hour. After each hour interval, males and virgin females of *E. aurantiana* were again localized and counted to compensate for individuals that had moved away or had been preyed upon. Therefore, it was possible to calculate the number of males and virgin females that stayed in the same place each hour. Preliminary studies with marked and unmarked adults in the greenhouse did not show any interference of the fluorescent powder with insect longevity or behavior, including mating. Three replicates of 24 hr each were performed, with a total of 250 pairs being released during a 24-hr period on February 9 and 24 and March 9, 1999. Temperature and relative humidity were measured with a thermohygrograph set up inside the field cage in the shade 1.0 m above ground during the assays. Official times of sunrise and sunset were provided by Instituto Astronômico e Geofísico da Universidade de São Paulo, São Paulo-SP, Brazil.

Female Attractiveness. Experiments 3–6 were designed to provide evidence for a long-range sex pheromone in *E. aurantiana*. Traps (stick Delta traps, Fuji Flavor Co., Tokyo, Japan) were baited with small cages ($2.5 \times 2.5 \times 2$ cm) containing two virgin females, two males, a rubber septum with 0.5 ml of female extract (25 female-equivalents), or a rubber septum with 0.5 ml solvent (control). The female extract was obtained on two consecutive days by cutting the last segments of the abdomen (segments VIII and IX) of 250 three-day-old virgin females and dipping (3-5 min) in hexane (5 ml). The time of the extractions was between 6:00 and 8:00 PM, coinciding with the mating time of *E. aurantiana*, previously observed in a greenhouse.

Experiments 3–6 were carried out in field cages, and tested virgin females versus empty trap (control) (experiment 3); virgin females versus males (experiment 4); virgin females versus female extract (experiment 5); and female extract versus solvent (control) (experiment 6). Each assay was carried out separately, and each treatment was replicated three times over two consecutive days in a completely randomized block design, with the treatments being renewed after 24 hr. Traps were placed in the upper crown of the tree (1.9–3 m) with an intertrap distance of 4 m. A total of 328, 290, 350, and 280 two-day-old virgin pairs were released between 4:00 and 5:00 PM on March 15, 22, and 29, and on April 5, 1999. Response to sex pheromone was quantified by counting the males captured in the traps each day. Capture data were transformed to log $(x + 1)$, and the differences between means were tested for significance by analysis of variance, with treatments being compared by the Tukey test at the 5% level of probability.

Attraction of Males to Pheromone Source in the Field. Experiment 7 was conducted in a 1200-ha commercial citrus grove (Ficher Co.) near Gavião Peixoto, SP, Brazil on April 7 and 8, 1999 to confirm the presence of sex pheromone. The experiment was conducted on the Hamlin orange variety, consisting of 12-to 15-year-old, 4-m-high trees spaced 4 m apart, with a 6-m space between rows. Delta sticky traps (Fuji Flavor Co., Tokyo, Japan) containing two virgin females, female extract (40 female-equivalents), and empty traps (control) were used. Each treatment was replicated four times over two consecutive days in a completely randomized block design, with the treatment being renewed after 24 hr. Each trap was placed in the upper third of the crown (2.5–3.5 m), with a 50-m space between traps and between rows and traps. The time when the experiment was started and the method for evaluation were the same as described for the field cage. Similarly, the natural extract of *E. aurantiana* was obtained as described previously, except that it was obtained on only one day from 400 virgin females using 5 ml of hexane. Capture data were transformed to log $(x + 1)$, and the differences between means were tested for significance by analysis of variance, with treatments being compared by Tukey test at the 5% level of probability.

RESULTS

Age, Time, and Duration of Mating. In a greenhouse, about 80% of the mating behavior of *E. aurantiana* was observed on the third and fourth days of life, corresponding to 44.7 and 36.8%, respectively (Figure 1A). Of the 40 pairs studied, 95% mated by the seventh day of life. Most mating behavior (60%) occurred between 7:00 and 8:00 PM, although the mating interval ranged from 5:00 to 9:00 PM (Figure 1B). The mean duration of copulation was 101.5 ± 62.1 min ($X \pm$ SEM; $N = 38$; range: 3–312 min).

Diel Distribution and Activities of Adult Moths. Field cage experiments showed that during the day most *E. aurantiana* moths were found in the lower and middle crown of citrus trees, with the presence of at least 34% and 38% of males, and 37% and 42% of virgin females, respectively (Table 1). This period was characterized by reduced flight activity with the moths usually resting on the surface of leaves, seemingly due to the high temperatures and low relative humidity (Figure 2). In the evening, about 88% and 75% of males and virgin females, respectively, were observed in the upper crown of citrus trees. This locomotion occurred between 6:00 and 7:00 PM, just after dusk (Figure 2). It was in the upper crown of the trees that all copulations took place (Table 1), peaking one hour after dusk, between 7:00 and 8:00 PM (64% of all episodes) (Figure 2).

During the night, after 8:00 PM, the locomotion and number of matings decreased quickly (ca. 8%; Figure 2). Most moths remained at rest, especially

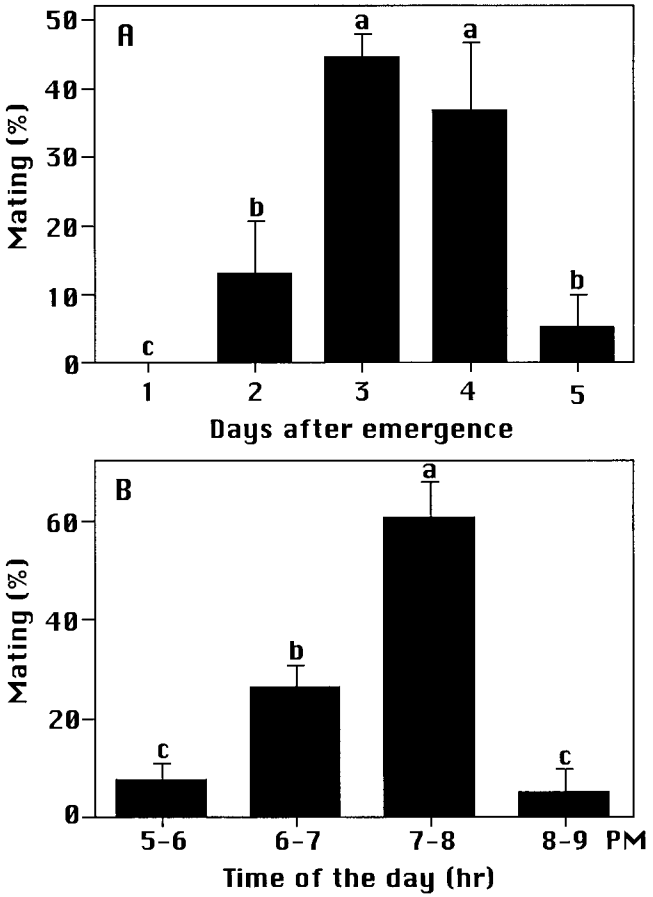


FIG. 1. Mating activities of *E. aurantiana* as function of age (A), and time (B), under greenhouse conditions. Bars with the same letter are not significantly different at the 5% level (Tukey test).

on leaves, occasionally engaging in walks and short flights. Many moths were observed flying for short distances after mating, although these data were not quantified. Adults were never observed feeding, although these data are not conclusive, requiring confirmation in future studies.

Just before dawn, a new peak of flight activity was recorded (Figure 2), and several moths were observed flying into the inner part of trees, especially towards the lower and middle crown of the host tree, reestablishing the same pattern of distribution as observed on the preceding day.

TABLE 1. DAY AND NIGHT DISTRIBUTIONS OF *E. aurantiana* IN CROWN OF CITRUS TREES UNDER FIELD CAGE CONDITIONS

Sex and status	Period	Height (m)		
		0.5–1.0	1.1–1.8	1.9–3.0
Male	Day	10.8 (34.7)[a]	12.1 (38.9)	8.2 (26.4)
	Night	0.5 (1.4)	3.7 (10.1)	32.3 (88.5)[b]
Virgin female	Day	12.2 (37.2)	14.0 (42.7)	6.6 (20.1)
	Night	1.0 (2.5)	8.8 (22.3)	29.7 (75.2)
Mating couples	Night[c]	0 (0)	0 (0)	22.5 (100)

[a] Percentages in parentheses.
[b] Includes individuals of mating couples.
[c] See Figure 2 for time.

Reduced numbers of males and virgin females were recorded on weeds or on the ground/screen during the experiment (Table 2). Although no counts were made, most moths found on the ground were weakened or dead.

Mating Behavior. Males of *E. aurantiana* hovering beside leaves with females in the upper crown of citrus trees were responding to female sex pheromone. Traps containing virgin females or a natural female extract in a solvent (25 female-equivalents) caught on average 8 and 6 male/trap/day in field cages (Figure 3). The same results were obtained in the field in a commercial area, with 13 and 8 males caught per trap per day in traps containing females and natural female extract in a solvent (40 female-equivalents), respectively (Figure 4).

DISCUSSION

The distribution (i.e., observed average number) and behavior of males and virgin females of *E. aurantiana* were synchronized and varied with respect to

TABLE 2. DISTRIBUTION OF *E. aurantiana* IN DIFFERENT PARTS OF CITRUS TREES OVER A 24-HR PERIOD

Place	Males	Virgin females	Total number (% mean)
Citrus			
Leaves	25.9 (88.7)	33.9 (90.8)	59.8 (89.7)
Fruits	0.7 (2.6)	0.4 (1.2)	1.1 (1.9)
Branches/trunk	0.5 (1.8)	0.8 (2.2)	1.3 (2.0)
Weeds	0.2 (0.7)	0.5 (1.4)	0.7 (1.1)
Soil/Netting	1.8 (6.2)	1.1 (4.4)	2.9 (5.3)

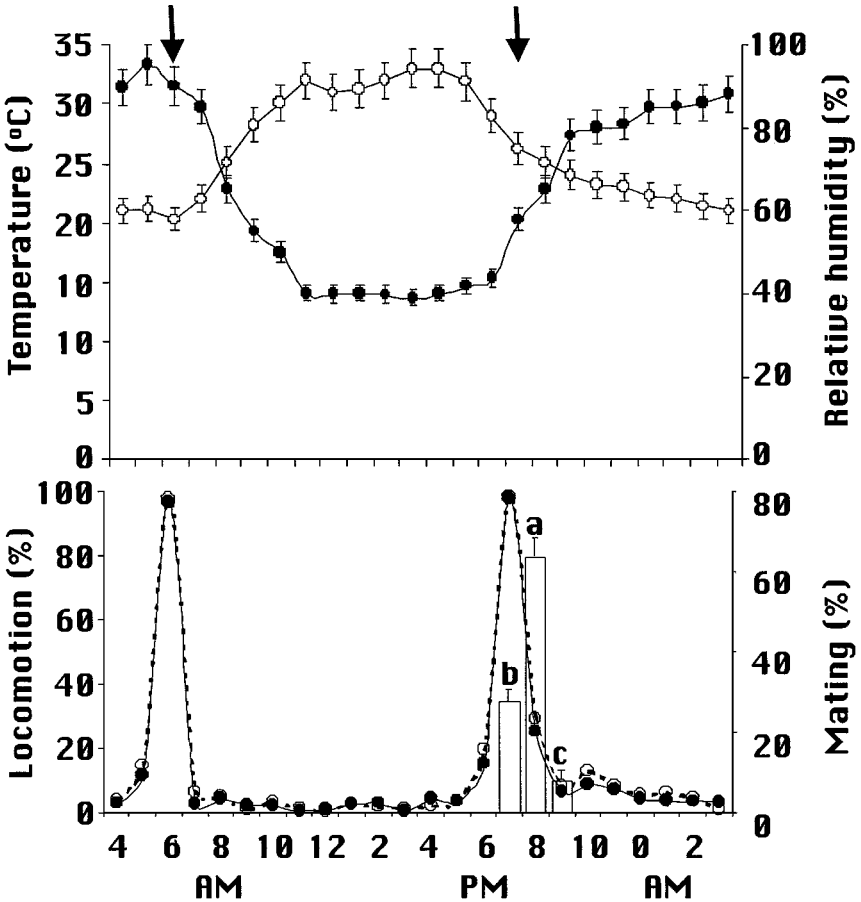


FIG. 2. Diel periodicity of locomotion and mating in adult *E. aurantiana* in relation to air temperature and relative humidity. (Top) Average temperature (—○—) and relative humidity (—●—) with SEM (bars); (Bottom) females (—○—) and males (—●—) locomotion. Arrows indicate time of sunrise and sunset. Bars with the same letter are not significantly different at the 5% level (Tukey test).

location in the crown of citrus trees and time of day. About 1 hr before dawn, many moths started to fly, and flight activity peaked just before dawn, as also reported for many other tortricids (Lewis and Taylor, 1964; Dreisig, 1986; Quiring, 1994). For *E. aurantiana*, the increase in flight activity during this period was not associated with mating or oviposition. Temperature increased quickly during the morning, and probably moth flight activity was due to the search for microhabitats with a reduced risk of hygrothermal stress. When temperature

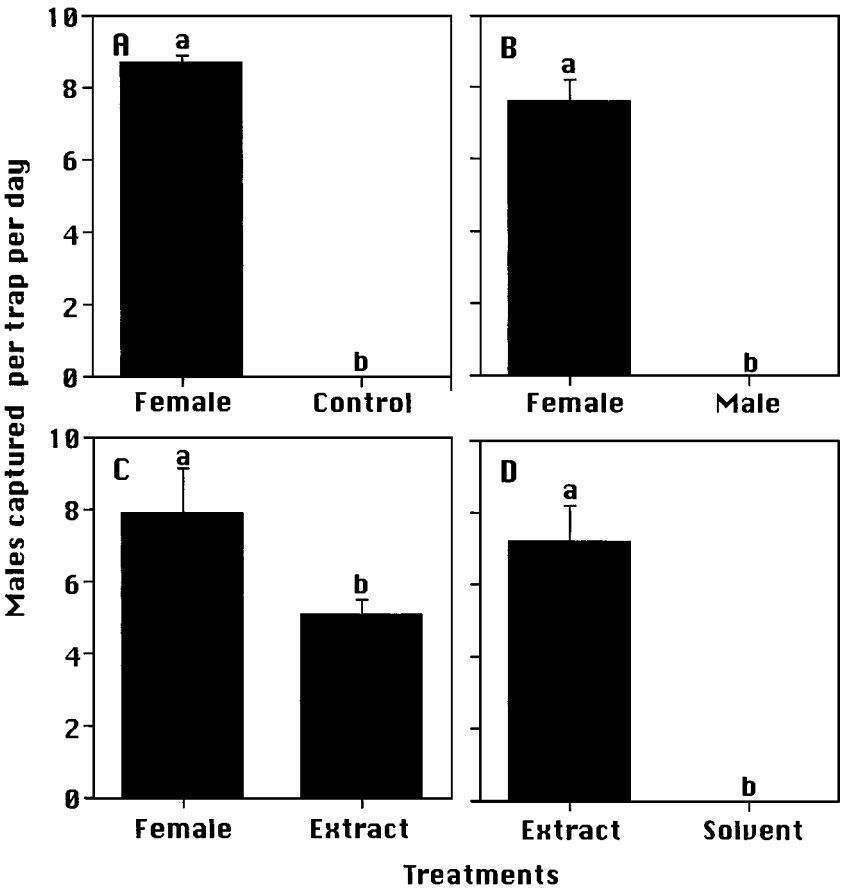


FIG. 3. Trap catches of male *E. aurantiana* in field cage experiments 3–6 baited with females (A), males (B), natural female extract in a solvent (C), or solvent alone (D). Bars with the same letter in each experiment are not significantly different at the 5% level (Tukey test).

was highest and relative humidity lowest, moths spent most of the day resting on leaves in the lower and middle crown of their host tree. Garcia (1999) reported that the viability and longevity of *E. aurantiana* is much reduced when the moth is exposed to temperatures above of 32°C and relative humidity below 50%.

During the day, some moths flew among the leaves of the tree or adjacent ones. However, these flights were sporadic and covered a short distance. Probably, they were motivated by the incidence of sunlight and the presence of different ant species and predators. Escaping predators may also explain the larger

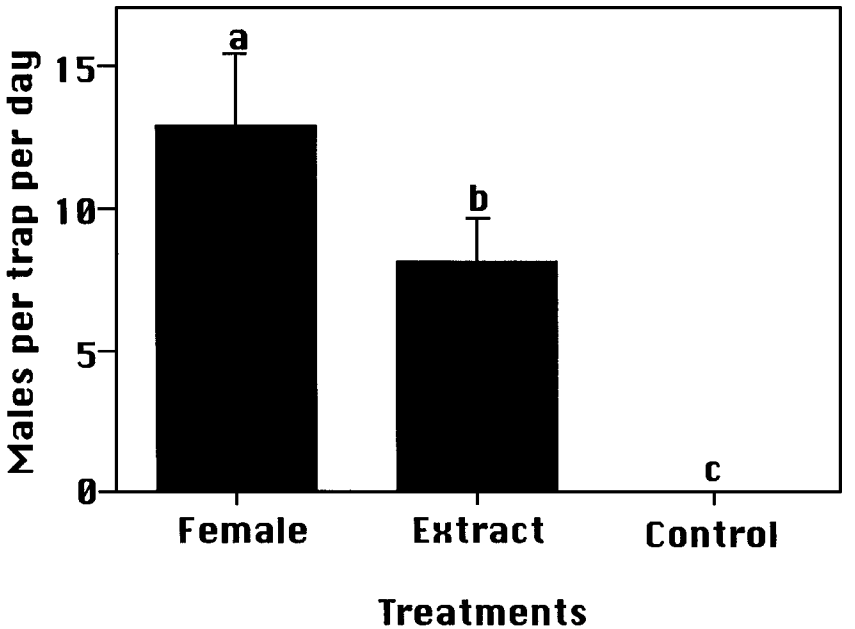


FIG. 4. Captures of male *E. aurantiana* in experiment 7 in traps baited with live virgin females, pheromone gland extract, or solvent controls. Bars with the same letter are not significantly different at the 5% level (Tukey test).

numbers of males and virgin females frequently found resting on leaves rather than on other parts of the trees. The large numbers of ants foraging on branches for nectar, oils, and honeydew throughout the day probably explain the escape behavior of the moths. Although this was not quantified, on several occasions various species of spiders also were observed preying on *E. aurantiana* adults on the leaves.

The second major peak of *E. aurantiana* activity started at dusk and continued for about 2 hr between 6:00 and 8:00 PM. Several moths were observed flying above the host tree canopy at dusk, as also reported for many other tortricids, an activity usually associated with oviposition and mating (see Pointing, 1961; Dustan, 1964; Turgeon, 1985; Turgeon et al., 1987; Quiring, 1994). Redfern and Di Giácomo (1991) and Garcia (1999) reported that oviposition of *E. aurantiana* is concentrated during the twilight period, with the latter author detecting about 60% of the eggs during this period.

In the present study, the flight activity of *E. aurantiana* in the evening was primarily associated with sexual behavior. In our experiment, we did not observe

oviposition because we only used males and virgin females. Most of the mating activity occurred about 1 hr after dusk and only in the upper crown of the host tree. This is an original finding concerning tortricid moths since there are no previous studies describing the exact site of copulation on host trees (Howell, 1995). The fact that the mating site may be related to the calling ability of females, or more probably to a better release of sex pheromone by them, may explain the capture of males in traps placed in the upper crown of trees. It was not possible to determine why copulation pairs were not observed at other sites on the tree even after repeated observations, although a likely hypothesis is that staying on the top of the tree represents an adaptive advantage. In addition, capture of tortricid moths with fermented products (Yothers, 1927), pheromone baited traps (Lewis and Macaulay, 1976; Riedl et al., 1979; McNally and Barnes, 1981; Ahmad and Al-Gharbawi, 1986; Weissling and Knight, 1995), and light traps (Borden, 1931; Hamilton and Steiner, 1939) has been reported to be more effective when the traps are set on tree tops.

The presence of males close to virgin females just before mating was due to long-distance attraction elicited by a female sex pheromone. Observations in greenhouses suggest that visual or short-range communication signals may play a role. Usually, males approached females within a distance of 1–2 cm, then presented a stereotyped sequence of behaviors, including vibration of the wings and hairpencil display toward the female, which frequently attracted the female towards them. The female then contacted the body or the end of the abdomen of the male, and this stimulus caused the male to turn quickly and attempt copulation. When copulation was successful, male and female remained attached through the abdomen in opposite direction for ca. 1 hr and 40 min. A similar courtship was described for the oriental fruit moth *Grapholita molesta* (Busck) (Baker and Cardé, 1979). On the other hand, mean copulation time varies widely among tortricids. Copulation time is 40–60 min in *Cydia pomonella* (L.) (Howell, 1995) and about 29 min in *G. molesta* (Dustan, 1964), while in *Zeiraphera canadensis* Mut. & Free, it is 4 hr and 20 min (Turgeon et al., 1987).

In summary, this study determined the time when mating takes place, and thus, when pheromone glands should be extracted for pheromone identification. In addition, observations that mating occurs on the top of trees from 6:00 to 9:00 PM are of importance not only for the design of trap experiments, but also for future utilization of sex pheromones for monitoring citrus fruit borer populations.

Acknowledgments—We thank Rosa M. S. Molina, Peter Kaster, Jr., and Neide G. Zério, Departamento Entomologia, Fitopatologia, Zoologia, Agrícola of Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ/USP), for assistance in rearing insects, and Luís A. Lopes, Centro de Energia Nuclear na Agricultura (CENA), for assistance in the field experiments. This work was partially supported by Fundo Paulista de Defesa da Citricultura (Fundecitrus).

REFERENCES

ANONYMOUS. 2000. Tecnologia contra o bicho-furão. *Rev. Fundecitrus* 96:8–10.

AHMAD, T. R. and AL-GHARBAWI, Z. A. 1986. Effects of pheromone trap design and placement on catches of codling moth males. *J. Appl. Entomol.* 102:52–57.

BAKER, T. C. and CARDÉ, R. T. 1979. Courtship behavior of the oriental fruit moth (*Grapholitha molesta*): Experimental analysis and consideration of the role of sexual selection in the evolution of courtship pheromones in the Lepidoptera. *Ann. Entomol. Soc. Am.* 72:173–188.

BORDEN, A. D. 1931. Some field observations on codling moth behavior. *J. Econ. Entomol.* 24:1137–1145.

DREISIG, H. 1986. Timing of daily activities in adult Lepidoptera. *Entomol. Gen.* 12:25–43.

DUSTAN, G. G. 1964. Mating behaviour of the oriental fruit moth, *Grapholitha molesta* (Busck) (Lepidoptera: Olethreutidae). *Can. Entomol.* 96:1087–1093.

FONSECA, J. P. da. 1934. Combate a lagarta das laranjas *Gymnandrosoma aurantiarum* Costa Lima. *Chac. Quint.* 50:215–216.

GARCIA, M. S. 1999. Bioecologia e potencial de controle biológico de *Ecdytolopha aurantiana* (Lima, 1927) (Lepidoptera: Tortricidae), o bicho-furão-dos-citros, através de *Trichogramma pretiosum* Riley, 1879. Piracicaba, 1998. PhD thesis. Escola Superior de Agricultura "Luiz de Queiroz," Universidade de São Paulo (in Portuguese), 118 pp.

GARCIA, M. S. and PARRA, J. R. P. 1999. Comparação de dietas artificiais, com fontes protéicas variáveis, para a criação de *Ecdytolopha aurantiana* (Lima) (Lepidoptera: Tortricidae). *An. Soc. Entomol. Brasil* 28:219–232.

GARCIA, M. S., PARRA, J. R. P., IAROSSI, A. R., and KASTEN, P. JR., 1998. Bioecologia do bicho-furão e perspectivas de controle. *Laranja* 19:249–260.

HAMILTON, D. W. and STEINER, L. F. 1939. Light traps and codling moth control. *J. Econ. Entomol.* 32:867–872.

HOWELL, J. F. 1995. Reproductive biology, pp. 157–174, *in* L. P. S. van der Geest and H. H. Evenhuis (eds.). Tortricid Pests: Their Biology, Natural Enemies and Control, Vol. 5. Elsevier, Amesterdam.

LEWIS, T. and MACAULAY, E. D. M. 1976. Design and elevation of sex-attractant traps for the pea-moth, *Cydia nigricana* (Steph.), and the effect of plume shape on catches. *Ecol. Entomol.* 1:175–187.

LEWIS, T. and TAYLOR, L. R. 1964. Diurnal periodicity of flight by insects. *Trans. R. Entomol. Soc. London* 116:393–476.

LIMA, A. C. 1927. Sobre um novo microlepidoptero, cuja lagarta é praga das laranjeiras no Distrito Federal. *Chac. Quint.* 36:33–35.

LIMA, A. C. 1945. Insetos do Brasil. Lepidópteros. 1° Parte. Rio de Janeiro, Escola Nacional de Agronomia. 5:346–353. (Série didática, 7). 379 pp.

MCNALLY, P. S. and BARNES, M. M. 1981. Effects of codling moth pheromone trap to placement, orientation and density on trap catches. *Envion. Entomol.* 10:22–26.

MEYRICK, E. 1931. Exotic microlepidoptera. Vol. 4. Parts 3–12. [Pp. 129–160]. Taylor & Francis, [London]. [1930–1937]: 642 pp.

NAKANO, O. and SOARES, M. G. 1995. Bicho-furão: Biologia, hábitos e controle. *Laranja* 16:187–208.

PICKLES, A. 1936. Insect pests of Trinidad crops with some suggestions for their control. *Proc. Agric. Soc. Trinidad Tobago* 36:339–348.

POINTING, P. J. 1961. The biology and behaviour of the European pine shoot moth, *Rhyacionia buoliana* (Schiff.), in southern Ontario. I. Adult. *Can. Entomol.* 93:1098–1112.

PRATES, H. S. and PINTO, W. B. S. 1988. Ocorrência da mariposa das laranjas (*Gymnandrosoma aurantiarum* Lima, 1927) na citricultura paulista. *Laranja* 9:117–124.

PRATES, H. S. and PINTO, W. B. S. 1991. Bicho-furão, um grave problema para a citricultura paulista. *Laranja* 12:425–434.

PRATES, H. S. and PINTO, W. B. S. 1995. Ocorrência do bicho-furão nas principais regiões citrícolas paulistas. *Laranja* 16:237–242.

QUIRING, D. T. 1994. Diel activity pattern of a nocturnal moth, *Zeiraphera canadensis*, in nature. *Entomol. Exp. Appl.* 73:111–120.

REDFERN , J. W. JR. and DI GIÁCOMO, O. A. S. 1991. Conheça o bicho furão para controlálo melhor. *Inf. Coopercitrus* 52:12–14.

RIEDL, H., HOYING, S. A., BARNETT, W. W., and DETAR, J. E. 1979. Relationship of within-tree placement of the pheromone trap to codling moth catches, *Environ. Entomol.* 8:765–769.

SCHULTZ, E. T. 1939. La mariposa de los naranjos (*Gymnandrosoma* sp.). *Rev. Ind. Agric. Tucuman* 29:87–90.

SOUTHWOOD, T. R. E. 1995. Ecological Methods: With Particular Reference to the Study of Insect Populations, 2nd ed. Chapman & Hall, London, 524 pp.

TURGEON, J. J. 1985. Life cycle and behavior of the spruce budmoth, *Zeiraphera canadensis* (Lepidoptera: Olethreutidae), in New Brunswick. *Can. Entomol.* 117:1239–1247.

TURGEON, J. J., NELSON, N., and KETTELA, E. G. 1987. Reproductive biology of the spruce budmoth, *Zeiraphera canadensis* Mut. & Free. (Lepidoptera: Tortricidae: Olethreutidae), in New Brunswick. *Can. Entomol.* 119:361–364.

WEISSLING, T. J. and KNIGHT, A. L. 1995. Vertical distribution of codling moth adults in pheromone-treated and untreated plots. *Entomol. Exp. Appl.* 77:271–275.

WHITE, G. L. 1993. Outbreak of *Ecdytolopha aurantianum* (Lima) on citrus in Trinidad. *FAO Plant Prot. Bull.* 41:130–132.

YOTHERS, M. A. 1927. Summary of three years' tests of trap baits for capturing the codling moth. *J. Econ. Entomol.* 20:567–575.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

IDENTIFICATION AND SYNTHESIS OF MALE-PRODUCED SEX PHEROMONE COMPONENTS OF THE STINK BUGS *Chlorochroa ligata* AND *Chlorochroa uhleri*

HSIAO-YUNG HO[1,2] and JOCELYN G. MILLAR[1,*]

[1]*Department of Entomology*
University of California
Riverside, California, 92521

[2]*Institute of Zoology*
Academia Sinica
Taipei, Taiwan

(Received February 26, 2001; accepted June 3, 2001)

Abstract—The reproductive behaviors of the stink bugs *Chlorochroa ligata* and *C. uhleri* were studied in the laboratory. Adults of both species became sexually mature about 12–14 days after the final molt, and both sexes mated multiple times during their lifetimes. The mean duration of copulation was 54 ± 24 min for virgin bugs and 46 ± 33 min for experienced bugs for *C. ligata* and 78 ± 55 min for field-collected *C. uhleri* of unknown mating status. Male *C. ligata* were found to transfer a significant fraction of their body mass (19%) to females during mating. Sexually mature *C. uhleri* males produced three sex-specific compounds, methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, and methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, in a ratio of 100:0.9:0.6. These three compounds were also produced by sexually mature male *C. ligata* in a ratio of 100:0.5:0.4. Identifications of the compounds were confirmed by synthesis. Production of the male-specific compounds peaked in late afternoon to early evening, coincident with the peak period of reproductive activity. Laboratory and field bioassays demonstrated that female bugs were attracted to odors from live males and to reconstructed blends of the male-specific compounds.

Key Words—*Chlorochroa ligata*, *Chlorochroa uhleri*, Pentatomidae, pheromone, methyl (*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, reproductive behavior.

*To whom correspondence should be addressed; e-mail: jocelyn.millar@ucr.edu

INTRODUCTION

Phytophagous pentatomid bugs are pests of a broad spectrum of agriculturally important crops including fruits, nuts, row crops, and vegetables (McPherson and McPherson, 2000). These insects are characterized by piercing and sucking mouth parts, which both immatures and adults use to inject saliva into plants, liquefying plant tissues. This feeding damage causes premature abortion or deformation of seeds and fruiting bodies and also transmits disease. The adult insects are strong fliers and can migrate from field to field as hosts are harvested or senesce (McPherson and McPherson, 2000). Current monitoring methods include beating tray or sweep-net sampling (McPherson and McPherson, 2000), both of which are labor intensive and time consuming. There is an urgent need for better monitoring methods, and traps baited with attractant pheromones may provide such methods. However, sex specific compounds have been identified for only a few stink bugs, including *Nezara viridula* (L.) (Aldrich et al., 1987; Baker et al., 1987; Brézot et al., 1994), *Euschistus spp*. (Aldrich et al., 1991, 1994; Borges et al., 1998a), *Plautia stali* Scott (Sugie et al., 1996), *Thyanta pallidovirens* Stål (Millar, 1997), *Piezodorus hybneri* Gmelin (Leal et al., 1998), *P. guildinii* (Westwood) (Borges et al., 1998b), and *Biprorulus bibax* Breddin (James et al., 1994). The complexities involved in the identification and development of applications of stink bug pheromones have been summarized by McBrien and Millar (1999) and are further complicated by the growing body of evidence that stink bug reproductive behaviors are mediated by both chemical and substrate-borne vibrational signals (e.g., Ota and Çokl, 1991; Çokl et al., 2001).

The conchuela stink bug, *Chlorochroa ligata* (Say), is found in the western regions of the United States and Canada, and it has been reported as far east as Missouri, Kansas, and South Dakota (Buxton et al., 1983; Henry and Froeschner, 1988). *C. ligata* adults are usually large grayish black bugs but the species exhibits a wide variation in color. Overall, the species is generally dark in color, with the lateral margins of the thorax, the basal third to two thirds of the costal margins of the wing corium, and the tip of the scutellum varying in color from pale yellow to bright crimson (Morrill, 1910; Buxton et al., 1983). The strain infesting apple and pear orchards in California is commonly black with reddish marginal borders and a reddish spot in the middle of the back (Ohlendorf, 1999), whereas *C. ligata* found in blackberry fields in Washington State were black with pale yellow borders (Fish and Alcock, 1973).

A congeneric species, *Chlorochroa uhleri* (Stål), commonly known as Uhler's stink bug, is distributed through the western United States, where it damages wheat (Caffrey and Barber, 1919), tomato (Ohlendorf, 1990), cotton (Morrill, 1910), and alfalfa (Russell, 1952). In California, it can have several generations per year, and details of its life history have been reported by Morrill (1910) and Russell (1952). The color of *C. uhleri* is green above and paler and yellower ventrally. The

callosities at the base of the scutellum are moderate to small. *C. uhleri* is morphologically similar to another congener, *Chlorochroa sayi* Stål, and the two species frequently have been confused (McPherson and McPherson, 2000), particularly as both *C. sayi* and *C. uhleri* can be found on the same host plants. The most useful character for separating the two species is the purple flecks on the membrane of the hemelytra of *C. sayi*, which are absent in *C. uhleri* (Buxton et al., 1983).

We recently reported the identification of pheromones from *C. sayi* (Ho and Millar, 2001a). Here, we report the results of investigations of the reproductive behavior and semiochemistry of *C. uhleri* and *C. ligata.* Our specific objectives were: (1) to describe the reproductive behaviors of *C. uhleri* and *C. ligata*; (2) to identify volatile chemicals produced by each species, focusing particularly on sex-specific compounds that might be sex pheromone components; and (3) to test reconstructed blends of sex-specific compounds as stink bug attractants in laboratory and field bioassays.

METHODS AND MATERIALS

Insects. A colony of *C. uhleri* was started from egg masses obtained from Paul da Silva, Kearney Agricultural Station, Parlier, California. Additional bugs were collected from Russian thistle (*Salsola iberica* Sennen) in Riverside County in the summers of 1998 and 1999. A colony of *C. ligata* was started from adult bugs provided by Dr. Les Ehler, University of California, Davis, California. A second colony of *C. ligata* was started in the summer of 1999 from specimens provided by Christian Krupke, Washington State University Tree Fruit Research and Extension Center, Wenatchee, Washington. Both species were reared on green-bean pods grown without pesticides, raw shelled peanuts and sunflower seeds, and bouquets of alfalfa and seasonal weed species, exactly as previously described for *C. sayi* (Ho and Millar, 2001a). Bugs were kept under a 16L:8D cycle, at 23 $\pm$ 2°C, and $>$50% relative humidity.

Observations of Reproductive Behavior. Mating behaviors of sexually mature adult *C. ligata* and *C. uhleri* were video-taped in time lapse mode as described for *C. sayi* (Ho and Millar, 2001a), using individual pairs of bugs in plastic tubes, with green-beans provided for food. Observations of mating behaviors were carried out using virgin, laboratory-reared *C. ligata.* For *C. uhleri*, due to the low number of bugs in the laboratory colony at the time the experiments were conducted, field collected insects of unknown age and mating status were used, after first segregating the bugs by sex for 4 days. A total of 27 matings were observed from 13 pairs of *C. ligata* in 14 days, and 28 matings from 12 pairs of *C. uhleri* over eight days. The time each copulation started, duration of each copulation, and the total numbers of copulations per pair were recorded. The numbers of matings initiated per 2 hr time period were plotted.

Weight Change during Mating. The experimental setup to examine weight change during mating was as previously described for *C. sayi* (Ho and Millar, 2001a). For *C. ligata*, 122 virgin pairs and 52 experienced pairs were observed between March 27 to April 10, 2000. Copulations were observed among a total of 13 virgin pairs and 11 experienced pairs. Because there were not enough *C. uhleri* in the laboratory colony, weight changes during mating experiments were not recorded.

Collection and Analysis of Insect-Produced Compounds. Volatile chemicals were collected and analyzed as previously described for *C. sayi* (Ho and Millar, 2001a). Briefly, volatiles were collected on activated charcoal traps from groups of sexed virgin adult bugs held in glass aeration chambers with green-beans. Volatiles from green-beans were collected as a control. Compounds were eluted from the traps with pentane and ether, and analyzed by splitless gas chromatography on a DB-17 column (30 m $\times$ 0.25 mm, J&W Scientific, Folsom, California), with a temperature program of 50°C for 1 min, 10°C/min to 250°C. Injector and detector temperatures were 250°C and 280°C, respectively, with helium carrier gas. Extracts were also analyzed by splitless coupled gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard 5890 GC fitted with a DB5-MS column (20 m $\times$ 0.2 mm id.), and interfaced to an H-P 5970B mass selective detector (electron impact ionization, 70 eV). The GC was programmed from 40°C/1 min, 10°C/min to 250°C, with injector and transfer line temperatures of 250 and 280°C, respectively. Compounds were tentatively identified from interpretation of the mass spectra, and from matches with the NBS-NIH mass spectral data base. Identifications were confirmed by comparison of retention times and spectra with those of authentic standards.

Bioassays Using Odors from Live Male Bugs, Extracts of Bug Volatiles, or Synthetic Chemicals as Test Stimulants. Laboratory bioassays were carried out with a vertical glass Y-tube olfactometer, exactly as described previously (Ho and Millar, 2001a), using sexually mature virgin adults. Bugs were used only once. Not all cohorts of virgin male *C. uhleri* produced male-specific compounds, as determined by GC analysis of collected bug odors. Consequently, only live males from *C. uhleri* cohorts that had been demonstrated to be producing male-specific compounds were used as sources of attractant in bioassays, with female responders.

Field Bioassays. The complete blend of male-specific compounds from each species (see below) was tested in field bioassays as described by Ho and Millar (2001a). Lures consisted of solutions of 20 mg of the racemic major compound, methyl (*E*)-6-2,3-dihydrofarnesoate, together with the minor components, methyl (2*E*,6*E*)-farnesoate (0.12 mg) and racemic methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate (0.18 mg) absorbed onto 10 cm lengths of natural latex rubber tubing. Controls consisted of sections of tubing treated with CH_2Cl_2. Lures and controls were hung in Russian thistle plants, with the number of bugs on each plant counted daily during the late afternoon. Lures and controls were rerandomized

among plants after each count. In the first sets of trials (see below), responding bugs were collected, counted, and released, whereas in later trials, all responding bugs were collected and taken back to the laboratory to augment the laboratory colonies.

For trial 1, August 3–16, 1999, lures with the three-component blend of *C. uhleri* together with solvent-treated controls were deployed at test sites in Ontario, Moreno Valley, Redlands, and Riverside, California, with three replicates of each treatment in each block. Bioassays were evaluated as described above, with the counted bugs being released.

For trial 2, August 19–24, 1999, six experimental blocks were set up in Russian thistle patches in Moreno Valley and Riverside, testing the three-component blend of *C. uhleri*, and a solvent control. Each treatment and control was replicated three times at each site. Bioassays were evaluated each evening from ~17:00 till dusk, collecting all responding bugs.

For both trials, where appropriate, the numbers of responding bugs were transformed, using the square root $(x + 0.5)$ transformation, and the transformed data were subjected to two-way ANOVA, with differences between treatments and controls determined by Dunnett's method. For those data sets that did not satisfy the assumptions of ANOVA, differences between treatments and controls were determined with the nonparametric multiresponse permutation procedure (Biondini et al., 1985; McCune and Mefford, 1999).

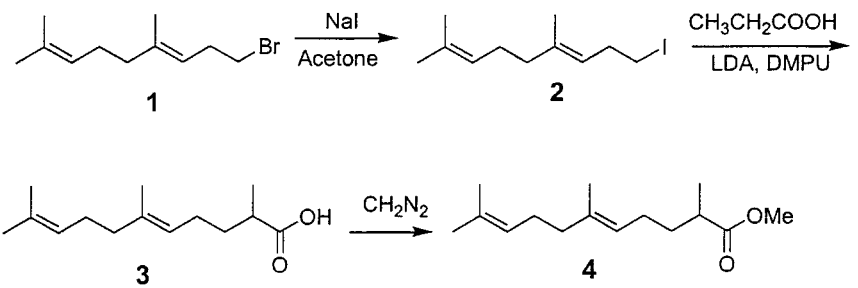
Chemicals. (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-hexenyl acetate, undecane, dodecane, tridecane, tetradecane, and pentadecane were purchased from Aldrich Chemical (Milwaukee Wisconsin), and (*E*)-2-octenyl acetate was obtained from Bedoukian Research (Danbury, Connecticut). (*Z*)-2-Octenyl acetate was synthesized by acetylation of (*Z*)-2-octenol with acetyl chloride and triethylamine in ether. Racemic methyl (*E*)-6-2,3-dihydrofarnesoate was synthesized as previously described (Ho and Millar, 2001a). (*E*)-4-Oxo-2-hexenal was synthesized as described by Ho and Millar (2001b).

Synthesized Compounds. Flash chromatography was carried out with 0.04 to 0.063-mm silica gel (Aldrich Chemical Co., Milwaukee, Wisconsin). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 300 and 75.48 MHz, respectively, using a General Electric QE 300 instrument. GC-MS analyses (electron impact ionization, 70 eV) were performed on a Hewlett-Packard 5890 gas chromatograph interfaced to an HP 5970B mass selective detector (Palo Alto, California). A DB-5MS column was used (20 m $\times$ 0.2 mm, 0.25 μm; J&W Scientific, Folsom, California). Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl immediately before use. Routine work-up of reactions included drying solutions over anhydrous Na_2SO_4 and concentrating by rotary evaporation under partial vacuum (~80 mm Hg). All reactions were carried out under argon atmosphere in oven-dried glassware unless otherwise specified.

Synthesis of Methyl (2E,6E)-Farnesoate. A solution of trimethylphosphonoacetate (2.18 g, 12 mmol; Lancaster Synthesis, Windham, New Hampshire)

in THF (20 ml) was cooled to −30°C under Ar, and *n*-butyllithium (4.8 ml of 2.5 M in hexane, 12 mmol) was added dropwise. The mixture was warmed to 0°C, and geranyl acetone (1.94 g, 10 mmol) was added dropwise. The mixture was warmed to room temperature, stirred 2 hr at room temperature, during which time considerable precipitate formed, then heated to reflux for 1 hr. The mixture was then cooled, poured into 1 M aq. $NaHCO_3$, and extracted three times with hexane. The combined hexane extracts were backwashed with brine, dried over anhydrous Na_2SO_4, and concentrated, giving a crude yield of 3 g of a ∼2:1 mixture of (2*E*,6*E*)- and (2*Z*,6*E*)-methyl farnesoates. The crude mixture was purified by flash chromatography on silica gel (5 × 25 cm, eluting with 1 liter each of 1%, 1.5%, and 2% ether in hexane). The combined fractions containing each component were concentrated and Kugelrohr distilled (oven temperature 120°C, 0.2 mm Hg vacuum), yielding 1.2 g and 0.3 g of pure (2*E*,6*E*)- and (2*Z*,6*E*)-methyl farnesoates, respectively. Methyl (2*E*,6*E*)-farnesoate: ^{1}H NMR: δ 5.68 (br. s, 1H, H2), 5.09 (br. t, 2H, $J = \sim 8$ Hz, H6,10), 3.69 (s, 3H, OCH_3), ∼2.18 (m, 4H, H4, H5), 2.18 (d, 3H, $J = 1.23$ Hz, H3′), 2.12–1.95 (m, 4H, H8, H9), 1.69 (br. s, 3H, H12), 1.61 (br. s, 6H, H7, H12′). ^{13}C NMR (CDCl3, 75.48 MHz): δ 167.27, 160.19, 136.16, 131.41, 124.20, 122.85, 115.20, 50.78, 40.94, 39.67, 26.66, 25.69, 25.94, 18.84, 17.69, 16.02. EI-MS: 250 (M^+, 1), 207 (2), 136 (4), 121 (13), 114 (24), 81 (22), 69 (100), 53 (18), 41 (85). Methyl (2*Z*, 6*E*)-farnesoate: ^{1}H NMR: δ 5.68 (br. s, 1H, H2), 5.18 (br. t, 1H, J = 6.2 Hz, H6), 5.10 (br. tt, 1H, $J = 6.7$, 1.1 Hz, H10), 3.69 (s, 3H, OMe), 2.65 (t, 2H, $J = 7.8$ Hz, H4), 2.18 (overlapped dt, 2H, $J \sim 7.8$, 7.7 Hz, H5), 2.12–1.95 (m, 4H, H8, H9), 1.90 (d, 3H, $J = 0.8$ Hz, H3′), 1.68 (br. s, 3H, H12), 1.62 (br. s, 3H, H12′), 1.59 (s, 3H, H7′). EI-MS: 250 (M^+, 7), 235 (1), 218 (1), 207 (12), 175 (5), 149 (17), 137 (10), 121 (36), 114 (28), 109 (22), 81 (31), 69 (100), 53 (19), 41 (55). The ^{1}H NMR and mass spectral data for both compounds matched literature data [NMR: Savu and Katzenellenbogen (1981); MS: Wainwright et al. (1996)].

Synthesis of Racemic Methyl (E)-5-2,6,10-Trimethyl-5,9-Undecadienoate (Scheme 1). For homogeranyl iodide (**2**), NaI (2.3 g, 15 mmol) was dried in a Kugelrohr distillation apparatus at 100°C under vacuum for 2 hr. After cooling,

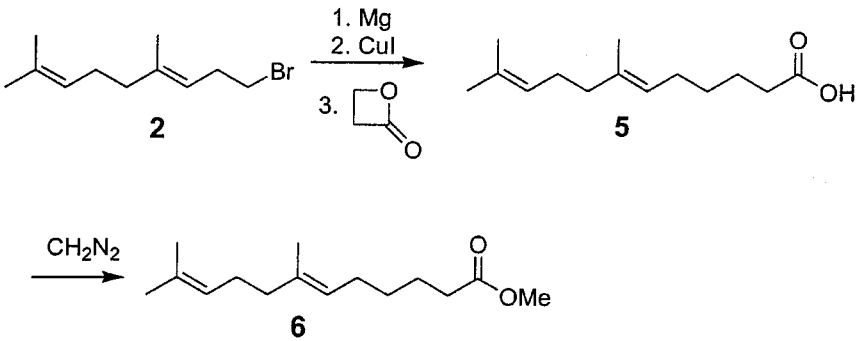


SCHEME 1.

25 ml acetone, homogeranyl bromide **1** (0.7 g, 3 mmol, prepared as described by Ho and Millar, 2001a), and 10 mg of Na_2CO_3 were added, and the mixture was stirred under Ar at room temp, monitoring the reaction by GC. When the reaction was complete (~4 hr), the mixture was poured into 150 ml water and extracted with hexane. The hexane layer was washed with aq. sodium bisulfite and brine, dried, and concentrated. The crude product was stored in the freezer until used, with a piece of copper wire added to retard degradation. EI-MS *m/z*: 278 (M^+, 0.7), 263 (1.3), 235 (9), 151 (2.7), 123 (12), 95 (150) 81 (16), 69 (100), 41 (74).

For (*E*)-5-2,6,10-*trimethyl-5,9-undecadienoic acid* (**3**), the procedure was adapted from that of Prashad et al. (1993). Lithium diisopropylamide (LDA) (1.5 M, 7.5 ml, 11.23 mmol) was added to 20 ml freshly distilled THF in an oven dried flask in an ice bath. Propionic acid (0.36 ml, 5 mmol) then was added dropwise, followed by 1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone (DMPU) (0.7 ml, 5.7 mmol) as cosolvent. The mixture was stirred at room temperature for 20 min, then cooled to 0°C, and crude homogeranyl iodide **2** (0.7 g, 2.5 mmol) in 2 ml THF was added over 10 min. The mixture was stirred at room temperature, monitoring by GC. When there was no further change in the product ratio, the mixture was poured into 100 ml 1 M HCl and extracted with hexane (1 × 100 ml, 2 × 50 ml). The combined hexane layers were extracted with 1 M NaOH (1 × 100 ml, 2 × 50 ml). The combined aq. NaOH layers were back-extracted with ether, then acidified with 100 ml 3 M HCl and extracted with ether (1 × 100 ml, 2 × 50 ml). The combined ether layers were backwashed with brine, dried, and concentrated, yielding 0.4 g of acid **3** (purity 95% by GC, 71% yield). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.1 (m, 2H, H5 and H9), 2.5 (sextet, 1 H, $J = 7$ Hz, H-2), 2.06 (m, 7H, H2, H4, H7, and H8), 1.8 (m, 1H, H3), 1.69 (s, 3H, H11), 1.61 (2 × s, 2 × 3H, H6′ and H11′), 1.45 (m, 1H, H3′), 1.19 (d, 3H, J = 7 Hz, H2′). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 182.99, 136.03, 131.41, 124.27, 123.33, 39.71, 38.75, 33.50, 26.62, 25.71, 25.48, 17.70, 16.86, 15.99. EI-MS *m/z*: 224 (M^+, 1), 209 (1.6), 181 (34), 163 (7.5), 137 (5.4), 109 (43), 81 (19), 69 (100), 41 (96). The spectra matched those reported by Coates and Freidinger (1970).

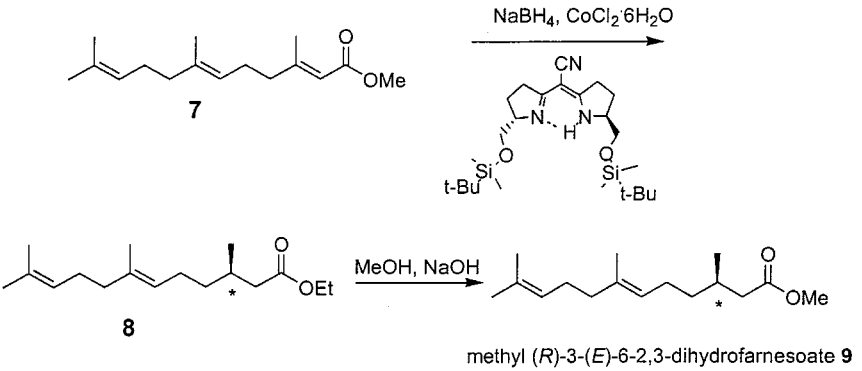
For *methyl* (*E*)-*5-2,6,10-trimethyl-5,9-undecadienoate* (**4**), acid **3** was dissolved in ~5 ml ether, cooled in an ice bath, and a freshly prepared solution of diazomethane in ether (prepared according to Aldrich Tech. Information Bulletin AL-180, Aldrich Chem. Co., Milwaukee, Wisconsin) was added until the free acid was consumed (monitoring by persistence of the yellow color of excess diazomethane). Nitrogen was bubbled vigorously through the solution to discharge traces of excess diazomethane; then the mixture was concentrated and Kugelrohr distilled at 0.1 mm Hg, oven temperature 70°C, yielding 0.34 g of ester **4** (58% yield, 92% purity by GC). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.09 (t, 2H, $J = 7$ Hz, H5 and H9), 3.67 (s, 3H, OCH_3), 2.46 (sextet, 1H, $J = 6.9$ Hz, H2), 1.88–2.15 (m, 7H, H2, H4, H7 and H8), 1.6–1.8 (m, 1H, H3), 1.68 (s, 3H, H11), 1.60 and 1.58



SCHEME 2.

(2 × s, 2 × 3H, H11′ and H6′), 1.35–1.5 (m, 1H, H3′), 1.15 (d, 3H, $J = 6.9$ Hz, H2′). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 177.33, 135.82, 131.37, 124.28, 123.50, 51.48. 39.73, 38.89, 33.79, 26.65, 25.71, 25.57, 17.69, 17.09, 15.95. EI-MS *m/z*: 238 (M^+, 0.7), 223 (3.3), 195 (19), 163 (10), 123 (7.4), 109 (75), 88 (22), 69 (84), 55 (20), 41 (100).

Synthesis of Racemic Methyl (*E*)*-6-7,11-Dimethyl-6,10-Dodecadienoate (Scheme 2).* For (*E*)-6-7,11-dimethyl-6,10-dodecadienoic acid (**5**), the procedure was adapted from those described by Fujisawa et al. (1980) and Sato et al. (1980). Homogeranyl bromide **2** (0.9 g, 3.9 mmol, purity 82%) was dissolved in ~10 ml of freshly distilled THF in a dropping funnel. One milliliter of the solution was added to a flask containing Mg turnings (0.2 g, 8 mmol), with several small crystals of iodine and 5 drops of dibromoethane. The mixture was stirred until the iodine color disappeared, and then the rest of the solution was added dropwise over ~2 hr, warming gently to 30–40°C with an oil bath. When the addition was complete, the solution was heated at 40°C for 1 hr, then cooled to room temperature The resulting solution of Grignard reagent was transferred by syringe into a solution of β-propiolactone (0.25 g/0.22 ml, 4 mmol; Aldrich Chemical) and powdered CuI (0.04 g, 0.2 mmol) in 5 ml dry THF at ~−10°C (ice-salt bath) in a 100-ml flask under Ar over 30 min. The mixture was warmed to 0°C and stirred until the reaction was complete. The reaction mixture was poured into 100 ml 1 M HCl and extracted with hexane (1 × 100 ml, 2 × 50 ml). The combined organic layers were extracted with 1 M NaOH (1 × 100 ml, 2 × 50 ml), and the combined aq. NaOH extracts were then back-extracted once with ether. The aq. NaOH extract was then acidified with 100 ml 3 M HCl, and extracted with ether (1 × 100 ml, 2 × 50 ml), the combined ether extracts were washed with brine, dried, and concentrated, yielding 0.4 g of acid **5**. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.75 (br s, 1 H, COOH), 5.11 (m, 2H, H6 and H10), 2.36 (t, 2H, $J = 7.4$ Hz, H2), 1.9–2.1 (m, 6H, H5, H8, H9), 1.68 (s, 3H, H12), 1.60 (s, 6H, H7′ and H12′), 1.5–1.7 (m, 2H, H3), 1.3–1.5



SCHEME 3.

(m, 2H, H4). ^{13}C NMR ($CDCl_3$, 75.48 MHz): δ 180.52, 135.49, 131.38, 124.36, 123.99, 39.78, 34.11, 29.25, 27.52, 26.73, 25.74, 24.32, 17.73, 16.05. EI-MS *m/z*: 224 (M^+, 0.5), 209 (1.8), 181 (28), 137 (5), 123 (15), 109 (12), 95 (36), 69 (100), 41 (77).

For *methyl* (*E*)*-6-7,11-dimethyl-6,10-dodecadienoate* (**6**), a portion of (*E*)-6-7,11-dimethyl-6,10-dodecadienoic acid **5** was esterified with diazomethane as described for 4. EI-MS *m/z*: 238 (M^+, 0.5), 223 (0.6), 195 (18), 163 (6), 137 (9), 123 (11), 109 (21), 95 (49), 69 (82), 41 (100).

Synthesis of Methyl (R)-3-(E)-6-2,3-Dihydrofarnesoate (Scheme 3). The reaction was carried out exactly as described by Pfaltz (1989) and Leutenegger et al. (1989). A successful reaction required rigorous degassing of solvents and starting materials, and exclusion of oxygen. Thus, methyl (2*E*,6*E*)-farnesoate **7** (0.25 g, 1 mmol) in a 25-ml three-necked round-bottom flask equipped with a magnetic stir bar, with the necks fitted with a stop-cock and two new rubber septa, was degassed by several cycles of pumping under vacuum and refilling the flask with N_2. All solvents and solutions were degassed by sonicating while pumping under house vacuum, then refilling with N_2; this was repeated several times. Then 0.5 ml ethanol were added to the flask by syringe, followed by 0.25 ml of a solution of $CoCl_2 \bullet 6H_2O$ (100 mg, 0.42 mmol) in ethanol (5.5 ml), a solution of (1*S*,9*S*)-1,9-bis[(*t*-butyldimethylsiloxy)-methyl]-5-cyanosemicorrin (10 mg, 0.022 mmol; Fluka #14556) in 0.5 ml ethanol (mixture turns deep blue), and a solution of $NaBH_4$ [85 mg (2.2 mmol) in 0.8 ml dimethylformamide] (mixture turns brown, cloudy). The mixture was degassed again by several freeze–pump–thaw cycles, using a Dry Ice–acetone bath. The flask was then sealed tightly and the mixture was stirred for 45 hr at room temperature. A sample taken indicated that the reaction was complete, with no trace of starting material, but that the product had been 60% transesterified to the ethyl ester. The mixture was worked up by

pouring into 100 ml water and extracting with 3 × 50 ml CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with water and brine, dried, and concentrated. The crude ethyl ester **8** was then transesterified by stirring with methanol (10 ml) and one pellet of NaOH (∼250 mg) overnight at room temperature. The mixture was poured into 50 ml water and extracted with hexane, washing the hexane solution with brine. The solution was dried, concentrated, and Kugelrohr distilled (oven temperature 110°C, 0.3 mm Hg), yielding 229 mg of methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate **9**, >98.5% pure by GC. The enantiomeric excess was determined to be 89.6% by hydrolysis of the acid followed by formation of the diastereomeric amides by reaction with (*S*)-(–)-α-methylbenzylamine and GC analysis (see below).

Determination of Absolute Configuration of Insect-Produced Methyl (E)-6-2,3-Dihydrofarnesoate. This procedure was adapted from a method reported by Huffer and Schreier (1990), scaling down the apparatus and quantities of reagents used. Thus, 10 μg of synthetic racemic or methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate was hydrolyzed in 20 μl of EtOH and 2 μl of aq. 4 M NaOH in a Keele vial (Wheaton Scientific Products, Millville, New Jersey), heating in a 50°C water bath for 2 hr. After cooling, the EtOH was evaporated under a gentle stream of N_2. Then 20 μl of 1 M HCl were added to the dry vial, and the mixture was extracted with pentane (3 × 100 μl) to recover the free (*E*)-6-2,3-dihydrofarnesoic acid. The pentane solution was dried by filtering through a Pasteur pipet containing a 0.5-cm layer of anhydrous $MgSO_4$, then transferred to a 250-μl conical glass vial insert inside a 4-ml glass vial. The solution was evaporated just to dryness under a gentle stream of N_2 and 20 μl of $CHCl_3$ were added, followed by 2 μl of a $CHCl_3$ solution of (*S*)-(–)-α-methylbenzylamine (10 μg/μl; Aldrich Chemical) and 4 μl of a solution of dicyclohexylcarbodiimide (DCC) (6 μg/μl in $CHCl_3$). The vial was sealed tightly with a Teflon-lined screw-cap, and the vial was heated in a 50°C water bath for 30 min. The mixture was then analyzed by GC and GC-MS on DB-5 and DB5-MS columns (20 m × 0.32 or 0.2 mm, respectively, J&W Scientific, Folsom, California) in splitless mode. GC and GC-MS conditions were: 150°C/0 min, 4°C/min to 275°C, hold at 275°C for 10 min, injector temp 300°C, detector or transfer line temp 280°C.

A pooled aeration extract from mature male *C. uhleri* containing approximately 1.2 mg of the major defensive compound, tridecane, and 22 μg of the major male-specific compound, methyl (*E*)-6-2,3-dihydrofarnesoate was fractionated on a silica gel solid-phase extraction cartridge as previously described (Ho and Millar, 2001a). The fraction containing methyl (*E*)-6-2,3-dihydrofarnesoate was hydrolyzed, and the resulting (*E*)-6-2,3-dihydrofarnesoic acid was converted to the diastereomeric methylbenzylamide derivative and analyzed as described above. This procedure was repeated with pooled aeration extracts from *C. ligata* males containing approximately 64 μg of tridecane and 11 μg of the major male-specific compound, methyl (*E*)-6-2,3-dihydrofarnesoate.

RESULTS

Basic Reproductive Behaviors. The reproductive behaviors of *C. uhleri* and *C. ligata* were similar. For both species, the male bug began close-range courtship with antennation of the female bug, followed by head butting the posterior end of the female bug. If the female was receptive, she stopped walking, lowered her head, and raised her abdomen. Then, the male turned around, coupled his genitalia with those of the female, and copulated in an end-to-end position. During mating, both female and male bugs were in a 'head-stand' position, shaped as an inverted 'V', with the abdomen higher than the head. When copulation was complete, one of the bugs swung from side to side to disengage from the other. If the female was not receptive, she walked away. In some cases, even though the female bug stopped and raised her abdomen in the receptive position, the male would still head butt for some time before he turned around and mated with her. In cases where the female assumed the receptive position with her abdomen raised, and the male failed to couple with her genitalia, the male resumed head butting and started over again. In some cases, the male had to pursue the female with periodic bouts of head butting for hours, before the female acquiesced and copulated with the male. In other cases, the female mated with the male after a relatively brief courtship of only a few minutes of head butting.

Weight Change after Mating. Experiments with 122 virgin pairs and 52 experienced pairs of *C. ligata* resulted in a total of only 13 observations of copulation between virgin pairs and 11 copulations of experienced pairs. For virgin insects, females gained $28 \pm 1\,0$ mg in weight (mean $\pm$ SD, $11 \pm 4\%$ of body weight), and males lost 42 ± 11 mg ($19 \pm 5\%$ of body weight) during copulation (Figure 1a). There was negligible weight change in the control group, with females losing 3 ± 3 mg, and males losing 1 ± 2 mg ($N = 16$). There was a trend towards increasing weight change in both sexes with increasing duration of mating [Figure 1a, $R^2 = 0.1527$ (females) and 0.4926 (males)].

For experienced pairs, weight change was not correlated with duration of mating for either sex (Figure 1b, experienced pairs, females, $P = 0.72$ and males, $P = 0.42$). Experienced males lost an average of 42 ± 7 mg ($19 \pm 3\%$ of body weight) during mating, and females gained 24 ± 10 mg ($11 \pm 5\%$ of body weight). Overall, these results indicate that virgin and experienced males transfer material equivalent to a significant fraction of their body mass to females during mating. For both virgin and experienced pairs, there was a small overall net weight loss, probably due to defecation; drops of feces were frequently seen on the walls of the cages at the termination of the experiments.

We were not able to carry out analogous experiments with *C. uhleri* because of insufficient numbers of bugs in the laboratory colonies.

Diurnal Patterns in Mating Behavior. The diurnal rhythm of mating behavior of adult *C. ligata* is shown in Figure 2a. Of 23 sexually mature, lab-reared pairs

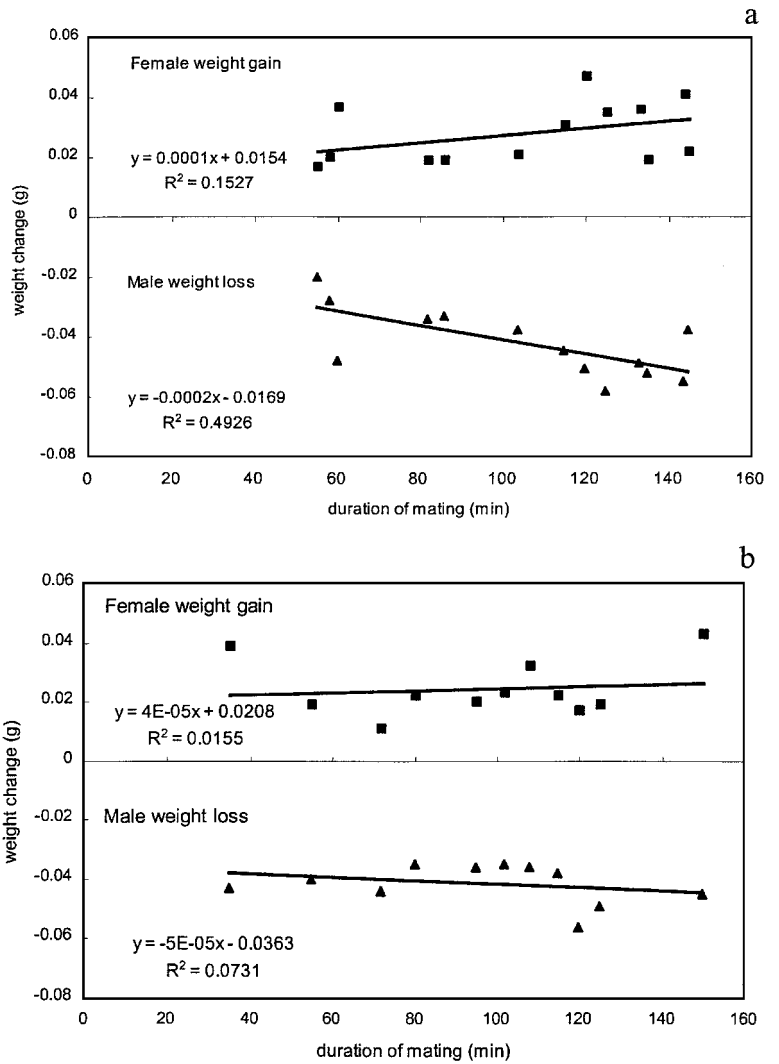


FIG. 1. Weight change of *C. ligata* after mating. (a) Virgin insects: females gained 0.028 ± 0.010 g (mean $\pm$ SD) (*t* test versus controls, $P = 6 \times 10^{-8}$, and males lost 0.042 ± 0.011 g in weight during mating (*t* test versus controls, $P = 1.8 \times 10^{-8}$) ($N = 13$). Linear regression was carried out on the weight change versus duration of mating for each sex. Female weight gain was not correlated with duration of mating ($P = 0.1868$), whereas male weight loss was correlated with mating duration ($P = 0.0075$). (b) Experienced insects: females gained 0.024 ± 0.010 g (mean $\pm$ SD) in weight (*t* test versus controls, $P = 1.3 \times 10^{-6}$), and males lost 0.042 ± 0.007 g with every mating (*t* test versus controls, $P = 1 \times 10^{-9}$) ($N = 11$). Linear regression was carried out on the weight change versus duration of mating for each sex. Neither female weight gain ($P = 0.7154$) nor male weight loss ($P = 0.4212$) was correlated with mating duration.

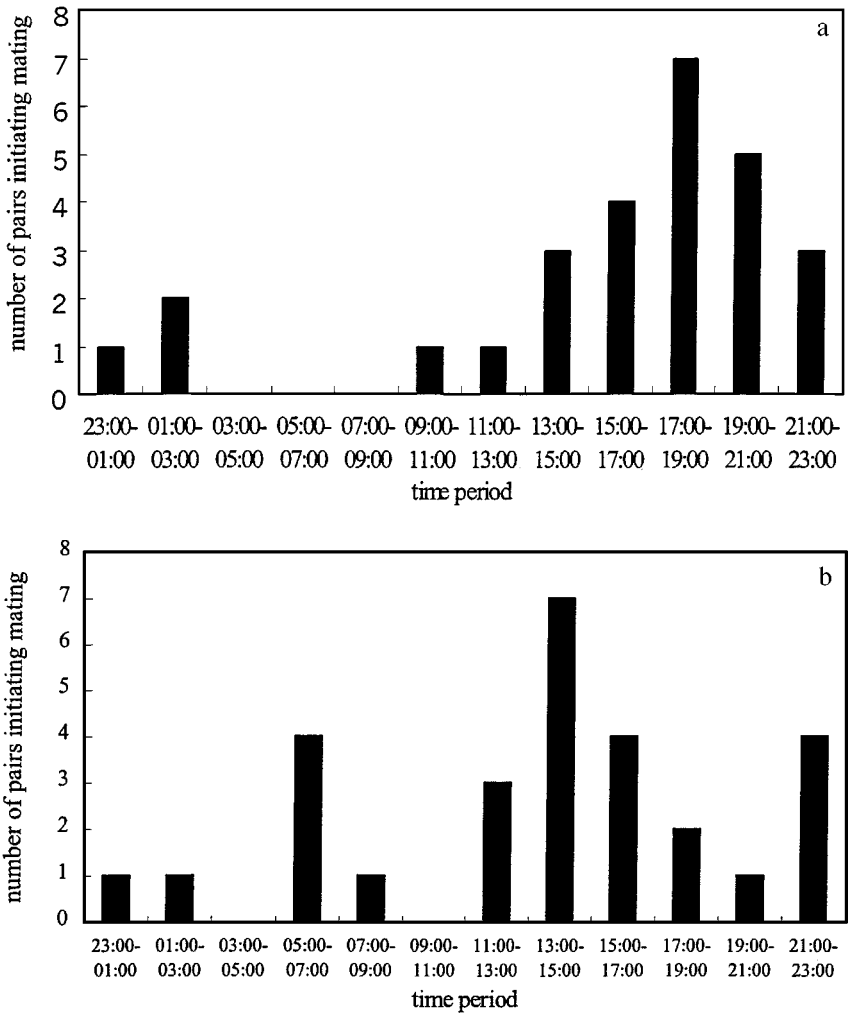


FIG. 2. Diurnal pattern of mating behavior of *C. ligata* (a) and *C. uhleri* (b), showing number of pairs that initiated copulation during successive 2-hr time periods. Lights were on from 06:00 to 20:00 hr each day. The *C. ligata* trial was run with 13 pairs of insects for 14 consecutive days, with 27 matings observed. The *C. uhleri* trial was run with 12 individual pairs of insects for 8 consecutive days, with 28 matings observed.

observed over a 14 day period, 13 pairs mated a total of 27 times. No mating was observed for the other 10 pairs because of the death of one or both bugs of the pair. For pairs that mated more than once, the mean latency between copulations was 50 ± 20 hr (range 22–92 hr). The duration of mating for virgin females

paired with virgin males (54 ± 24 min; $N = 12$), and for experienced females and males (46 ± 33 min; $N = 15$) was not different (two tailed t test, $df = 25$, $P = 0.44$). The largest percentage of copulations (>25%) were initiated between 17:00–19:00 hr (Figure 2a). The remainder of copulations generally were initiated in the afternoon and evening hours, from 13:00 to 23:00 hr, indicating that this species is sexually active primarily during the latter half of the photophase.

Twelve pairs of field-collected *C. uhleri* adults of unknown age and mating status, held in sexed groups for 4 days before beginning observations, mated a total of 28 times during the 8 day observation period. The overall pattern of reproductive activity is shown in Figure 2b. Mating duration ranged from 8 to 193 min, (mean ± SD, 78 ± 55 min). Eight of the 12 pairs mated more than once, one pair did not mate at all, and three pairs mated only once due to the death of one of the pair. Of the eight multiply-mating pairs, two pairs mated twice the same day, and then every one to two days. Another three pairs mated every other day. The interval between mating ranged from 10 to 51 hr (mean 26 ± 14 hr). Females usually laid eggs between copulations. Mating was initiated mostly in the afternoon, with 25% of the 28 matings observed being initiated between 13:00–15:00.

Identification of Compounds from Odors Produced by Adult C. uhleri and C. ligata. Typical gas chromatograms of aeration extracts from groups of 5–10 sexually mature, virgin adult female and male *C. uhleri* are shown in Figure 3. Several compounds appeared in both female and male aeration extracts, including (*E*)-2-hexenal, (*E*)-2-octenal, dodecane, (*E*)-2-octenyl acetate, tridecane, tetradecane, and pentadecane. Female extracts also contained the known bug defensive compound (*E*)-4-oxo-2-hexenal (Aldrich, 1995) and a small amount of undecane. All compounds except (*E*)-4-oxo-2-hexenal were tentatively identified by comparison of their mass spectra with those from the National Bureau of Standards GC-MS database. (*E*)-4-Oxo-2-hexenal was tentatively identified by comparison of the mass spectrum with a reference spectrum obtained from J. R. Aldrich. Identifications then were confirmed by comparison of retention times and mass spectra of the insect-produced compounds with those of authentic standards. The C_{11}-C_{15} hydrocarbons, (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-octenyl acetate, and (*E*)-4-oxo-2-hexenal are probably defensive compounds from the metathoracic glands (Ho and Millar, 2001b). Aeration extracts from green-bean controls were found to contain trace amounts of the common plant volatiles hexanol, (*E*)-2-hexenol, (*E*)-2-hexenal, and linalool.

Three male-specific compounds (Figure 3, peaks A, B, and C) in a ratio of 0.9:100:0.6, were first detected in aeration extracts 12 days after the final molt in each of two cohorts of virgin males. The mass spectrum of the most abundant of the three compounds, peak B, suggested a molecular mass of 252 with major fragment ions of *m/z* 69, 109, 123, 177, and 209. The compound was tentatively identified as methyl (*E*)-6-2,3-dihydrofarnesoate by comparison of its mass spectrum with the

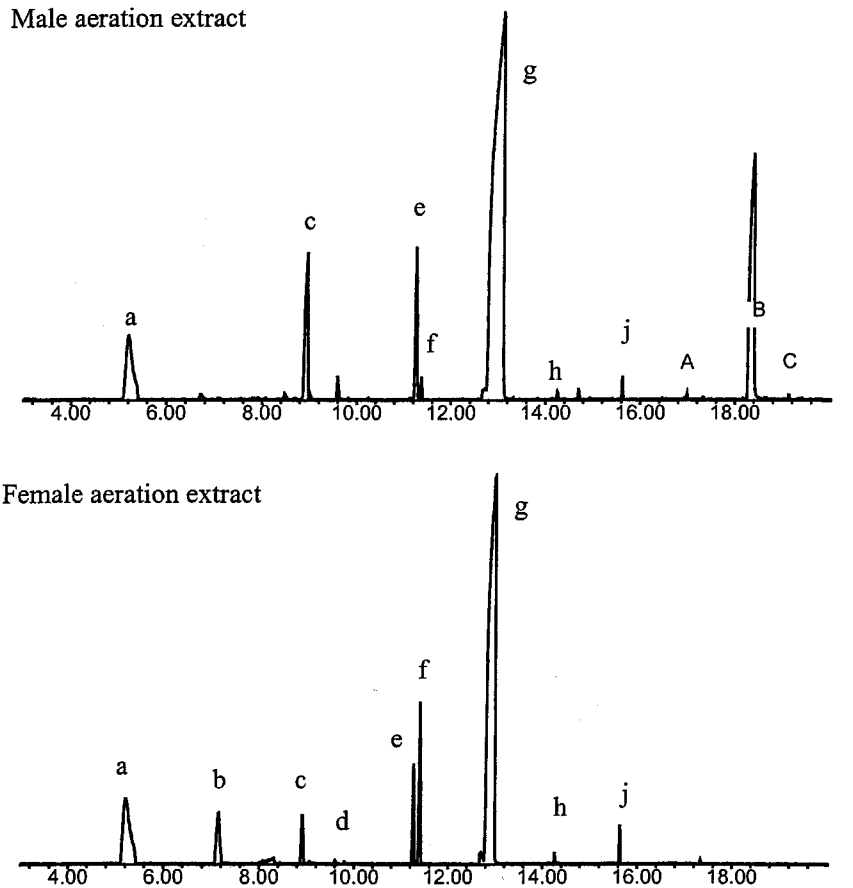


FIG. 3. Gas chromatograms of aeration extracts of sexually mature male (top trace) and female (bottom trace) *C. uhleri*. GC conditions: column DB5-MS (20 m × 0.2 mm ID), initial temperature 50°C (1 min), 10°C/min to 250°C, 250°C (10 min). Three compounds were found only in male aeration extract: A: methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate; B: methyl (*E*)-6-2,3-dihydrofarnesoate; C: methyl (2*E*,6*E*)-farnesoate. Other peaks: a: (*E*)-2-hexenal; b: (*E*)-4-oxo-2-hexenal; c: (*E*)-2-octenal; d: undecane; e: dodecane; f: (*E*)-2-octenyl acetate; g: tridecane; h: tetradecane; j: pentadecane.

mass spectral database. The identification was confirmed by synthesis of methyl (*E*)-6-2,3-dihydrofarnesoate, and match-up of mass spectra and GC retention times on two columns of different polarity (DB-5MS and DB-17).

Peak C was tentatively identified as methyl (2*E*,6*E*)-farnesoate based on its molecular ion at *m/z* 250 (corresponding to a possible molecular formula of

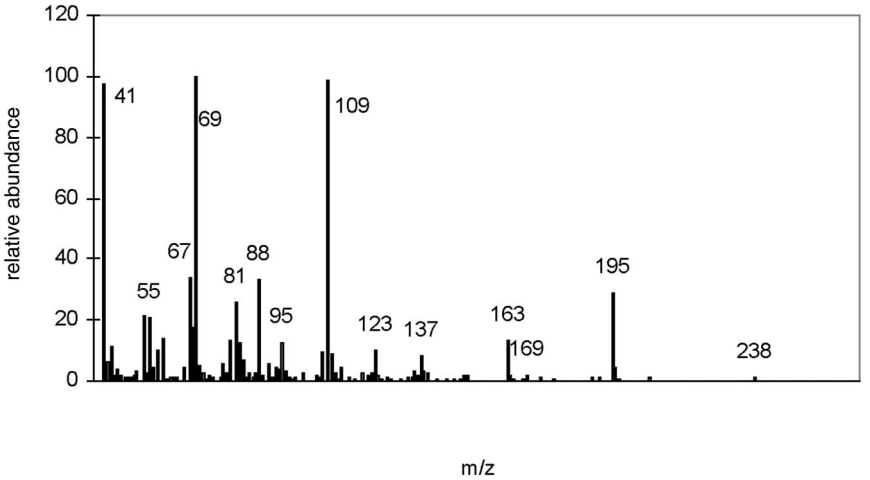


FIG. 4. Mass spectrum of (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate.

$C_{16}H_{26}O_2$); significant fragment ions of *m/z* 41, 69, 114, 136, and 235; and an excellent match with a spectrum of the authentic compound in the mass spectral database. The identification was confirmed by matching the mass spectrum, and retention times on DB-5MS and DB-17 GC columns, with those of a synthesized standard of methyl (2*E*,6*E*)-farnesoate.

The mass spectrum of the small peak A showed a possible molecular weight of 238 amu (Figure 4). The molecular weights of peak A and the major component, methyl (*E*)-6-2,3-dihydrofarnesoate, were 14 mass units different, and both gave similar mass spectral fragmentation patterns in the lower mass ranges. This suggested that the unknown might be a homolog of methyl (*E*)-6-2,3-dihydrofarnesoate, with one methylene group less. There were two obvious possibilities. First, the compound might be derived from chain shortening of methyl (*E*)-6-2,3-dihydrofarnesoate by one carbon, that is, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate. Alternatively, the structure might be methyl (*E*)-6-7,11-dimethyl-6,10-dodecadienoate, from removal of the methyl group on carbon 3 of methyl (*E*)-6-2,3-dihydrofarnesoate. Other possibilities, such as removal of a methyl group further along the chain, seemed unlikely because of the similarities between the mass spectra of this compound and methyl (*E*)-6-2,3-dihydrofarnesoate, whereas removal of an allylic methyl from carbons 7 or 11 of methyl (*E*)-6-2,3-dihydrofarnesoate would have shifted the masses of most of the major fragments by 14 mass units. To confirm the structure of the unknown, the two most likely compounds were synthesized. By comparison of the retention times and mass spectrum of the unknown with those of the synthetic compounds, compound A was identified

as methyl (*E*)-5-2,6,10-trimethyl- 5,9-undecadienoate. A diagnostic mass spectral fragment with *m/z* 88 (Figure 4), arising from McLafferty rearrangement of the methyl ester (Silverstein and Webster, 1998), aided the conclusive identification. This fragment was not seen in the spectrum of methyl (*E*)-6-7,11-dimethyl-6,10-dodecadienoate, and the retention time of this compound was different from that of the insect-produced compound.

The absolute configuration of the insect-produced methyl (*E*)-6-2,3-dihydrofarnesoate was determined by hydrolysis of the ester to the free acid, followed by derivatization with (*S*)-(–)-α-methylbenzylamine. GC analysis of the resulting amide diastereomer determined that its retention time matched that of the corresponding derivative prepared from synthetic methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate. The pair of derivatives from derivatization of racemic (*E*)-6-2,3-dihydrofarnesoic acid were resolved to baseline on a DB-5MS column [(*R*)-3- enantiomer, 22.76 min; (*S*)-3 enantiomer, 22.94 min], unequivocally indicating that the bugs produce methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate.

There was also a chiral center in the minor component, (*E*)-5-methyl-2,6,10-trimethyl-5,9-undecadienoate. This compound was present in trace amounts in the fraction containing methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, but after the two-step hydrolysis and derivatization procedure, a peak corresponding to this compound could not be located in the chromatograms.

Typical gas chromatograms of aeration extracts from female and male *C. ligata* are shown in Figure 5. The compounds shared by both females and males included (*E*)-2-octenal, dodecane, tridecane, and pentadecane. Extracts from females also contained (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, undecane, and tetradecane. Sexually mature males also produced the same three male-specific compounds as *C. uhleri*, in a similar ratio [methyl (*E*)-6-2,3-dihydrofarnesoate, methyl (2*E*,6*E*)-farnesoate, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, 100:0.4:0.5]. Furthermore, GC analysis of the diastereomeric derivative formed from the *C. ligata*-produced methyl (*E*)-6-2,3-dihydrofarnesoate determined that it too had the (*R*)-3 configuration. As with *C. uhleri*, the amount of the minor component methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate was too small to be able to determine its absolute configuration by the derivatization method. Thus, the male-specific blends of compounds from *C. uhleri* and *C. ligata* appear to be remarkably similar, even to the extent of the major, chiral component having the same absolute configuration.

Dynamics of Production of Male-Specific Compounds. Less than half the cohorts of virgin male *C. uhleri* produced male-specific compounds. For example, between November 1997 and November 1998, of 28 cohorts of virgin males $>$10 days old that were aerated continuously for at least 8 days, only 13 cohorts produced detectable amounts of male-specific compounds. In a second set of aerations conducted between February 6 and March 24, 1999, only three of 17 cohorts

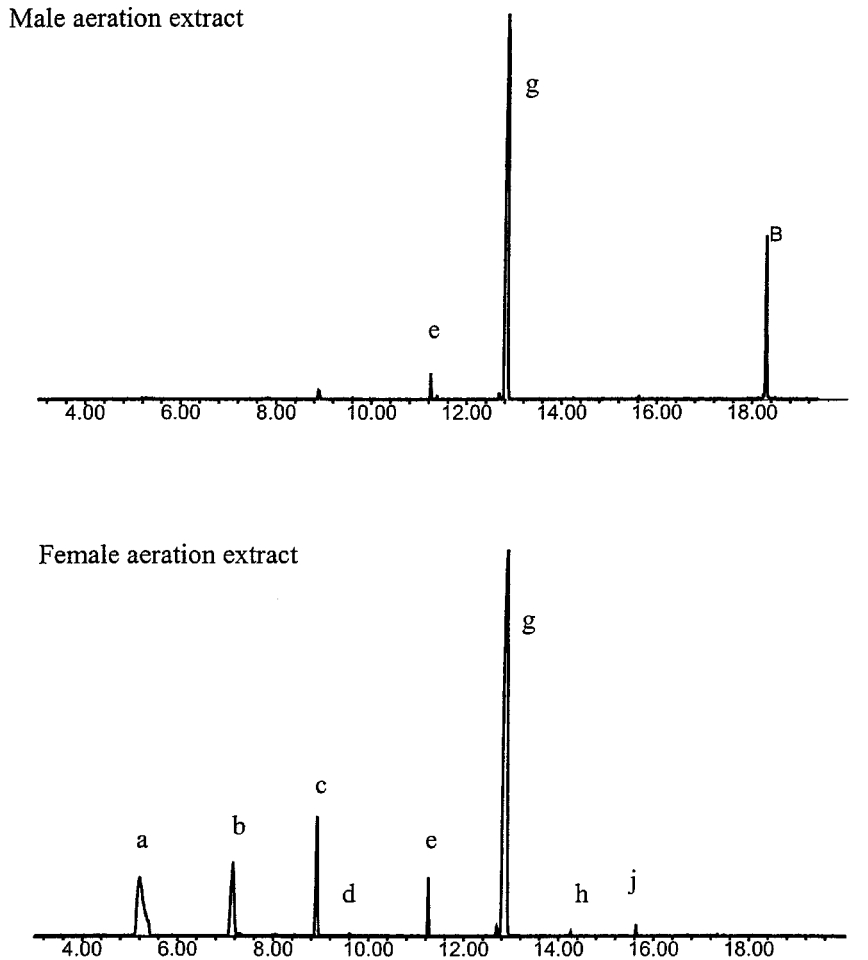


FIG. 5. Gas chromatograms of male (top trace) and female (bottom trace) *C. ligata* aeration extracts. GC conditions: column DB5-MS (20 m × 0.2 mm ID), oven temperature: 40°C (1 min), 10°C/min to 250°C, 250°C (10 min). Peak B: methyl (*E*)-6-2,3-dihydrofarnesoate. Other peaks: a: (*E*)-2-hexenal; b: (*E*)-4-oxo-2-hexenal; c: (*E*)-2-octenal; d: undecane; e: dodecane; f: (*E*)-2-octenyl acetate; g: tridecane; h: tetradecane; j: pentadecane.

of virgin males >12 days old aerated for periods of one week produced male-specific compounds. This second set of aerations was conducted with individuals reared from eggs laid by bugs collected in late fall of 1998.

For *C. ligata*, male-specific compounds were first detected from three cohorts of virgin males 13, 14, and 14 days, respectively, after the final molt. Unlike

C. uhleri, all cohorts (>20 total) of males that were aerated produced male-specific compounds.

Diurnal Rhythm of Production of Male-Specific Compounds. Aeration data from seven cohorts of male *C. ligata* were pooled and analyzed by 2-way ANOVA to determine the diurnal rhythm of production (Figure 6a). Maximum production of male-specific compounds occurred from 19:00 to 21:00 hr, and the quantities produced were significantly greater than for the next most productive periods between 17:00 and 19:00 and 21:00 and 23:00 hr. During peak production, males produced about 35 ng/bug/hr of the major male-specific compound.

For cohorts of male *C. uhleri* that did produce male-specific compounds, production followed a diurnal cycle, increasing from midday and peaking in the late afternoon at 17:00–19:00 (Figure 6b). The mean amount of the most abundant compound, methyl (*E*)-6-2,3-dihydrofarnesoate, produced during the peak production period was 8.8 ± 4.7 ng/bug/hr. Lower levels of this compound were detected during the entire 24 hr cycle.

Laboratory Bioassays. In vertical Y-tube bioassays, female *C. uhleri* were more attracted to odors from live males than to clean air controls (male odor stimulus, 24 responders, control, 10 responders, 3 nonresponders; χ^2 test, $P = 0.016$). In subsequent bioassays testing different doses of aeration extracts of males (doses of 8–32 ng of the major male specific compound), female bugs showed no preference for the odor extract from males versus the solvent control (extract, 14 responders, control, 18 responders). Because the effects of the pheromone might have been confounded by the presence of defensive chemicals in the crude extracts, no further bioassays were conducted with extracts.

When tested as a single component, 2 μg of the synthesized major male-specific compound, methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate, attracted 26 females, versus 12 females attracted to the solvent control (χ^2 test, $P = 0.023$), with 10 nonresponders. In further bioassays testing the effects of the minor male-specific components, and using racemic (*E*)-6-2,3-dihydrofarnesoate instead of the (*R*)-3 enantiomer, results were unclear. With 4-μg doses of the three-component blend, there were no significant differences in responses of females to the treatment or the control, ($N = 16$; treatment = 6, control = 8, 2 nonresponders). However, a second set of bioassays using 40 μg of the three-component blend resulted in attraction of female bugs to the treatment (treatment = 25, control = 10, 5 nonresponders; $P = 0.011$). In a direct test of the attractiveness of the three-component blend versus the single major component methyl (*E*)-6-2,3-dihydrofarnesoate (40 μg doses), female bugs responded equally to each stimulus (three-component blend = 10, single component = 14, 4 nonresponders; χ^2, $P = 0.414$). This result suggested that the two minor components of the male-specific volatiles are not required for attraction and that they are not pheromone components.

In a final set of laboratory bioassays, mature male *C. uhleri* were repelled by 40 μg of the three-component pheromone blend. Of the 20 bugs tested, 3 went to

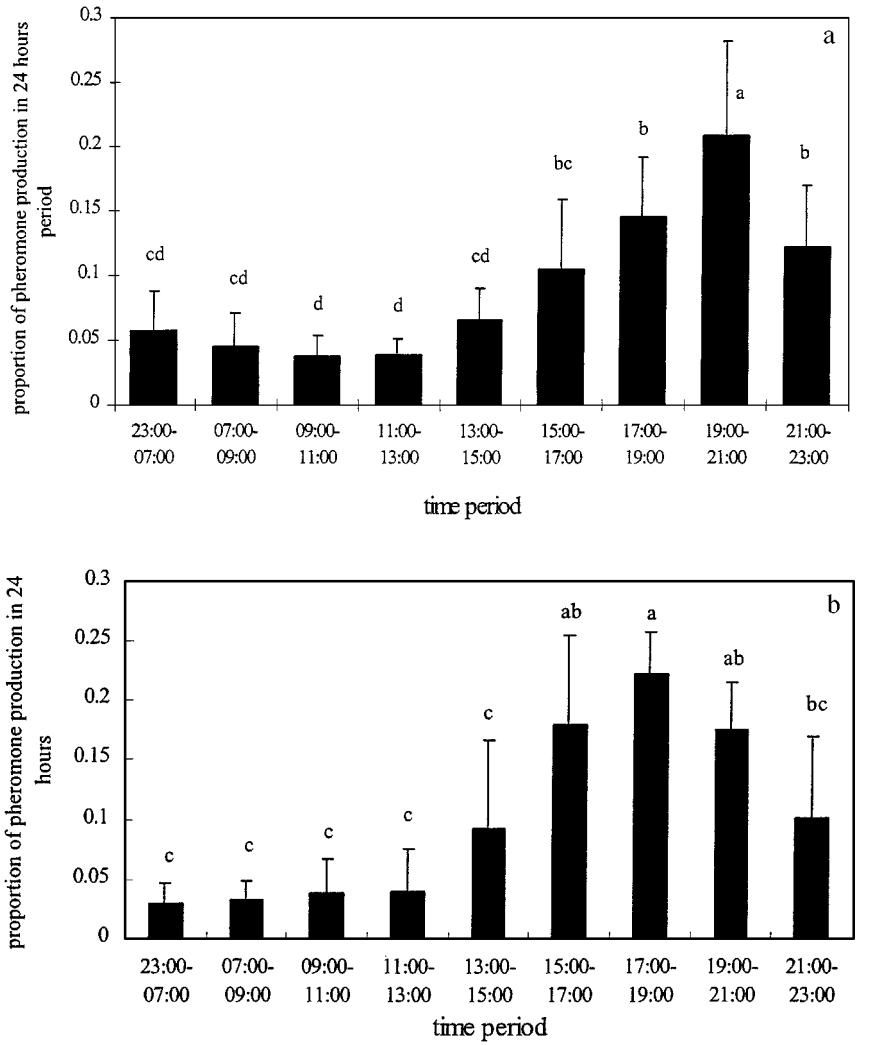


FIG. 6. Diurnal rhythm of production of the major male-specific compound, methyl (*E*)-6-2,3-dihydrofarnesoate, by 7 cohorts of *C. ligata* (a) and 4 cohorts of *C. uhleri* (b) males. *C. ligata*: 2-way ANOVA, for cohort effect, $F = 0.495$, $df = 6, 48$, $P = 0.8086$; for time interval effect $F = 13.326$, $df = 8, 48$, $P < 0.0001$. The maximum amount produced, from 19:00–21:00 hr, was 35.3 ± 32.2 ng/bug/hr. *C. uhleri*: 2-way ANOVA for cohort effect, $F = 0.519$, $df = 3, 24$, $P = 0.6732$; for time interval effect $F = 3.750$, $df = 8, 24$, $P = 0.0056$. The maximum amount, produced from 17:00 to 19:00 hr, was 8.8 ± 4.7 ng/bug/hr. Bars surmounted by the same letter(s) are not significantly different (Student-Newman-Keuls test, $P < 0.05$).

the arm containing the test treatment, 14 went to the control arm, and 3 bugs did not respond (χ^2, $P = 0.008$).

In the vertical Y-tube olfactometer, virgin female *C. ligata* were attracted to odors from live males (male odors versus control, 20 vs. 8 responders, χ^2 test, $P = 0.02$). The results were further categorized into response times of less than or greater than 1 min. This more detailed analysis indicated that when bugs responded more slowly, circling around and taking time to sample the airstreams (i.e., response time >1 min), they chose the arm with the odor of the male bug a large percentage of the time (treatment = 13, control = 2, χ^2, $P = 0.0045$). Furthermore, some of these bugs started up the control arm, then turned back and finally moved all the way to the end of the treatment arm of the olfactometer. In contrast, bugs that made a choice in less than 1 min showed no significant discrimination between the treatment and control arm (seven and six bugs, respectively).

Attempts to demonstrate attraction of sexually mature virgin *C. ligata* females to extracts of male volatiles were not successful, using several different aeration extracts containing varying amounts of the male-specific compounds and the defensive compounds. Possible reasons for this lack of attraction include incorrect dose or interference from the defensive compounds present in the extracts. Consequently, all further laboratory bioassays were conducted with synthetic compounds, in which the blend and dose could be completely controlled.

In the first bioassay of synthetic compounds with female *C. ligata* responders, there were indications that methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate as a single component (2 μg dose) might be attractive to female bugs (treatment = 17, control = 9, nonresponders = 8; χ^2 test, $P = 0.11$). Further bioassays conducted with the two-component blend of methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate with methyl (2*E*,6*E*)-farnesoate (100:1, total dose 2.02 μg) indicated that the two-component blend was more attractive than the control (treatment = 19, control = 6, 6 nonresponders, χ^2 test, $P = 0.009$). In a third series of bioassays, female bugs were more attracted to a three-component blend consisting of racemic methyl (*E*)-6-2,3-dihydrofarnesoate, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate and methyl (2*E*,6*E*)-farnesoate (100:0.8:0.4, total dose 4 μg) than to the control (treatment = 15, control = 1, 4 nonresponders, χ^2 test, $P = 0.0004$). Bioassays directly comparing the attraction of female bugs to the three-component blend versus methyl (*E*)-6-2,3-dihydrofarnesoate as a single component (2 μg doses) were inconclusive due to the limited number of bugs available for bioassays. Of the 11 bugs available, 6 went to the arm containing the single component and 3 went to the arm containing the three-component blend, and 2 did not respond.

In an abbreviated series of bioassays, there was no indication that male *C. ligata* were attracted to or repelled by the three-component blend (treatment = 5, control = 5, nonresponders = 7), but the numbers tested were too low to reach any firm conclusion.

Field Bioassays with C. uhleri *and* C. ligata. In the first field trials conducted August 3–16, 1999, more female *C. uhleri* were attracted to lures containing a three-component blend of the synthesized male-specific components than to solvent treated controls (67 to treatment, 7 to control; two-way ANOVA for treatment effect $F = 7.42$, $df = 2,10$, $P = 0.011$; for block effect $F = 3.48$, $df = 5,10$, $P = 0.043$). Although three times as many males were found on plants with lures versus control plants, the numbers of males attracted were not different between the treatment and control (36 to treatment and 12 to control, multiresponse permutation procedure (MRPP), $P > 0.05$). Interpretation of field test results was complicated by large variations in bug populations among blocks.

A second trial conducted in six blocks from August 19 to 24, 1999, attracted only low numbers of bugs. Significantly more female bugs were collected on baited plants than on control plants (9 vs. 0, MRPP test, $P = 0.01558$). Of the 9 males collected, 7 were on baited plants, and 2 were on control plants.

Because we did not find significant populations of *C. ligata* in the Riverside area in 1999, no specific field experiments were deployed for this species. However, two female *C. ligata* were collected from plants baited with lures for *C. uhleri*. Because male *C. ligata* and *C. uhleri* produce virtually identical blends of male-specific compounds, this was not unexpected. However, it is noteworthy because no other *C. ligata* were seen or collected at that site using visual observations and sweep-netting sampling.

DISCUSSION

Reproductive Behavior. The close-range courtship of phytophagous stink bugs follows characteristic steps, including males approaching females (over short distances), followed by antennation and head-butting of females by males, and end-to-end copulation (Fish and Alcock, 1973; Borges et al., 1987; Wang and Millar, 1997; Ho and Millar, 2001a). The daily rhythms of reproductive activity also appear similar among species, with courtship and copulation being initiated primarily in the afternoon and evening [e.g., *Chlorochroa ligata* (Fish and Alcock, 1973), *Nezara viridula* (Harris and Todd, 1980), *Euschistus heros* (Borges et al., 1998a), *Thyanta pallidovirens* (Wang and Millar, 1997), *Acrosternum hilare* and *T. pallidovirens* (H. McBrien and J. Millar, unpublished data)]. Thus, late afternoon and evening were chosen as the optimum time to conduct both laboratory and field bioassays.

The patterns of *C. uhleri* and *C. ligata* reproductive activity coincided with the maximum production of male-specific compounds. However, male-specific compounds were detected throughout the 24-hr monitoring periods, suggesting that the compounds were produced continuously with only the production rate varying. This pattern may have developed for several possible reasons. First, parasitoids use stink bug pheromones as kairomones (Aldrich, 1995), and timing peak production to parts of the day with waning light levels may reduce the risk of

parasitization (Harris and Todd, 1980). Second, because of the long copulation times, pheromone production in late afternoon may be timed so that males and females find each other around dusk and then continue copulation under the cover of darkness. Third, mating activities may be timed for optimum temperature and humidity conditions. Reproductive activities in the heat of the day increase the risk of desiccation, whereas cooler nighttime temperatures may hinder movement and activity.

Male *C. ligata* transferred substantial weights of material to females during mating, with body weights of both sexes changing significantly after copulation. Weight changes occurred with both virgin and experienced insects, and mean duration of copulation was similar for virgin and experienced insects. Similar weight changes after mating occur with *T. pallidovirens* (Wang and Millar, 1997), and it has been documented that pentatomid females obtain nutrients from males, often in the form of large, nonfertilizing sperm (Schrader, 1960; Mitchell and Mau, 1969; Mau and Mitchell, 1978; Kasule, 1986). For *T. pallidovirens*, these materials resulted in enhanced egg production by females, increasing the reproductive output of both sexes (Wang and Millar, 1997). This increase in reproductive output should in turn encourage multiple copulations by both sexes, which is indeed a characteristic of pentatomid reproduction.

There are several possible explanations for male stink bugs being the pheromone-producing sex. For example, adult stink bugs are polyphagous and mobile, and they move into and out of habitats quickly (McPherson and McPherson, 2000). However, the details of the dynamics of these migrations are unknown, and it is possible that males are the pioneering sex in colonizing new habitats. Under such a scenario, males, upon finding a good habitat, might produce pheromones to attract females, with oviposition commencing within a day or so after copulation to the mutual benefit of both sexes. Alternatively, because parasitoids use bug semiochemicals as kairomones to locate their hosts (Aldrich, 1995), selective pressures may have favored males as the pheromone-producing gender.

Male Specific Compounds. The male-specific compounds produced by *C. uhleri* and *C. ligata* included two known terpenoid compounds, methyl (*R*)-3-(*E*)-6-2,3-dihydrofarnesoate and methyl (2*E*,6*E*)-farnesoate, and a new terpenoid natural product, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate. The blends of male-specific compounds produced by the two species were remarkably similar, even to the extent that the absolute configuration (3*R*) of the major compound, methyl (*E*)-6-2,3-dihydrofarnesoate, was the same. In contrast, the two species are quite different morphologically, with *C. ligata* adults being dark brown or black with a band of orange or yellow outlining the body, whereas *C. uhleri* are green in color. Although detailed distribution records and habitat preferences are not available, these two species appear to share or at least overlap in ranges and host plants, and we have caught bugs of both species in the same field plots on the same day. The fact that the two species are sympatric, coupled with the apparently identical

pheromone blends, suggests that communication with pheromones is only part of the mate location system. Research on these (McBrien and Millar, unpublished data) and other species (Harris et al., 1982; Çokl, 1985; Kon et al., 1988; Ota and Çokl, 1991; Ryan and Walter, 1992; Jeraj and Walter, 1998; Miklas et al., 1999, Çokl et al., 2001) suggests that over shorter distances, mate location by phytophagous pentatomids involves species-specific and possibly even strain-specific substrate-borne vibrational signals (Ryan et al., 1996; Jeraj and Walter, 1998).

The blend of male-specific compounds produced by the congener *C. sayi*, which is morphologically very similar to and is frequently confused with *C. uhleri* (McPherson and McPherson, 2000), is entirely different from that produced by *C. uhleri* and *C. ligata.* The *C. sayi* blend is composed primarily of the monoterpenoid methyl geranate, with traces of methyl citronellate and methyl (*E*)-6-2,3-dihydrofarnesoate (Ho and Millar, 2001a), the latter being the only shared compound between *C. sayi* and the other two species. To our knowledge, these are the only reports of methyl (*E*)-6-2,3-dihydrofarnesoate being found in nature. It was first synthesized for treating liver disease (Yamatsu et al. 1981), and because of its structural similarity to insect juvenile hormones, it also has been used in the study of juvenile hormones (Campbell et al., 1998). The homolog, methyl (*E*)-5-2,6,10-trimethyl-5,9-undecadienoate, has never been reported from natural sources. Methyl farnesoate has been found to function as a juvenile hormone in crustaceans (Homola and Chang, 1997), but to our knowledge, it has never before been reported as an insect pheromone component.

In studying the dynamics of production of the male-specific compounds, it is unclear why they were detected in all of the *C. ligata* cohorts aerated but less than half of the *C. uhleri* cohorts. One possible explanation is that reproductive diapause, and the cessation of pheromone production, might be triggered easily in *C. uhleri*, despite the fact that the temperature, humidity, diet, and the length of the photophase (16L:8D) in the rearing room were constant. Even cohorts that failed to produce detectable levels of pheromone were not in complete reproductive diapause because mating did occur in the laboratory colonies. However, the close confines and relatively crowded conditions under which the bugs were reared may have precluded any need for the use of pheromones.

Laboratory Bioassays. Laboratory and field bioassays were complicated by the fact that females did not respond strongly to odors from conspecific males, to extracts from males, or to reconstructed blends of male specific compounds. Analogous problems have been reported during attempts to bioassay possible pheromone components for other phytophagous bug species. For example, blends of two male-specific compounds, *trans*- and *cis*-(*Z*)-bisabolene epoxide, were weakly attractive to female *Nezara viridula* in laboratory bioassays (Brézot et al., 1994). Despite the importance of this bug as a worldwide agricultural pest (Panizzi, 1997), there has been only a single short report of field bioassays of the pheromone, in which low numbers of bugs were attracted (Aldrich et al., 1993). Male-specific compounds

also have been identified from a number of *Euschistus* spp. (Aldrich et al., 1991), but field bioassays resulted in collection of less than one bug per trap-day (Aldrich et al., 1991; Borges et al., 1998b). Laboratory bioassays with *Euschistus heros* (Borges et al., 1998a), *E. obscurus* (Borges and Aldrich, 1994), and *Piezodorus hybneri* (Leal et al., 1998) also demonstrated that attraction of female bugs to male volatiles or synthetic pheromone components was relatively weak, with less than 50% of the individuals tested being attracted to the test treatments.

Field Bioassays. Several reports indicated that commonly used insect trap designs were not effective for phytophagous stink bugs and that bugs were frequently attracted to the vicinity of traps but not right into traps (Aldrich et al., 1991; Sugie et al., 1996). In perhaps the most extreme example, James et al. (1996) caught no spined citrus bugs (*Biprorulus bibax*) in pheromone-baited traps placed in citrus trees, but found that large numbers of bugs were attracted to trees containing traps. Consequently, instead of using traps of unknown efficacy, we decided to use a "trap plant" concept, in which the numbers of bugs on plants baited with pheromone lures or solvent controls were counted. Before deploying bioassays, in a pilot experiment run to determine the optimum time to conduct bug counts, female bugs were not seen close to lures in the early morning when temperatures were still cool, nor were they seen in the upper half of the plants close to the lures during the heat of the day. The optimal period for attraction, in agreement with the laboratory studies and with previously reported data from other species, appeared to be late afternoon through to darkness. Consequently, field tests were evaluated from about 17:00 hr until dark.

Field test results followed the same general pattern as that seen in the laboratory, with weak attraction of adult female bugs to lures. Several other points emerged. First, there were indications that lures also attracted male bugs; the number of males on baited plants was always higher than the number near controls, in all tests run. However, there may be an alternative explanation for this apparent attraction of males to pheromone lures. Males foraging for females may be attracted to or arrested on plants with females in response to cues associated with females, such as the shorter range substrate-borne vibrational cues described above. Further studies will be required to determine whether males are indeed attracted to the synthetic male pheromone or whether the apparent attraction is actually arrestment or attraction in response to female-produced cues. There was no indication that the lures were attractive to immature bugs, as has been reported for at least one other stink bug species (Aldrich et al., 1987).

It must be mentioned that the trap plant concept has limitations. First, bugs that were attracted to lure-baited plants in the late afternoon had disappeared by the next morning, indicating that the attraction is transient. Thus, if used as a bug monitoring strategy, trap plants would need to be checked consistently during late afternoon each day. Second, because bugs are free to move away from the lure-baited plants, counting the bugs on a trap plant provides a snapshot of one point

in time, rather than a cumulative count of all the bugs that have been attracted. Consequently, a trap plant strategy may be less sensitive than a method that retains most or all of the bugs attracted.

In total, the laboratory and field bioassay data provide strong evidence that one or more of the *C. uhleri* and *C. ligata* male-specific compounds are sex pheromone components. Because of the weak attraction to the male-produced compounds, and despite laboratory and field bioassay attempts extending over several years, it remains to be determined whether the male-produced pheromones consist of the single component methyl (*E*)-6-2,3-dihydrofarnesoate, or a blend of this compound with minor male-specific components. Efforts to address this question are continuing, in parallel with trap development and study of stink bug substrate-borne vibrational signals.

Acknowledgments—H.-Y. Ho thanks the National Science Council Taiwan and Academia Sinica, Taiwan, for financial support during this work. The authors also gratefully acknowledge financial support from the California Pistachio Commission, the Washington State Tree Fruit Agreement, and the University of California IPM Project.

REFERENCES

ALDRICH, J. R. 1995. Chemical communication in the true bugs and parasitoid exploitation, pp. 318–363 *in* R. Cardé and W. Bell (eds.). Chemical Ecology of Insects 2, Chapman & Hall, New York.

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and LOCKWOOD, J. A. 1987. Pheromone strains of the cosmopolitan pest, *Nezara viridula* (Heteroptera: Pentatomidae). *J. Exp. Zool.* 244:171–175.

ALDRICH, J. R., HOFFMANN, M. P., KOCHANSKY, J. P., LUSBY, W. R., EGER, J. E., and PAYNE, J. A. 1991. Identification and attractiveness of a major pheromone component for Nearctic *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae). *Environ. Entomol.* 20:477–483.

ALDRICH, J. R., NUMATA, H., BORGES, M., BIN, F., WAITE, G. K., and LUSBY, W. R. 1993. Artifacts and pheromone blends from *Nezara* spp. and other stink bugs (Heteroptera: Pentatomidae). *Z. Naturforsch.* 48C:73–79.

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and BORGES, M. 1994. Identification of male-specific volatiles from Nearctic and Neotropical stink bugs (Heteroptera: Pentatomidae). *J. Chem. Ecol.* 20:1103–1111.

BAKER, R., BORGES, M., COOKE, N. G., and HERBERT, R. H. 1987. Identification and synthesis of (*Z*)-(1′*S*,3′*R*,4′*S*)-(–)-2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methyl-hepta-2,5-diene, the sex pheromone of the southern green stink bug, *Nezara viridula* (L.). *J. Chem. Soc. Chem. Commun.* 1987:414–416.

BIONDINI, M. E., BONHAM, C. D., and REDENTE, E. F. 1985. Secondary successional patterns in a sagebrush (*Artemisia tridentata*) community as they relate to soil disturbance and soil biological activity. *Vegetatio* 60:25–36.

BORGES, M. and ALDRICH, J. R. 1994. An attractant pheromone for the Nearctic stink bug, *Euschistus obscurus* (Heteroptera: Pentatomidae): Insight into a Neotropical relative. *J. Chem. Ecol.* 20:1095–1102.

BORGES, M., JEPSON, P. C., and HOWSE, P. E. 1987. Long-range mate location and close range courtship behaviour of the green stink bug, *Nezara viridula* and its mediation by sex pheromones. *Entomol. Exp. Appl.* 44:205–212.

BORGES, M., MORI, K., COSTA, M. L. M., and SUJII, E. R. 1998a. Behavioural evidence of methyl-2,6,10-trimethyltridecanoate as a sex pheromone of *Euschistus heros* (Heteroptera: Pentatomidae). *J. Appl. Entomol.* 122:335–338.

BORGES, M., SCHMIDT, F. G. V., SUJII, E. R., MEDEIROS, M. A., MORI, K., ZARBIN, P. H. G., and FERREIRA, J. T. B. 1998b. Field responses of stink bugs to the natural and synthetic pheromone of the Neotropical brown stink bug, *Euschistus heros* (Heteroptera: Pentatomidae) *Physiol. Entomol.* 23:202–207.

BRÉZOT, P., MALOSSE, C., MORI, K., and RENOU, M. 1994. Bisabolene epoxides in sex pheromone in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Role of *cis* isomer and relation to specificity of pheromone. *J. Chem. Ecol.* 20:3133–3147.

BUXTON, G. M., THOMAS, D. B., and FROESCHNER, R. C. 1983. Revision of the species of the Sayi-group of *Chlorochroa* Stål (Hemiptera: Pentatomidae). *Occas. Pap. Entomol.* 29:1–23.

CAFFREY, D. J. and BARBER, G. W. 1919. The Grain Bug. Bulletin No.779, June 1919, United States Department of Agriculture, Bureau of Entomology, Washington, D.C.

CAMPBELL, P. E., OAKESHOTT, J. G., and HEALY, M. J. 1998. Purification and kinetic characterisation of juvenile hormone esterase from *Drosophila melanogaster. Insect Biochem. Mol. Biol.* 28:501–515.

COATES, R. M., and FREIDINGER, R. M. 1970. Total synthesis of (±) sesquicarene. *Tetrahedron* 26: 3487–3493.

ÇOKL, A. 1985. Problems of sound communication in a land bug species *Nezara viridula* (L.) (Heteroptera: Pentatomidae), pp. 163–168, *in* K. Kalmring and N. Elsner (eds.). Acoustic and Vibrational Communication in Insects: Proceedings XVII International Congress on Entomology, Hamburg, 1984. Verlag Paul Parey, Berlin.

ÇOKL, A., MCBRIEN, H. L., and MILLAR, J. G. 2001. Comparison of substrate-borne vibrational signals of two stink bug species, *Acrosternum hilare* (Say) and *Nezara viridula* (L.) (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 94:471–479.

FISH, J. and ALCOCK, J. 1973. The behavior of *Chlorochroa ligata* (Say) and *Cosmopepla bimaculata* (Thomas) (Hemiptera: Pentatomidae). *Entomol. News.* 84:260–268.

FUJISAWA, T., SATO, T., KAWARA, T., NODA, A., and OBINATA, T. 1980. A novel method for terpene synthesis by the ring-opening reaction of β-methyl-β- propiolactone. *Tetrahedron Lett.* 21:2553–2554.

HARRIS, V. E. and TODD, J. W. 1980. Male-mediated aggregation of male, female, and 5th-instar southern green stink bugs and concomitant attraction of a tachinid parasite, *Trichopoda pennipes. Entomol. Exp. Appl.* 27:117–126.

HARRIS, V. E., TODD, J. W., WEBB, J. C., and BENNER, J. C. 1982. Acoustical and behavioral analysis of the songs of the southern green stink bug, *Nezara viridula. Ann. Entomol. Soc. Am.* 75:234–249.

HENRY, T. J. and FROESCHNER, R. C. (eds.). 1988. Catalog of the Heteroptera, or true bugs, of Canada and the continental United States. E. J. Brill, Leiden.

HO, H.-Y. and MILLAR, J. G. 2001a. Identification and synthesis of a male-produced sex pheromone from the stink bug *Chlorochroa sayi. J. Chem. Ecol.* 27:1177–1201.

HO, H.-Y. and MILLAR, J. G. 2001b. Compounds in the metathoracic glands of adults and the dorsal abdominal glands of nymphs of the stink bugs *Chlorochroa uhleri, C. sayi*, and *C. ligata* (Hemiptera: Pentatomidae). *Zoological Studies* 40:193–198.

HOMOLA, E. and CHANG, E. S. 1997. Methyl farnesoate: Crustacean juvenile hormone in search of function. *Comp. Biochem. Physiol., B: Biochem. Mol. Biol.* 117B:347–356.

HUFFER, M. and SCHREIER, P. 1990. High resolution gas chromatographic resolution of chiral primary alcohols and acids as their diastereomeric 1-phenylethylamides. *J. Chromatogr.* 519:263–267.

JAMES, D. G., MORI, K., ALDRICH, J. R. and OLIVER, J. E. 1994. Flight-mediated attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to natural and synthetic aggregation pheromone. *J. Chem. Ecol.* 20:71–80.

JAMES, D. G., HEFFER, R., and AMAIKE, A. 1996. Field attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to synthetic aggregation pheromone and (*E*)-2-hexenal, a pentatomid defense chemical. *J. Chem. Ecol.* 22:1697–1708.

JERAJ, M. and WALTER, G. H. 1998. Vibrational communication in *Nezara viridula*: Response of Slovenian and Australian bugs to one another. *Behav. Processes* 44:51–58.

KASULE, F. K. 1986. Repetitive mating and female fitness in *Dysdercus cardinalis* (Hemiptera: Pyrrhocoridae). *Zool. J. Linn. Soc.* 88:191–199.

KON, M., OE, A., NUMATA, H., and HIDAKA, T. 1988. Comparison of the mating behavior between two sympatric species, *Nezara antennata* and *N. viridula* (Heteroptera: Pentatomidae), with special reference to sound emission. *J. Ethol.* 2:91–98.

LEAL, W. S., KUWAHARA, S., SHI, X., HIGUCHI, H., MARINO, C. E. B., ONO, M., and MEINWALD, J. 1998. Male released sex pheromone of the stink bug *Piezodorus hybneri*. *J. Chem. Ecol.* 24:1817–1829.

LEUTENEGGER, U., MADIN, A., and PFALTZ, A. 1989. Enantioselective reduction of α,β-unsaturated carboxylates with $NaBH_4$ and catalytic amounts of chiral cobalt semicorrin complexes. *Angew. Chem. Int. Ed.* 28:60–61.

MAU, R. F. L. and MITCHELL, W. C. 1978. Development and reproduction of the oriental stink bug *Plautia stali* (Hemiptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 71:756–757.

MCBRIEN, H. L. and MILLAR, J. G. 1999. Phytophagous bugs, pp. 277–304, *in* J. Hardie and A.K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants, CAB International, Wallingford, United Kingdom.

MCCUNE, B. and MEFFORD, M. J. 1999. PC-ORD. Multivariate Analysis of Ecological Data, Version 4. MjM Software Design, Gleneden Beach, Oregon. http://www.ptinet.net/∼mjm

MCPHERSON, J. E. and MCPHERSON, R. M. 2000. Stink Bugs of Economic Importance North of Mexico. CRC Press, Boca Raton, Florida.

MIKLAS, N., RENOU, M., ÇOKAL, A., and VIRANT-DOBERLET, M. 1999. Songs and pheromone interplay in sexual communication in *Nezara viridula* (Heteroptera: Pentatomidae). Poster 111, 16th Annual Meeting, International Society of Chemical Ecology, Marseille, Nov. 13–17, 1999.

MILLAR, J. G. 1997. Methyl (2*E*,4*Z*,6*Z*)-deca-2,4,6-trienoate, a thermally unstable, sex-specific compound from the stink bug *Thyanta pallidovirens*. *Tetrahedron Lett.* 38:7971–7972.

MITCHELL, W. C. and MAU, R. F. L. 1969. Sexual activity and longevity of the southern green stink bug *Nezara viridula*. *Ann. Entomol. Soc. Am.* 62:1246–1247.

MORRILL, A.W. 1910. Plant-bugs injurious to cotton bolls. *USDA Bur. Entomol. Bull.* 86:23–73.

OHLENDORF, B. L. P. 1990. UC IPM Pest Management Guidelines. University of California Statewide Integrated Pest Management Project, University of California, Davis, Division of Agriculture and Natural Resources, publication 3339.

OHLENDORF, B. L. P. 1999. Integrated Pest Control Management for Apples and Pears, 2nd ed. University of California Statewide Integrated Pest Management Project, University of California, Davis, Division of Agriculture and Nature Resources, Resources; publication 3340.

OTA, D. and ÇOKL, A. 1991. Mate location in the southern green stink bug, *Nezara viridula* (Heteroptera: Pentatomidae) mediated through substrate-borne signals in ivy. *J. Insect Behav.* 4:441–447.

PANIZZI, A. R. 1997. Wild hosts of Pentatomids: Ecological significance and role in their pest status on crops. *Annu. Rev. Entomol.* 42:99–122.

PFALTZ, A. 1989. Enantioselective catalysis with chiral cobalt and copper complexes. *Mod. Synth. Methods* 5:198–248.

PRASHAD, M., KATHAWALA, F. G., and SCALLEN, T. 1993. *N*-Arylalkylfarnesyl-amines: New potent squalene synthesis inhibitors. *J. Med. Chem.* 36:1501–1504.

RUSSELL, E. E. 1952. Stink bugs on seed alfalfa in southern Arizona. Circular No. 903. United States Department of Agriculture, Washington, D.C.

RYAN, M. A. and WALTER, G. H. 1992. Sound transmission in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Further evidence that signal transmission is substrate-borne. *Experientia* 48: 1112–1115.

RYAN, M. A., ÇOKL, A., and WALTER, G. H. 1996. Differences in vibratory sound communication between a Slovenian and an Australian population of *Nezara viridula* (L.) (Heteroptera: Pentatomidae). *Behav. Processes* 36:183–193.

SATO, T., KAWARA, T., KAWASHIMA, M., and FUJISAWA, T. 1980. Copper-catalyzed reaction of Grignard reagents with β-propiolactones: A convenient method for the synthesis of β-substituted propionic acids. *Chem. Lett.* 571–574.

SAVU, P. M. and KATZENELLENBOGEN, J. A. 1981. Selective γ alkylation of copper enolates derived from α,β-unsaturated acids: Factors affecting scope and regio- and stereoselectivity. *J. Org. Chem.* 46:239–250.

SCHRADER, F. 1960. Evolutionary aspects of aberrant meiosis in some Pentatomidae (Heteroptera). *Evolution* 14:498–508.

SILVERSTEIN, R. M. and WEBSTER, F. X. 1998. Spectrometric Identification of Organic Compounds. Wiley, New York.

SUGIE, H., YOSHIDA, M., KAWASAKI, K., NOGUCHI, H., MORIYA, S., TAKAGI, K., FUKUDA, H., FUJIIE, A., YAMANAKA, M., OHIRA, Y., TSUTSUMI, T., TSUDA, K., FUKUMOTO, K., YAMASHITA, M., and SUZUKI, H. 1996. Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Appl. Entomol. Zool.* 31:427–431.

WAINWRIGHT, G., PRESCOTT, M. C., and REES, H. H. 1996. Mass spectrometric determination of methyl farnesoate profiles and correlation with ovarian development in the edible crab, *Cancer pagurus. J. Mass Spectrom.* 31:1338–1344.

WANG, Q. and MILLAR, J. G. 1997. Reproductive behavior of *Thyanta pallidovirens* (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 90:380–388.

YAMATSU, I., YUICHI, I., SHINYA, A., WATANABE, H., IGARASHI, T., SHIOJIRI, H., TANABE, Y., and KUNIKO, H. 1981. α,β-Dihydropolyprenyl derivatives, pharmacological preparations containing them and method for treating liver diseases. *Eur. Pat. Appl.* EP 41:235.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

SEX PHEROMONE OF TOMATO FRUIT BORER, *Neoleucinodes elegantalis*

AIVLÉ CABRERA,[1,2,*] ALVARO E. EIRAS,[3,5] GERHARD GRIES,[4] REGINE GRIES,[4] NEUDO URDANETA,[2] BEATRIZ MIRÁS,[1] CESAR BADJI,[5] and KLAUS JAFFE[1]

[1]*Laboratòriò de Comportamiento*

[2]*Departamento de Química*
Universidad Simón Bolívar
Apartado 89000, Caracas 1080-A, Venezuela

[3]*Departamento de Parasitologia, ICB*
Universidade Federal de Minas Gerais (UFMG), Belo Horizonte
CEP 31270-901, MG, Brazil

[4]*Centre for Environmental Biology*
Department of Biological Sciences
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

[5]*Laboratório de Proteção de Plantas, CCTA*
Universidade Estadual do Norte Fluminense
CEP 28045-640, Campos, RJ, Brazil

(Received December 26, 2000; accepted June 12, 2001)

Abstract—Five candidate pheromone components were identified by analyzing pheromone gland extracts by gas chromatography (GC), coupled GC-electroantennographic detection (EAD), and coupled GC-mass spectrometry (MS): (*E*)-11-hexadecenol(*E*11–16 : OH), (*Z*)-11-hexadecenol (*Z*11–16 : OH), (*E*)-11-hexadecenal, (*E*)-11-hexadecenyl acetate, and (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene (*Z*3,*Z*6,*Z*9–23 : Hy). In electroantennogram (EAG) recordings, synthetic *E*11–16 : OH elicited stronger antennal responses at low doses than other candidate pheromone components. Field tests demonstrated that synthetic *E*11–16 : OH as a trap bait was effective in attracting males, whereas addition of *Z*11–16 : OH inhibited the males' response. *Z*3,*Z*6,*Z*9–23 : Hy strongly enhanced attractiveness of *E*11–16 : OH, but was not attractive by itself. A pheromone blend with synergistic behavioral activity of an alcohol (*E*11–16 : OH) and hydrocarbon (*Z*3,*Z*6,*Z*9–23 : Hy) component is most unusual in the Lepidoptera. The synthetic two-component pheromone is approximately 60 times more attractive than the female-produced blend and might facilitate the control of this pest.

*To whom correspondence should be addressed; e-mail: acabrera@usb.ve

0098-0331/01/1000-2097$19.50/0

Key Words—*Neoleucinodes elegantalis*, Lepidoptera, Crambidae, sex pheromone, (*E*)-11-hexadecenol, (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene, (*Z*)-11-hexadecenol, GC-EAD, tomato.

INTRODUCTION

The tomato fruit borer, *Neoleucinodes elegantalis* (Lepidoptera: Crambidae), is one of the most important economic pests of tomato (*Lycopersicum esculentum*) crops in tropical South America. Current control tactics include frequent applications of pesticides (Reis and Souza, 1996) that may lead to the development of insecticide resistance. Moreover, once larvae have entered the fruit, pesticides and biological control agents are ineffective. Pheromone-based mating disruption represents an alternative tactic to manage this pest insect but requires identification of the female-produced sex pheromone. We know that virgin females attract males in the field (Mirás et al., 1997), and extracts of abdominal tips with pheromone glands from virgin females attract males in wind tunnel bioassays (Eiras, 2000). Here we report identification, electrophysiological studies, and field experiments of sex pheromone components of *N. elegantalis.*

METHODS AND MATERIALS

Insects. Tomato fruits infested with *N. elegantalis* larvae were collected in commercial tomato crops in the states of Lara and Aragua, Venezuela, and in Rio de Janeiro, Brazil. Fruits were kept in plastic containers until larvae emerged from the fruit to pupate. Adult insects were maintained according to Eiras (2000). During peak calling activity, abdominal tips with pheromone glands of 2-to 3-day-old virgin female moths were removed and extracted for 10–15 min in hexane. The supernatant was withdrawn and stored in microcapillaries at -15°C.

Chemical Analyses. Pheromone extracts were subjected to gas chromatographic (GC) and coupled GC-electroantennographic detection (EAD) analyses, using a Hewlett Packard 5890A gas chromatograph equipped with fused silica columns (30 m $\times$ 0.25 or 0.32 mm ID) coated with DB-5, DB-210, or DB-23 (J&W Scientific, Folsom, California). Forty female equivalents of pheromone gland extract were also analyzed by coupled GC-mass spectrometry (MS), employing a Perkin Elmer QMass-910 attached to a GC-Autosystem 2000, equipped with a DB-5 column (25 m $\times$ 0.18 mm ID, Quadrex, New Haven, Connecticut).

The position and geometry of a double bond in an EAD-active hexadecenol were determined by: (1) GC-EAD analyses of synthetic hexadecenols (*E*2 to *E*14 and *Z*2 to *Z*14); (2) derivatization of extract with *m*-chloroperbenzoic acid (Bierl-Leonhardt et al., 1980), followed by GC-MS analysis of the resulting epoxide; and (3) acetylation of pheromone extract with acetic anhydride and

pyridine, followed by GC analyses [HP-GC equipped with a SP-1000-coated column (30 m × 0.25 mm ID; Supelco, Bellefonte, Pennsylvania)] with improved chromatographic resolution of the acetylated alcohol and synthetic acetate standards.

Effluvia of calling, 1- or 2-day-old female *N. elegantalis* were adsorbed on a solid-phase microextraction (SPME) fiber coated with 100 μm polidimethylsiloxane (Supelco). The fiber was exposed to a female during the entire calling period and then desorbed at the capillary injection port (270°C) of the GC equipped with columns (30 m × 0.25 mm ID or 25 m × 0.18 mm ID; Quadrex, New Haven, Connecticut) coated with methyl–5% phenyl silicone or Carbowax 20 M. Retention characteristics of female-produced, fiber-desorbed compounds were compared with those of authentic standards.

Electrophysiology. Electroantennogram (EAG) recordings were carried out, using a Syntech EAG system (NL-1200 BM Hilversum; The Netherlands). Five compounds (*E*11–16 : OH, *Z*11–16 : OH, *E*11–16 : OAc, *E*11–16 : Ald, and *Z*3,*Z*6,*Z*9–23 : Hy) at increasing logarithmic doses (10 to 10^6 ng) were tested with each of five 48- to 72-hr-old male antennae. An antenna was stimulated by subjecting it to puffs (0.5 sec) of purified and humidified air (1.5 liters/min) delivered through a Pasteur pipet (15 cm long), containing a filter paper strip (1 × 5 cm) impregnated with 50 μl of a test solution. Air puffs from an empty pipet, pipet plus filter paper, or filter paper plus solvent served as control stimuli.

Syntheses. (*Z*)-9,(*Z*)-12,(*Z*)-15-octadecatrien-1-ol (99%) was synthesized from methyl linolenate after reduction with $LiAlH_4$. This alcohol was oxidized with pyridinium chlorochromate (PCC) to afford (*Z*)-9,(*Z*)-12,(*Z*)-15-octadecatrienal (98%). *n*-Pentyl magnesium bromide was added to a solution of this aldehyde in anhydrous ether to obtain 6-hydroxy-(*Z*)-14,(*Z*)-17,(*Z*)-20-tricosatriene. A solution of this compound in anhydrous pyridine was treated with methane sulfonyl chloride (Kocovský and Cerný, 1978). The obtained mesylate was reduced with zinc dust and sodium iodide (Kocovský and Cerný, 1978) to obtain (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene, which was purified (>99%) by flash silica column chromatography using *n*-hexane as eluent. ^{1}H NMR (ppm; δ): 5.35 (m), 2.80 (t), 2.05 (m), 1.25 (m), 0.95 (t) 0.85 (t); EI-MS [*m*/*z* (relative intensity)]: 79 (100); 108 (98); 67 (49); 93 (48); 262 (6); M^+ 318 (2).

Sources of Candidate Pheromone Components. (*E*)-11-Hexadecenol (*E*11–16 : OH) was obtained from the Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands; (*Z*)-11-hexadecenol (*Z*11–16 : OH) was purchased from Aldrich Chem. Co. (Milwaukee, Wisconsin); (*E*)-11-hexadecenyl acetate (*E*11–16 : OAc) was prepared by acetylation of *E*11–16 : OH with acetic anhydride and pyridine; (*E*)-11-hexadecenal (*E*11–16 : Al) was prepared by oxidation of E11-hexadecenol with PCC in dichloromethane; (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene (*Z*3,*Z*6,*Z*9–23 : Hy) was synthesized as described above. All compounds were >98% chemically pure.

Field Experiments. Field experiments were conducted in commercial tomato plantations in Aragua State, Venezuela, employing a complete randomized block design. Water traps (Mirás et al., 1997) were suspended ~1 m above ground at 20-m intervals and baited with red rubber septa (Aldrich Chemical Co., Milwaukee, Wisconsin, catalog No. *Z*12,434-6) that were Soxhlet-extracted with ethanol for 24 hr, and impregnated with candidate pheromone components in HPLC-grade dichloromethane. Location, experimental period, and number of replicates for each experiment are reported in the captions of Figures 3 and 4 below.

Experiment 1 tested traps baited with *E*11–16 : OH (1 mg) and *Z*11–16 : OH (1 mg), singly and in binary combination at ratios of 1 : 0.1 and 1 : 0.01 mg. Solvent-impregnated rubber septa served as baits in control traps. Both experiments 2 and 3 tested traps baited with two virgin females or *E*11–16 : OH (1 mg). Experiment 4 tested traps baited with *Z*3,*Z*6,*Z*9–23 : Hy (1 mg) or *E*11–16 : OH (1 mg) singly, and *E*11–16 : OH (1 mg) in binary combinations with *Z*3,*Z*6,*Z*9–23 : Hy (0.025, 0.05, or 0.1 mg) and in penternary combination with *Z*11–16 : OH (0.07 mg), *E*11–16 : Ald (0.02 mg), *E*11–16 : OAc (0.02 mg), and *Z*3,*Z*6,*Z*9–23 : Hy (0.04 mg) at ratios as determined in pheromone gland extract. Experiment 5 tested traps baited with *E*11–16 : OH (1 mg) in binary combinations with *E*11–16 : OAc (0.05 mg), *E*11–16 : Ald (0.05 mg), or *Z*3,*Z*6,*Z*9–23 : Hy (0.05 mg), in ternary combination with *E*11–16 : OAc (0.05 mg), and *E*11–16 : Ald (0.05 mg), and in quaternary combination with *E*11–16 : OAc (0.05 mg), *E*11–16 : Ald (0.05 mg), and *Z*3,*Z*6,*Z*9–23 : Hy (0.05 mg). Experiment 6 tested traps baited with *E*11–16 : OH (1mg) singly, and in combination with *Z*3,*Z*6,*Z*9–23 : Hy (0.05 mg).

Statistical Analysis. Multivariate analysis with the using Mann-Whitney test (Spiegel, 1991) was performed to analyze data from field tests.

RESULTS

Pheromone Identification. GC-EAD analysis of pheromone gland extracts of female *N. elegantalis* revealed six components that elicited an antennal response (Figure 1). The most abundant and EAD-active component **2** (Figure 1) was hypothesized to be a hexadecenol, based on its retention indices on DB-5 (1865), DB-210 (2078), and DB-23 (2406) columns. Position and geometry of the double bond was approximated by comparative GC and GC-EAD analyses of synthetic hexadecenols. (*E*)-11- and (*E*)-12-hexadecenol (*E*11- and *E*12–16 : OH) had retention times identical to, and EAD-activity comparable with, female-produced **2**. Epoxidation (Bierl-Leonhardt et al., 1980) of female-produced **2**, and GC-MS analysis of the epoxy alcohol revealed fragmentation ions at *m/z* 99 and 199 (due to α-cleavage of the epoxy group), confirming the double bond at C-11. GC-EAD analyses of pheromone extract on a DB-23 column, which separates the *E* and *Z* isomers, provided tentative evidence also for trace amounts of the *Z* isomer.

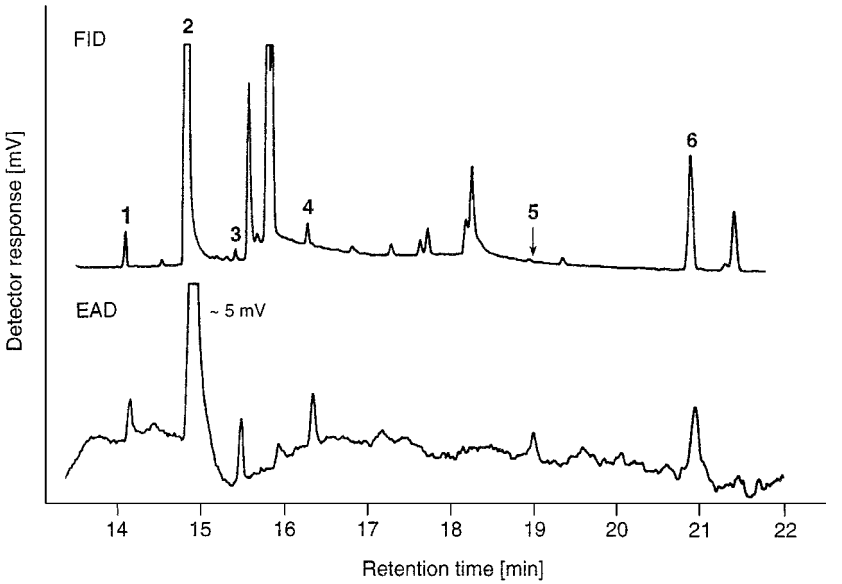


FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: male *N. elegantalis* antenna) responses to one female equivalent of pheromone gland extract, chromatograph on a DB-5 column; temperature program: 100°C (1 min) then 10°C/min to 240°C. Compound identity as follows: 1 = (*E*)-11-hexadecenal (*E*11–16 : Ald); 2 = (*E*)-11-hexadecenol (*E*11–16 : OH); 3 = unknown; 4 = (*E*)-11-hexadecenyl acetate (*E*11–16 : OAc); 5 = unknown; 6 = (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene (*Z*3, *Z*6, *Z*9-23 : Hy). Note: GC-EAD analyses of pheromone extract on a DB-23 column, which separated *E* and *Z* isomers of candidate pheromone components, provided tentative evidence also for trace amounts of (*Z*)-11-hexadecenol (*Z*11–16 : OH).

EAD-active **1** and **4** (Figure 1) were hypothesized and, through comparative GC analyses of authentic standards, confirmed to be *E*11–16 : Ald (**1**) and *E*11–16 : OAc (**4**). Mass spectrum and retention indices of EAD-active **6** were indicative of an triunsaturated C_{23} hydrocarbon, such as *Z*3,*Z*6,*Z*9–23 : Hy (Bell and Meinwald, 1986). Identical retention and mass spectrometric characteristics of an authentic standard and female-produced **6** confirmed this structural assignment. EAD-active compounds **3** and **5** occurred below detection threshold of the flame ionization detector and are yet to be identified.

In GC analyses of SPME-desorbed volatiles, small amounts of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy could be detected.

Electrophysiology. Synthetic equivalents of candidate pheromone components evoked significant EAG responses. *E*11–16OH was most EAG-active and, unlike other test stimuli, elicited >5 mV EAG responses from male antennae already at a 10 ng dose (Figure 2). *Z*11–16 : OH, *E*11–16 : OAc, *E*11–16 : Ald,

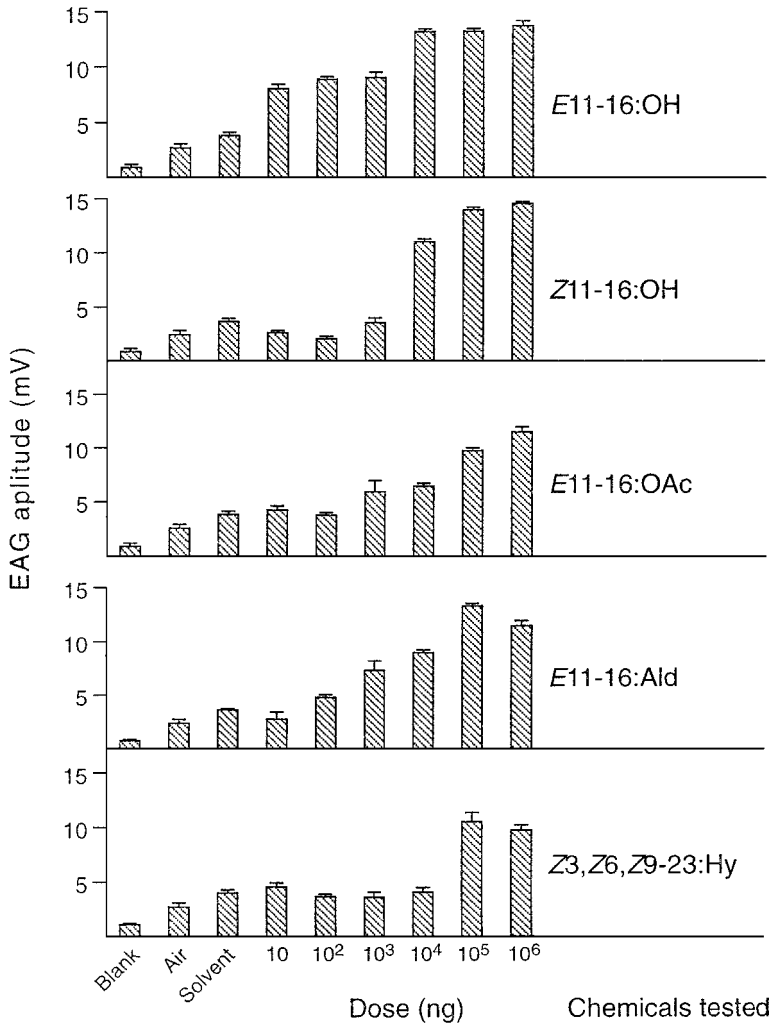


FIG. 2. Responses by male *N. elegantalis* antennae in electroantennogram (EAG) recordings to increasing amounts of five candidate pheromone components; compound nomenclature as in Figure 1.

and *Z*3,*Z*6,*Z*9–23 : Hy required doses of 10^4, 10^3, 10^2, and 10^5 ng, respectively, to induce EAG voltages greater than those of control stimuli (Figure 2).

Field Tests. Traps baited with *E*11–16 : OH captured significant numbers of male moths (Figure 3, experiment 1). *Z*11–16 : OH, in contrast, was not attractive and, when added to *E*11–16 : OH, strongly reduced captures of males. Traps baited

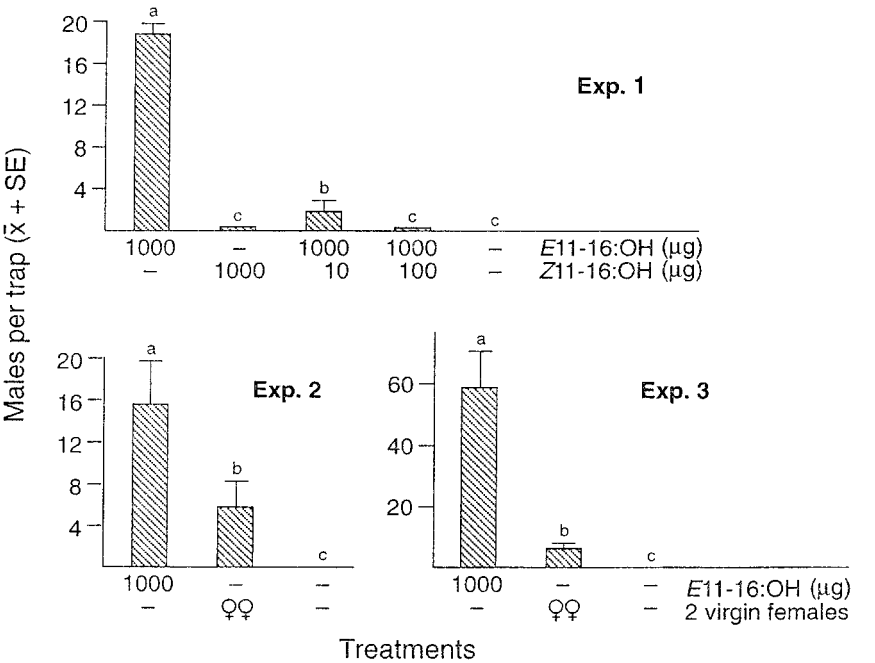


FIG. 3. Captures of male *N. elegantalis* in experiments 1–3 in water traps baited with two virgin female *N. elegantalis* or candidate pheromone components singly and in various combinations. Experiment 1: Casablanca, Aragua State; October 16–December 1, 1996; four replicates. Experiment 2: Casablanca, Aragua State; December 5–19, 1996; 7 replicates. Experiment 3: Múcura, Aragua State; February 15–20, 1997; 4 replicates. In each experiment, bars with the same letter superscript are not significantly different, $P > 0.05$.

with *E*11–16 : OH at 1 mg captured more males than those baited with two virgin female moths (Figure 3, experiments 2, and 3). *Z*3,*Z*6,*Z*9–23 : Hy enhanced attractiveness of *E*11–11 : OH, but was not attractive by itself (Figure 4, experiment 4). *E*11–16 : OH plus *Z*3,*Z*6,*Z*9–23 : Hy was the most attractive blend among several two-, three-, or four-component blends of candidate pheromone components (Figure 4, experiment 5). Synergistic activity of *Z*3,*Z*6,*Z*9–23 : Hy was confirmed in experiment 6 (Figure 4).

DISCUSSION

Our data show that *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy are sex pheromone components in *N. elegantalis*. Evidence in support of this conclusion includes the following: (1) both compounds were present and EAD-active in pheromone

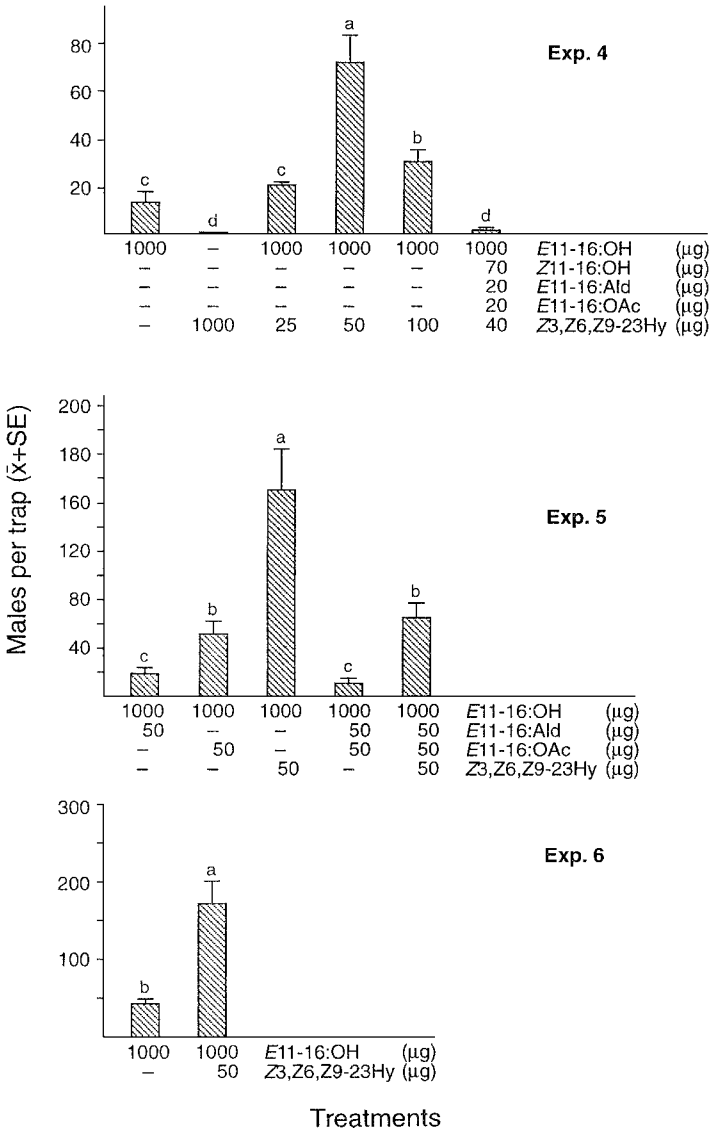


FIG. 4. Captures of male *N. elegantalis* in experiments 4–6 in water traps baited with candidate pheromone components singly and in various combinations. Experiment 4: Pao de Zárate, Aragua State; June 23–August 3, 1998; 5 replicates. Experiment 5: Valle de Tucutunemo, Aragua State; February 5–March 26, 1999; 5 replicates. Experiment 6: Pao de Zárate, Aragua State; July 3–25, 2000, 10 replicates. In each experiment, bars with the same letter superscript are not significantly different, $P > 0.05$.

gland extracts and effluviums of female moths (Figure 1); (2) identifications were based on comparative GC, GC-MS, and GC-EAD analyses of female-produced compounds and authentic standards; (3) both compounds elicited stronger antennal responses from male antennae in EAG recordings than did control stimuli (Figure 2); and (4) blends of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy attracted significant numbers of males (Figure 4). *E*11–16 : OH is the major pheromone component, because it was most abundant in gland extracts (Figure 1), elicited the strongest EAG response at low doses (10 or 100 ng) (Figure 2), and, unlike *Z*3,*Z*6,*Z*9–23 : Hy, was attractive by itself as a bait (Figures 3 and 4).

*E*11–16 : OH has also been identified as a sex pheromone component in other moths. It is the major pheromone component of the pod worm, *Leucinodes orbonalis* (Zhu et al., 1987; Attygalle et al., 1988), and a minor pheromone component of female pickleworms *Diaphania hyalinata* and *D. nitidalis* (Raina et al., 1986; Klun et al., 1986). It was also present in pheromone gland extracts of eggfruit caterpillar, *Sceliodes cordalis* (Clearwater et al., 1986) and of sugarcane borer, *Sesamia grisescens* (Whittle et al., 1995), but behavioral activity of *E*11–16 : OH in these two species is yet to be determined.

*Z*3,*Z*6,*Z*9–23 : Hy is reported here for the first time as a sex pheromone component in the Lepidoptera. While it was identified as a trace component in pheromone glands of the arctiid moth *Creatonotos gangis* (Bell and Meinwald, 1986), behavioral activity was not determined. A sex pheromone blend with synergistic behavioral activity between a triene hydrocarbon (*Z*3,*Z*6,*Z*9–23 : Hy) and an alcohol (*E*11–16 : OH), as demonstrated in our study, is most unusual. Most triene hydrocarbons as pheromone components in the Lepidoptera are either attractive by themselves (Millar et al., 1992) or serve as synergists of corresponding diene epoxides (Tóth et al., 1994).

Further compounds (*Z*11–16 : OH, *E*11–16 : Ald, *E*11–16 : OAc) in pheromone gland extracts of female *N. elegantalis* seem to have no pheromonal activity. This conclusion is based on their low EAG activity at low doses (Figure 2) and/or lack of attractiveness in field experiments. The role of *Z*11–16 : OH is interesting. Its low EAG activity but strong behavioral inhibition at a low dose (Figures 2 and 3) suggests that mate-seeking males may select calling females based on the presence or absence of *Z*11–16 : OH in the pheromone effluvium, providing a basis for a highly efficient system of sexual selection (Jaffe, 1996, 1998, 1999), as only very fit females would produce *E*11–16 : OH with small amounts of *Z*11–16 : OH. Alternatively, *Z*11–16 : OH may serve as a bifunctional pheromone component (Linn and Roelofs, 1995) in a sympatric species to enhance the specificity of the communication signal, although no such sympatric species is known.

The two-component pheromone blend of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy seems to work as a supernormal stimulus (Tinbergen and Perdeck, 1950), as it is approximately 60 times more attractive than the natural pheromone blend emitted by the females (Experiments 3 and 4, in Figures 3 and 4, respectively).

A synthetic mixture of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy as a trap bait is currently used by Brazilian and Venezuelan tomato growers to reduce infestations of *N. elegantalis*, promising efficient control in pheromone-based mass trapping programs.

Acknowledgments—This research was financial supported by BID-CONICIT QF-36, Venezuela and FAPERJ (Brazil). We thank L. Caraballo, C. Cárdenas, and J. Velásquez for field assistance. Grants from Venezuela (CONICIT and USB-Decanato de Postgrado) and Brazil (CNPq-300698/91-7 and IFS-F/2478-1) are acknowledged. A.E.E. acknowledges Dr. J. van der Peers for his collaboration with EAG recordings. The pheromone-lure system is protected by patent application 002488, Venezuela.

REFERENCES

ATTYGALLE, A., SCHWARZ, J., and GUNAWARDENA, N. E. 1988. Sex pheromone of brinjal ashoot and pod borer *Leucinodes orbonalis* Guenée (Lepidoptera: Pyralidae: Pyraustinae). *Z. Naturforsch.* 43c:790–792.

BELL, T. and MEINWALD, J. 1986. Pheromones of two arctiids moths (*Creatonotos transiens* and *C. gangis*): Chiral components from both sexes and achiral female components, *J. Chem. Ecol.* 12:385–410.

BIERL-LEONHARDT, B., DEVILBISS, E., and PLIMMER, J. 1980. Location of double-bond position in long-chain aldehydes and acetates by mass spectral analysis of epoxide derivatives. *J. Chromatogr. Sci.* 18:364–367.

CLEARWATER, J., GALBREATH, R., BENN, M., and YOUNG, H. 1986. Female-produced sexual pheromone of *Sceliodes cordalis* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 12:1943–1964.

EIRAS, A. E. 2000. Calling behaviour and extraction of sex pheromone of the tomato fruit borer, *Neoleucinodes elegantalis* Guenée (Lepidoptera:Crambidae). *An. Soc. Entomol. Bras.* 29:453–460.

JAFFE, K. 1996. The evolutionary dynamics of sex: Why the sexes are, in fact, always two? *Interciencia* 21:259–267.

JAFFE, K. 1998. Sex, mate selection and evolution, pp. 483–492, *in* V. W. Porto, N. Saravanan, D. Waagen, and A.E. Eiben (eds.). Lecture Notes in Computer Science 1447: Evolutionary Programming VII, Springer-Verlag, Berlin.

JAFFE, K. 1999. On the adaptive value of some mate selection strategies. *Acta Biotheoret.* 47:29–40

KLUN, J. A., LIONHARDT, B. A., SCHWARZ, M., DAY, A., and RAINA, A. K. 1986. Female sex pheromone of the pickleworm *Diaphania nitidalis* (Lepidoptera: Pyralidae) *J. Chem. Ecol.* 12:239–249.

KOCOVSKÝ, P. and CERNÝ, V. 1978. Reduction of some mesyloxy and tosyloxy steroids with sodium iodide and zinc dust. *Collect. Czechoslov. Chem. Commun.* 44:246–250.

LINN, C. E. JR. and ROELOFS, W. L. 1995. Pheromone communication in moths and its role in the speciation process, pp. 263–300, *in* D. M. Lambert and H. G. Spencer (eds.). Speciation and the Recognition Concept. Johns Hopkins University Press, Baltimore, Maryland.

MILLAR, J., GIBLIN, M., BARTON, D., and UNDERHILL, E. 1992. Sex pheromone components of the geometrid moths *Lobophora nivigerata* and *Epirrhoe sperryi*. *J. Chem. Ecol.* 18:1057–1068.

MIRÁS, B., ISSA, S., and JAFFE, K. 1997. Diseño y evaluación de trampas cebadas con hembras vírgenes para la captura del perforador del fruto del tomate. *Agron. Trop.* 47:315–330.

RAINA, A. K., KLUN, J. A., SCHWARZ, M., DAY, A., and LEONHARDT, B. A. 1986. Female sex pheromone of the melonworm *Diaphania hyalinata* (Lepidoptera:Pyralidae), and analysis of male responses to pheromone in a flight tunnel. *J. Chem. Ecol.* 12:229–237.

REIS, P. R. and SOUZA, J. C. 1996. Controle da broca-pequena, *Neoleucinodes elegantalis* (Guenée) (Lepidoptera: Pyralidae), com inseticidas fisiológicos, em tomateiro estaqueado. *An. Soc. Entomol. Bras.* 25:65–70.

SPIEGEL, M. 1991. Estadística. Mc Graw-Hill, Madrid, 556 p.

TINBERGEN, N. and PERDECK, A. C. 1950. On the stimulus situation releasing the begging response in the newly hatched herring gull chick (*Larus a. argentatus* Pont). *Behaviour* 3:1–38.

TÓTH, M., SZÖCS, G., FRANCKE, W., SCHMIDT, F., PHILLIPP, P., LÖFSTEDT, C., HANSSON, B., and FARAG, A. 1994. Pheromonal production of and response to optically active epoxydienes in some geometrid moths (Lepidoptera: Geometridae). *Z. Naturforsch.* 49c:516–521.

WHITTLE, C., VICKERS, R., KUNIATA, L., BELLAS, T., and RUMBO, E. 1995. Identification of an attractant for the caneborer *Sesamia grisescens* Walker (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 21:1409–1420.

ZHU, Z., KONG, F., YU, S., YU, Y., JIN, S., HU, X., and XU, J. 1987. Identification of the sex pheromone of eggplant borer *Leucinodes orbonalis* Guenée (Lepidoptera: Pyralidae). *Z. Naturforsch.* 42c:1347–1348.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

MATING DISRUPTION OF *Plodia interpunctella* IN SMALL-SCALE PLOTS: EFFECTS OF PHEROMONE BLEND, EMISSION RATES, AND POPULATION DENSITY

CAMILLA RYNE,* GLENN P. SVENSSON, and CHRISTER LÖFSTEDT

Lund University
Department of Ecology
SE-223 62 Lund, Sweden

(Received December 19, 2000; accepted June 1, 2001)

Abstract—An indoor mating disruption experiment was performed on the stored-product pest *Plodia interpunctella*. The female of this species emits a four-component pheromone blend consisting of *Z*9,*E*12–14 : OAc, *Z*9,*E*12–14 : OH, *Z*9,*E*12–14 : Ald, and *Z*9–14 : OAc. Mating of *Plodia interpunctella* was disrupted up to 93% by using synthetic pheromone in small-scale plot experiments. The study was performed in 2.5 m × 2.5 m × 2.5 m polythene cubicles housed in a greenhouse, and pheromones were released by MSTRS spraying every 15 min. The disruption effect was tested at different doses 0.075, 0.75, and 3.75 mg/spray (corresponding to 5, 50, and 250 μg/min), different pheromone formulas (one-component (*Z*9,*E*12–14 : OAc) and four-component), and at different population densities (10, 20, and 30 individuals, equivalent to 0.32, 0.64, and 0.96 individuals/m^2). The moths were released into the cubicles and recaptured 24 hr later. The females were checked for spermatophore presence indicating successful mating. The mating was significantly suppressed in all treatments compared to the control. There was, however, no difference in mating activity between the one-component and four-component disruptants. In addition, EAG measurements were conducted with a portable device to keep track of aerial concentrations of pheromone. The results show that the one-component formula disrupts mating as efficiently as the more complete four-component blend at doses applied in this study. This fact improves the prospects for mating disruption of indoor pyralids, since many pyralid species share the major component in their pheromones, and, thus, can probably be controlled simultaneously by using this compound only.

Key Words—*Plodia interpunctella*, Lepidoptera, Pyralidae, stored-product, EAG, mating disruption, electroantennography.

*To whom correspondence should be addressed; e-mail: Camilla.Ryne@ekol.lu.se

0098-0331/01/1000-2109$19.50/0

INTRODUCTION

Male upwind flight in response to the female sex pheromone release in moths can be disrupted by emission of large quantities of synthetic pheromones. Mating disruption has been successfully used for population control in many cases. The Oriental fruit moth (*Grapholita molesta*) is controlled by means of mating disruption in France, Spain, Italy, China, and Brazil (Cardé and Minks, 1995). Pink bollworm (*Pectinophora gossypiella*) populations have been successfully reduced with the mating disruption technique in the United States (Staten et al., 1987). The success, however, varies depending on dispensers (Thorpe et al., 1999), pheromone formula (Evenden et al., 1999), number and type of dispensers (Sauer and Karg, 1998), and population dynamics of the target species (Cardé and Minks, 1995).

Several mechanisms have been suggested to be responsible for mating disruption in moths. Sensory fatigue can be caused either by adaptation of the peripheral receptors on the antennae or habituation at the central nervous level. It could also be that the synthetic pheromones camouflage the females' pheromone plumes and, thus, make it more difficult for the male to locate a calling female. Pheromone point sources may compete with pheromone plumes from calling females, thus creating a "false-trail following" by the males (Cardé and Minks, 1995).

One of the world's most important stored-product pest insects is the Indian meal moth [*Plodia interpunctella* (Hübner); Lepidoptera: Pyralidae]. The larvae feed on fruits, nuts, cereals, legumes, and other food products, causing large economic losses due to infestations (Phillips, 1994). *P. interpunctella* is a species that is highly resistant to insecticides, such as pyrethrine and malathion, which makes it a target for alternative control methods (Arthur et al., 1988). Insecticide resistance (Halliday and Zettler, 1988) and restrictions on the use of insecticides because of human health concerns have stimulated research on the applicability of pheromones and integrated pest management (IPM).

The sex pheromone emitted by female *P. interpunctella* was first identified as (*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12–14 : OAc) by Kuwahara et al. (1971b) and Brady et al. (1971). Additional pheromone components have since been identified. However, a recent reinvestigation of the pheromone and associated behavioral studies revealed that the female emits at least three additional components (Zhu et al., 1999, and references therein). The three components are (*Z*,*E*)-9,12-tetradecadienol (*Z*9,*E*12–14 : OH), (*Z*,*E*)-9,12-tetradecadienal (*Z*9,*E*12–14 : Ald), and (*Z*)-9-tetradecenyl acetate (*Z*9–14 : OAc). The four components were reported in a blend ratio of 100 : 7 : 16 : 6, respectively, and are all important for maximum male attraction and upwind flight (Zhu et al., 1999).

Stored-product pest insects are characterized by inhabiting indoor localities, and if they occur outside storage facilities, they are not considered pests due to the

lower population levels. Few studies have focused on the use of mating disruption for control of stored-product pests in indoor facilities, even though the conditions for mating disruption indoors have some advantages. Outdoors, migrating males attracted to an area with a higher pheromone release can decrease the success of mating disruption. Gravid females, who are mated outside a treated area, may be able to immigrate into the area to oviposit. The storage facilities create natural boundaries that limit these outdoor constraints, even though contaminated food products transported into storage facilities may act as boundaries, producing vectors for adults and larvae. However, if an already established mating disruption method together with other methods for control are available, an increase in population would be made difficult.

Early studies on pheromone-based control of stored-product pests were conducted almost exclusively under laboratory conditions, using boxes or cages (Brady and Daley, 1975). One study by Mafra-Neto and Baker (1996) concerns mating disruption of the almond moth [*Cadra (Ephestia) cautella*], in which they were able to disrupt mating by 100% in free flying moths for 24 hr and by 92% for 72 hr. The study was conducted in rooms of $3 \times 3 \times 3$ m. The disruption was independent of moth population densities and the use of one or two dispensers (Mafra-Neto and Baker, 1996).

The attraction of males is usually improved with the identification of additional pheromone components in moth species (Zhu et al., 1999). The mechanism of disruption and the efficiency in mating disruption with more complete blends are not yet fully understood. However, indications that more complex blends are not necessarily more efficient in mating disruption have been reported. Evenden et al. (1999) found that the use of a more complex blend did not yield a higher mating disruption in the oblique-banded leafroller (*Choristoneura rosaceana*).

By using mating disruption techniques similar to those that Mafra-Neto and Baker (1996) used to disrupt *E. cautella*, we wanted to see whether it was possible to disrupt the mating of free-flying *P. interpunctella* and if a higher mating disruption effect was obtained by using the recently identified Four-component blend compared to the major pheromone component (*Z*9,*E*12–14 : OAc) alone at different emission rates and population densities.

METHODS AND MATERIALS

Insects. P. interpunctella moths were taken from a culture maintained at the Department of Ecology, Lund University, Lund, Sweden. The culture originates from the Danish Pest Infestation Laboratory, Lyngby, Denmark. Additional moths have been incorporated from a laboratory culture provided by The Central Science Laboratory, Slough, England. Larvae were reared on an artificial diet consisting of

100 g wheat germ, 10 g brewer's yeast, and 20 g glycerol. Insects were separated by sex during the pupal stage (Zhu et al., 1999). Emerged males and females were kept in separate environmental chambers at 23°C and 60% relative humidity with a 17L : 7D photoperiod.

Chemicals. The synthetic compounds *Z*9,*E*12–14 : OAc, *Z*9,*E*12–14 : OH, and *Z*9–14 : OAc were purchased from Bedoukian Research Inc., Danbury, US, Connecticut. *Z*9,*E*12–14 : Ald was synthesized from *Z*9,*E*12–14 : OH (described in Zhu et al., 1999) at the Department of Organic Chemistry, Royal Institute of Technology, Stockholm, Sweden.

Two disruptants were used: *Z*9,*E*12–14 : OAc alone or in a Four-component blend consisting of *Z*9,*E*12–14 : OAc, *Z*9,*E*12–14 : OH, *Z*9,*E*12–14 : Ald, and *Z*9–14 : OAc in the proportions 100 : 7 : 16 : 6, as described in Zhu et al. (1999). Disruptants were diluted in 95% ethanol to correspond to emission rates of 5, 50, and 250 μg/min of the main component *Z*9,*E*12–14 : OAc.

Pheromone Releasing System. The synthetic pheromone was emitted from MSTRS dispensers set at spraying approximately 70 μl/spray every 15 min. all day, giving off 0.075, 0.750, or 3.75 mg/spray of the main component (*Z*9,*E*12–14 : OAc) depending on the experiment (Table 1). The pheromone was sprayed onto an acrylic pad stretched out on a needlepoint frame (15 cm InD) positioned 20 cm from the nozzle of the pheromone canister (Mafra-Neto and Baker, 1996). The pad, in turn, emitted the pheromone between sprays. The MSTRS advantages are controlled emissions, even in conditions with high temperature fluctuations, and that the canister is airtight, which will decrease the risk of oxidizing sensitive pheromone components such as aldehydes.

Experimental Sites. The experiments were conducted in transparent polythene cubicles, measuring 2.5 × 2.5 × 2.5 m, with zip-lock entries (The Central Science Laboratory, Slough, England). Cubicles were in a greenhouse at the Swedish Agricultural University in Alnarp, Sweden. Different treatments were conducted in separate rooms (6 × 3 m^2), two cubicles per room. Two net-covered holes (10 × 15 cm^2) were cut out from the walls of the cubicles, one at the bottom and one at the top of the opposite wall of the cubicle, in order to create a passive ventilation system. The control cubicles did not contain any pheromone dispensers, except when testing 0.75 and 3.75 mg/spray of the Four-component blend (trial 3), where we put a dispenser spraying only 95% ethanol every 15 min to see if mating activity was affected by the solvent. Different blends, emission rates, and densities of moths were used as described in Table 1. Due to the limited number of cubicles and rooms, we used the same cubicles for different pheromone treatments. However, we always went from lower to higher doses and from One-component to Four-component blends when reusing the same cubicles. A month passed between changing from the emission rate of 3.75 to 0.75 mg/spray of Four-component blend, during which the cubicles were thoroughly aired out (between trials 3 and 4, Table 1).

TABLE 1. ORDER OF TREATMENTS AND ANIMAL DENSITIES[a]

	Treatment A			Treatment B		
Trial	Disruptant	Emission rate (mg/spray)	Density (males + females)	Disruptant	Emission rate (mg/spray)	Density (males + females)
1	1-component	0.075	10 + 10	4-component	0.075	10 + 10
2	1-component	0.75	10 + 10	1-component	3.75	10 + 10
3	4-component	0.75	10 + 10	4-component	3.75	10 + 10
4	1-component	0.75	5 + 5	1-component	0.75	15 + 15
5	4-component	0.75	5 + 5	4-component	0.75	15 + 15

[a] Treatments and control consisted of two cubicles each. Doses refer to amount of *Z*9,*E*12–14 : OAc in both one-component and four-component blends. The controls contained no synthetic pheromone. The control cubicles in trial 3 contained a MSTRS spraying only the solvent (ethanol).

Experimental Design. One MSTRS device was placed in the middle of each treatment cubicle on a stand approximately 1 m high and was set to spray synthetic pheromone at least 24 hr before introducing insects. Male and female *P. interpunctella* were randomly picked from the laboratory culture at 2–4 days old. The females and males were held separately until introduced into the cubicles. The animals were left in the cubicles for 24 hr, and they were then picked from the walls and put in individual vials. New males and females were then released, and the procedure was repeated for five or six consecutive days. The recaptured animals were immediately brought to the laboratory, where they were frozen, and the females were later dissected under a microscope to determine the presence of spermatophore, indicating mating (Mafra-Neto and Baker, 1996). The disruption effect was calculated as: [(mating frequency$_{\text{control}}$ − mating frequency$_{\text{treatment}}$)/mating frequency$_{\text{control}}$] × 100 Three different population densities were tested—10, 20, and 30 individuals in total—with males and females in equal numbers.

EAG Recordings. A portable EAG device was provided by Syntech/VDP Laboratories, Hilversum, The Netherlands. We used the apparatus to monitor pheromone accumulation and relative doses in different treatment cubicles as well as for checking that the control cubicles were not contaminated. A freshly excised antenna from a 0-to 1-day-old male was mounted in the airtight holder and the two ends of the antenna covered with electrically conductive gel (Spectra 360, Parker Laboratories, Orange, New York), making a bridge over the central hole. A rubber septum acting as a reference stimulus was treated with 10 μg of *Z*9,*E*12–14 : OAc. A continuous flow (0.5 m/sec) of air, cleaned through an activated charcoal filter, maintains the nonstimulated condition of the antenna, forming the baseline. Each recording consisted of reference recording (R_1) (0.3 sec), ambient air recording (A) (0.3 sec), and reference recording (R_2) (0.3 sec) with 5-sec intervals. Relative EAG responses were calculated as: $\{(A/[(R_1 + R_2)/2])\} \times 100$.

For detailed information about the EAG device, see Van der Pers and Minks (1998). The EAG responses were continuously recorded as a frequency modulated signal on a portable audio tape recorder (SONY TDC-D7) and later stored and analyzed on a PC equipped with an IDAC card and the program EAG version 2.2a (both from Syntech, Hilversum, The Netherlands).

All insects were removed from the cubicles prior to recordings. Eight recordings were made for each treatment (four in each cubicle), starting in control cubicles and moving into cubicles with higher concentrations or from one-component to four-component treatments. A time period between two recordings was at least 30 sec to let the antenna recover from adaptation, and the time to move from one cubicle to another was approximately 1–2 min . Since the four measurements in one cubicle are pseudoreplicates, only the mean value of the recordings was calculated and used. Measurements were conducted at the beginning and at the end in 0.75 mg/spray of one-component and 0.75 mg/spray of four-component treatments (trials 2 and 3).

Statistical Analysis. The proportional difference in mating frequency among treatments as well as the difference in disruption effects was analyzed with ANOVA, on arcsin $\sqrt{x}$-transformed data.

RESULTS

Blend and Emission Rate Effects. No statistical differences in mating disruption effect could be observed between the one-component and four-component disruptants, in either 0.075, 0.750, or 3.75 mg/spray (Figure 1). The single component in the 0.075 mg/spray suppressed mating by 80 ± 3%, and the four-component blend disrupted the mating by 78 ± 6% (Figure 1). The mating frequency was lower in both treatments compared to the control (ANOVA; $F_{2,32} = 113.69$; $P < 0.001$). Tukey's post-hoc test showed a significant effect between treatments and control in mating frequency ($P < 0.001$), but a non-significant effect among the treatments. The mating frequencies in the different treatments were: 79 ± 3% (control), 16 ± 3% (one-component), 17 ± 4% (four-components).

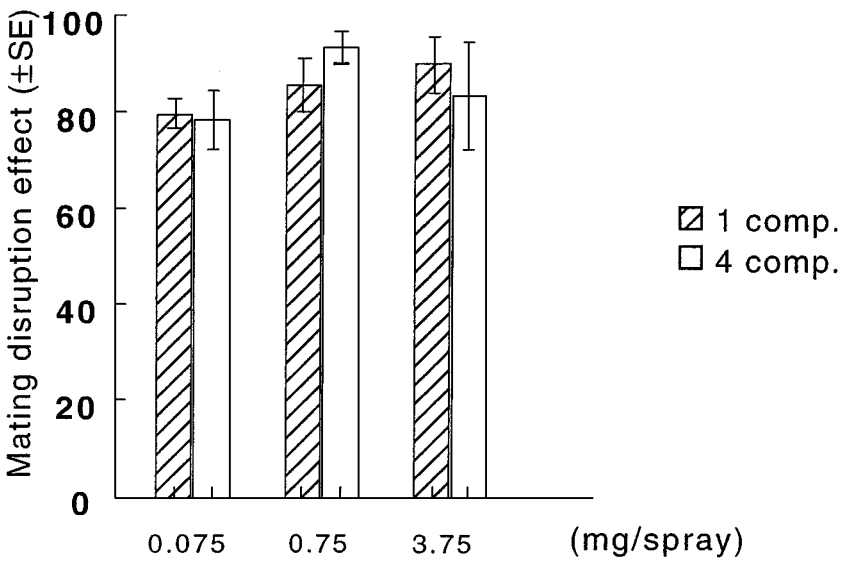


FIG. 1. Mating disruption effect (mean ± SE) in an environment treated with 0.075, 0.75, and 3.75 mg/spray of *Z*9,*E*12–14 : OAc ($N = 10$) and of a four-component blend at the same emission rate of *Z*9,*E*12–14 : OAc ($N = 10$). All females were checked for spermatophore presence, indicating mating. No statistically significant difference between the treatments (ANOVA $F_{5,56} = 1.379$; $P > 0.05$).

There were no differences in mating frequency in the controls among the trials where we had a MSTRS device spraying only ethanol and no MSTRS at all. Hence, the controls were pooled in the statistical tests. The sprayer was removed from the control in later trials. By increasing the emission rate from 0.075 mg/spray to 0.75 mg/spray, the mating was suppressed by $88 \pm 5\%$ (one-component) and $93 \pm 3\%$ (four-component), and the mating frequency was different from the control (ANOVA; $F_{4,54} = 38.72$; $P < 0.001$). Tukey's *post-hoc* test showed a significant effect on mating frequency between control and treatments ($P < 0.01$) and a non-significant effect among treatments. The mating disruption effect did not differ among treatments (ANOVA; $F_{5,56} = 1.379$; $P > 0.05$) (Figure 1). By increasing the dose to 3.75 mg/spray, we did not achieve higher disruption compared to the 0.75 mg/spray, rather disruption stayed at the same level ($93 \pm 5\%$ (one component) and $89 \pm 11\%$ (four components)) as for the 0.75 mg/spray (Figure 1). The mating frequencies in the treatments were $58 \pm 7\%$ (control), $7 \pm 3\%$ (0.75 mg/spray of one component), $4 \pm 2\%$ (3.75 mg/spray of one component), and $47 \pm 6\%$ (control), 3 ± 2 % (0.75 mg/spray of four components) and $5 \pm 2\%$ (3.75 mg/spray of four-components).

Density Effects. Population densities were tested at the emission rate of 0.75 mg/spray of one- and four-component blends, and five females and five males (0.32 individuals/m^2), or 15 females and 15 males (0.96 individuals/m^2) were released into the cubicles. The treatments were significantly different from the control considering mating frequency (10 individuals; ANOVA; $F_{2,27} = 16.869$; $P < 0.001$, 30 individuals; ANOVA; $F_{2,27} = 36.257$; $P < 0.001$). Tukey's post-hoc test showed no difference in mating frequencies between one-component and four-component treatments in both density trials and a significant effect between control and treatments ($P < 0.001$) (Figure 2). At a population density of 10 individuals, the mating was disrupted by $58 \pm 14\%$ in one-component- and by $84 \pm 10\%$ in four-component-treated a cubicle. At a density of 30 individuals, mating was suppressed by $67 \pm 11\%$ in one-component- and $78 \pm 4\%$ in four-component-treated cubicles. The mating frequencies in the different population densities were for 10 individuals: $54 \pm 9\%$ (control), $14 \pm 5\%$ (one component), $4 \pm 3\%$ (four components), and for 30 individuals: 57 ± 5 (control), $16 \pm 4\%$(one component), $12 \pm 2\%$ (four components).

EAG Recording. In spite of the passive ventilation, EAG recordings showed an accumulation of the disruptants in the treatment cubicles during the experiments. No severe contamination of pheromones in the control cubicles could be observed from the EAG recordings (Figure 3). Antennal responses showed a high level of adaptation in the high doses. The adaptation could be observed since the responses to the reference decreased markedly and were unstable in different measurements within a treatment cubicle (example in Figure 3b). The decrease in reference responses, combined with a maximal response to ambient air, increased the relative EAG values from 343 to 669 in 0.75 mg/spray trials. This effect was even larger in

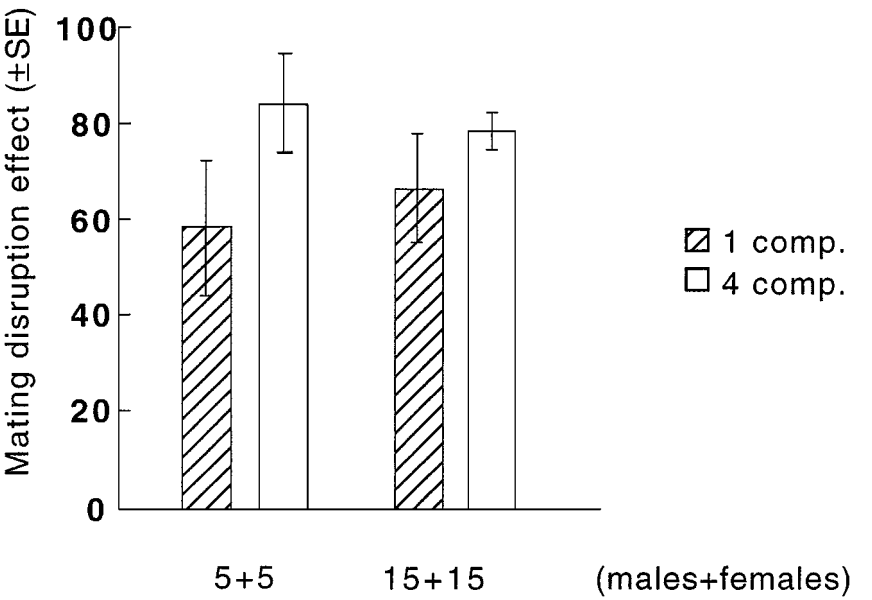


FIG. 2. Disruption effects (mean ± SE) in different population densities in an environment of 0.75 mg/spray of *Z*9,*E*12–14 : OAc and of a four-component blend at the same emission rate of *Z*9,*E*12–14 : OAc. (a) Five males and five females were released each day and retrieved 24 hr later ($N = 10$). (b) Fifteen males and 15 females were released and recaptured 24 hr later ($N = 10$). All females were checked for spermatophores under microscope. No significant difference between the treatments and densities (ANOVA $F_{3,34} = 0.656$; $P > 0.05$).

higher doses, which resulted in unreliable EAG responses and are, therefore, not shown.

DISCUSSION

In this study, mating was disrupted up to 93% by using high emission rates of synthetic pheromones. The disruption effect was measured by the presence of spermatophores in females' bursa copulatrix, which is the most relevant measure of mating activity. Counting spermatophores in the field, however, can be a misleading measurement due to underestimation (Drummond, 1984). The male can fail to pass a spermatophore and still have a successful mating, which was reported in multiply mated males of *Grapholita molesta* (George and Howard, 1968). An underestimation is also possible due to deterioration of the spermatophore if the

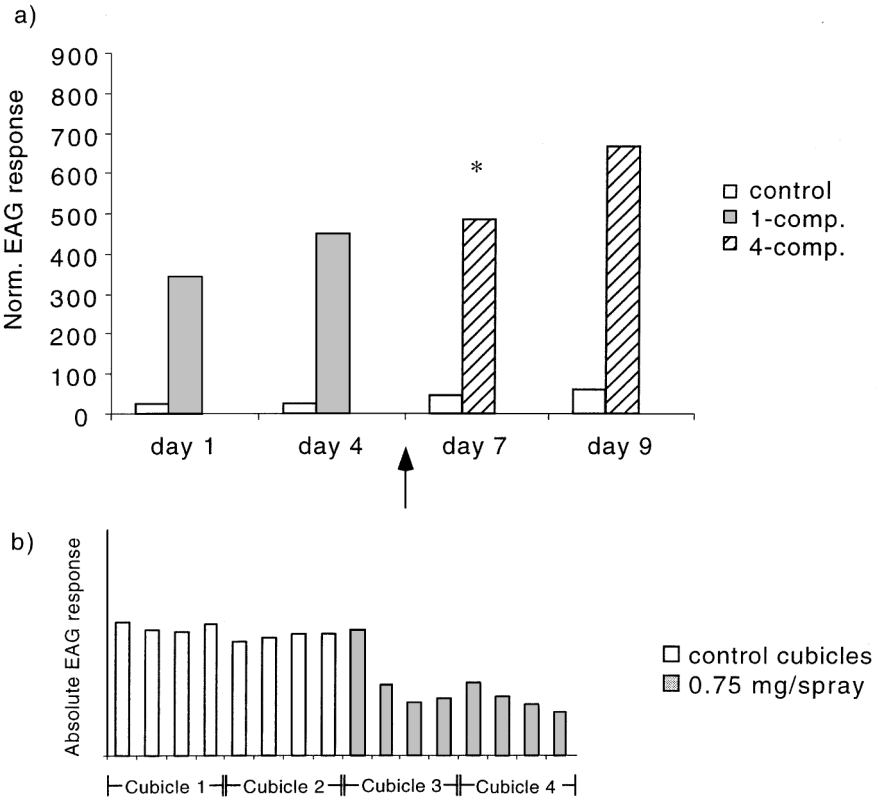


FIG. 3. (a) Pheromone accumulation recorded as electroantennographic responses (mean of four recordings in the same cubicle and the mean of two cubicles for each recording period) of *Plodia interpunctella* conducted on a portable EAG device inside plastic cubicles. The graph shows the sampling sequence, at the beginning and end of each treatment. The asterisk indicates where the MSTRS was spraying only the solvent (ethanol) in the control cubicles. The arrow shows when the treatment was changed from one-component to four-component mixtures. Responses are normalized to a response to a reference containing 10 μg of *Z*9,*E*12–14 : OAc. (b) A typical response to the reference, indicating that the antenna is adapted by the high pheromone concentrations. Responses are stable and of equal size through out the test (four tests in four cubicles; 16 bars). The measurements were taken with a 30-sec recovery time within each cubicle. Moving between the cubicles created a time lapse of 2–3 min in which the antenna can be seen to recover. The grey bars are responses to the reference inside cubicles with 0.75 mg/spray of *Z*9,*E*12–14 : OAc. The white bars are responses to the reference inside control cubicles containing no pheromone dispensers. Bars are decreasing in size for each antennal response, indicating adaptation.

time between the mating and the dissection is too long (Drummond, 1984). However, in this test, an underestimation is not likely since male *P. interpunctella* pass a large spermatophore in all first matings, and during the 24 hr, the males mate only once (Ryne et al., unpublished results). The time between collection and freezing of the females did not exceed 2 hr, and all the spermatophores observed were large in size. Other methods to measure mating activity are by tethered females (e.g., Evenden et al., 1999), by sticky traps with pheromone dispensers (e.g., McBrien et al., 1998), or by crop damage estimations (e.g., Mazomenos et al., 1999). These data are easier to collect, but they do not show the actual mating activity. The risk of the tethered female being disturbed by the handling, and thus ceasing to call is high, and the probability of a tethered female being mated is higher than that of a free-flying female. The efficacy of a pheromone dispenser is decreased by other calling females in the vicinity, which will also attract males.

In the study by Mafra-Neto and Baker (1996), the mating of *E. cautella* was suppressed by 100% in the low population densities (1.27 moths/m^2) with one MSTRS emitting 50 μg/spray of the major component every 15 min. The mating activity in that study was also measured by the presence of spermatophores in females. The higher mating disruption level obtained, even though higher dosages of pheromone was used in our study, was most probably due to the fact that they had constant darkness during the testing period (T. C. Baker, personal communication). Since our cubicles were placed in a greenhouse, a normal day and night rhythm was obtained, thus enabling the moths to use vision for mate finding. The difference in mating suppression between these two closely related species, using similar pheromone disruption conditions, suggests that these moths are using additional cues other than long-range sex pheromones and odor perception for tracking and finding mates. It also suggests that higher doses of pheromone do not necessarily produce a higher mating disruption effect. Other studies have shown that pyralid males [*P. interpunctella* (Mankin et al., 1999) and *E. cautella* (Mankin and Hagstrum, 1995)] perceive and orient toward pheromones at 40–50 cm from the source. At closer distances, males and females can recognize each other by visual and/or acoustic cues. Trematerra and Pavan (1995) showed that female *P. interpunctella*, *E. kuehniella*, and *E. cautella* use ultrasound produced by the male's wing-fanning in order to identify the courting male. The exact utilization of the visual cues by the moths has never been investigated, but it has been shown that male and female *P. interpunctella* orient toward figures of definite shapes, indicating that vision is used in finding sites for settling, calling, and mating (Levinson and Hoppe, 1983).

The moths were often observed aggregating in corners in the cubicles. Females were observed calling in treatment cubicles, with other males and females in close vicinity, without being mated. Aggregation to specific areas in the environment may increase an individual's chance of being mated. This behavior may even occur without the aid of pheromone cues. The cubicles in this study consisted

of straight walls, ceilings, and floors, and the only object in the tents was the MSTRS, standing in the middle of the cubicle. Thus, the environment in which the moths were placed was very simple. As the complexity of the room increases, the number of possible aggregation spots increases. The number of moths in the different possible aggregation spots will be fewer, and the chance of finding a mate in one of those spots will decrease, thus increasing the mating disruption effect. If the behavior of aggregating in corners and crevices enhances the chance of mate finding, then the result of a 93% mating disruption effect, as was the case with 0.75 mg/spray of the four-component blend (Figure 1), is an exceptionally good result, considering the non-complex environment.

Population densities of Phycitinae are sometimes reported as number of insects per square meter of ceiling and walls, since mating behavior occurs on those surfaces (Sower and Whitmer, 1977; Hodges et al., 1984; Mafra-Neto and Baker, 1996). For simplicity, we chose to refer to the number of animals as the true number, but for easier comparison with other studies (e.g., Mafra-Neto and Baker, 1996), the densities are 0.32, 0.64, and 0.96 individuals/m^2. The population densities used in this experiment are referred to as low, intermediate, and high. However, we are not positive that the population densities we chose are relevant in true indoor conditions with infestations. Mafra-Neto and Baker (1996) performed their tests with twice as many males as females, while we used equal numbers of males and females in the cubicles. This was partly due to the fact that a female-biased sex ratio tends to increase male mating, i.e., males increase their mating activity and mate more frequently when the number of available females increases (Brower, 1975). This means that in our study, moths had a higher possibility of mating because of the equal sex ratio compared to a 2 : 1 male–female sex ratio.

The most commonly suggested mechanisms behind mating disruption are either local adaptation of the antennal receptors, habituation on a central level, false trail following, or camouflage of the female's pheromone plume (Cardé and Minks, 1995). High levels of antennal adaptation were clearly observed in the EAG traces (Figure 3), and the adaptation was as high for the one-component as for the four-component treatment. It is difficult to draw any conclusions from the EAG measurements since the high concentrations of disruptant in the cubicles made the antennal responses to the reference septum lower and more unstable than in control cubicles. The portable EAG device would be more useful in conditions where the pheromone concentrations are much lower than in our treatments. While measuring antennal responses in the cubicles, there was a trade-off between minimizing the time between recording due to the short life-time of the antenna and maximizing the time to enable the antenna to fully recover. In order to retrieve statistically sound data, a series of recordings from many antennae is necessary.

The minor increase observed in EAG responses in control cubicles between the beginning of the trial period and the end (Figure 3a) can be explained by pheromones still present in the cubicles from earlier, removed calling females, as

well as contamination from one room to thr other. The larger increase of pheromone concentration between the beginning and the end of the trial period in the one-component trial (Figure 3a) is most probably due to an accumulation of pheromone during the trial period. Pheromone is likely to be absorbed into the plastic of the cubicles, which would then act as secondary dispensers and create a stable background pheromone concentration. It is not known whether the EAG responses by the antenna were derived from pheromones only or whether the antenna also detected the ethanol solvent. The large increase in the EAG responses are probably deriving from the pheromones present in the tents and not the ethanol solvent. Figure 3a shows normalized EAG responses when the control tents had a MSTRS spraying only ethanol, and no elevated responses can be seen. Cossé et al. (1994) reported receptors sensitive to ethanol in females of the pyralid *Ectomyelois ceratoniae*, but whether or not ethanol-sensitive receptors occur in male *P. interpunctella* has not been investigated.

Only a few males were seen orientating towards, or sitting on, the pads of the MSTRS, which indicates a low level of false trail following in both laboratory and field studies (Valeur and Löstedt, 1996; McBrien et al., 1998). The mechanisms by which males are disrupted may depend on the concentration level of the disruptant and the sensitivity of the male receptors. The importance of using a more complete blend in a specific dose is of interest for commercial purposes, and a more complete blend is often more attractive to a male than an off-blend (Zhu et al., 1999). Cardé and Minks (1995) suggested that a complete blend would by definition be the most effective in mating disruption since it evokes the whole behavioral repertoire and, therefore, the complete blend would possess the lowest threshold for most or all behaviors. On the other hand, an off-blend might, evoke a sensory imbalance effect (Cardé and Minks, 1995). Flint and Merkle (1983) showed that by disrupting pink bollworm (*P. gossypiella*) with an off-blend or a partial blend, the male moths experienced an imbalance in sensory input, which impaired the male's ability to respond correctly to the natural blend.

A small-scale experiment of this kind is valuable since it allows for control plots, which are almost impossible to achieve in field experiments. However, the true efficiency of mating disruption can only be seen when conducting the experiments in storage facilities. Since our experiments resulted in a 93% mating disruption in 24 hr, it is likely that the population would decrease. The emission rates in this experiment are comparable to the study by Mafra-Neto and Baker (1996). It can be argued that the emission rates were too high in the small experimental sites. The high concentration may be the reason for not achieving a difference in disruption between the one-component and four-component trials [a female gland contains approximately 4 ng of the main component (Zhu et al., 1999)]. However, we chose to concentrate on the effect of blends in a situation where the mating disruption was efficient, rather than to investigate the impact of blends when disruption effect was low and not acceptable for IPM.

Pheromone-based mating disruption conditions may produce a high selection pressure on the communication channel in the target species in which more sensitive males and/or females producing off-blends are favored. If large variation exists in pheromone production among females, individuals emitting large quantities or blends not similar to the disruption blend may be favored. Moreover, selection could be acting on the males who are able to locate a female in high ambient pheromone concentrations. A project is now in progress to evaluate the evolutionary consequences of the selection pressures imposed on the pheromone-based mating-system of *P. interpunctella* in high atmospheric concentrations of synthetic pheromones. The implementation of mating disruption indoors, and to some extent mating disruption outdoors, may be prevented by the fact that toxicity tests are needed before using pheromones in "high" doses in close contact to personnel and food products.

The potential for indoor mating disruption of *P. interpunctella* is promising. Our study shows that it is possible to achieve a high degree of disruption. The fact that the one-component (*Z*9,*E*12–14 : OAc) formula is used as part of the pheromone blend in many pyralid species (Phelan and Baker, 1990) makes the applicability of mating disruption with the one-component formula even greater. The compound is shared by *E. kuehniella* (Kuwahara et al., 1971a), *Ephestia (Cadra) cautella* (Kuwahara et al., 1971b; Brady, 1971), *P. interpunctella*, and other pyralid species, and since the species can occur in the same habitat, it may on these occasions be possible to control the pests with only one pheromone formula.

Acknowledgments—We would like to thank Dr. Rikard C. Unelius and Ellen Mattos Santangelo, Sweden, for synthesizing the aldehyde used in the experiments. Prof. Jan Löfquist, Sweden, for letting us use the greenhouses for the cubicles, Dr. Jan van der Pers, for lending us the portable EAG device, and Dr. P. A. Nilsson for valuable discussions on earlier versions of the manuscript. This study was funded by Cerealia Research and Development, MISTRA, the Swedish Council for Forestry and Agricultural Research, and the Knut and Alice Wallenberg foundation.

REFERENCES

ARTHUR, F., ZETTLER, J. L., and HALLIDAY, W. R. 1988. Insecticide resistance among populations of Almond moth and Indian meal moth (Lepidoptera: Pyralidae) in stored peanuts. *J. Econ. Entomol*. 81:1283–1287.

BRADY, U. E. and DALEY, R. C. 1975. Mating activity of *Cadra cautella* during exposure to synthetic sex pheromone and related compounds in the laboratory. *Environ. Entomol*. 4:445–447.

BRADY, U. E., TUMLINSON, J. H., BROWNLEE, R. G., and SILVERSTEIN, R. M. 1971. Sex stimulant and attractant in the Indian meal moth and almond moth. *Science* 171:802–804.

BROWER, J. H. 1975. *Plodia interpunctella*: Effect of sex ratio on reproductivity. *Ann. Entomol. Soc. Am*. 68:847–851.

CARDÉ, R. T. and MINKS, A. K. 1995. Control of moth pests by mating disruption: successes and constraints. *Annu. Rev. Entomol*. 40:559–585.

COSSÉ, A. A., ENDRIS, J. J., MILLAR, J. G., and BAKER, T. C. 1994. Identification of volatile compounds from fungus-infested date fruit that stimulate upwind flight in female *Ectomyelois ceratoniae*. *Entomol. Exp. Appl*. 72:233–238.

DRUMMOND, B. A. 1984. Multiple mating and sperm competition in the Lepidoptera, pp. 291–370, *in* R. L. Smith (ed.). Sperm Competition. Academic Press, London.

EVENDEN, M. L., JUDD, G. J. R., and BORDEN, J. H. 1999. Pheromone-mediated disruption of *Choristoneura rosaceana*: Is the most attractive blend really the most attractive? *Entomol. Exp Appl*. 90:37–47.

FLINT, H. M. and MERKLE, J. R. 1983. Pink bollworm (Lepidoptera: Gelechiidae): communication disruption by pheromone composition imbalance. *J. Econ. Entomol*. 76:40–46.

GEORGE, J. A. and HOWARD, M. G. 1968. Insemination without spermatophores in the Oriental fruit moth, *Grapholita molesta* (Lepidoptera, Tortricidae). *Can. Entomol*. 100:190–192.

HALLIDAY, A. F. and ZETTLER, J. L. 1988. Insecticide resistance among populations of almond moth and Indian meal moth (Lepidoptera: Pyralidae) in stored peanuts. *J. Econ. Entomol*. 81: 1283–1287.

HODGES, R. J., BENTON, F. P., HALL, D. R., and SANTOS SERODIO, R. D. 1984. Control of *Ephestia cautella* (Walker) (Lepidoptera: Phycitidae) by synthetic sex pheromones in the laboratory and store. *J. Stored Prod. Res*. 20:191–197.

KUWAHARA, Y., HARA, H., ISHII, S., and FUKAMI, H. 1971a. The sex pheromone of the Mediterranean meal moth. *Agric. Biol. Chem*. 35:447–448.

KUWAHARA, Y., KITAMURA, C., TAKAHASHI, S., HARA, H., ISHII, S., and FUKAMI, H. 1971b. Sex pheromone of the almond moth and the Indian meal moth: *cis*-9, *trans*- 12-tetradecadienyl acetate. *Science* 171:801–802.

LEVINSON, H. Z. and HOPPE, T. 1983. Preferential flight of *Plodia interpunctella* and *Cadra cautella* (Phycitinae) toward definite shape and position with notes on the interaction between optical and pheromone stimuli. *Z. Angew. Entomol*. 96:491–500.

MAFRA-NETO, A. and BAKER, T. C. 1996. Timed, metered sprays of pheromone disrupt mating of *Cadra cautella* (Lepidoptera: Pyralidae). *J. Agric. Entomol*. 13:149–168.

MANKIN, R. W. and HAGSTRUM, D. W. 1995. Three-dimensional orientation of male *Cadra cautella* (Lepidoptera: Pyralidae) flying to calling females in a windless environment. *Environm. Entomol*. 24:1616–1626.

MANKIN, R. W., ARBOGAST, R. T., KENDRA, P. E., and WEAVER, D. K. 1999. Active spaces of pheromone traps for *Plodia interpunctella* (Lepidoptera: Pyralidae) in enclosed environments. *Environ. Entomol*. 28:557–565.

MAZOMENOS, B. E., ORTIZ, A., MAZOMENOS-PANTAZI, A., STEFANOU, D., STAVRAKIS, N., KARAPATI, C., and FOUNTOULAKIS, M. 1999. Mating disruption for the control of the olive moth, *Prays oleae* (Bern) (Lep., Yponomeutidae) with the major sex pheromone component. *J. Appl. Entomol*. 4:247–254.

MCBRIEN, H. L., JUDD, G. J. R., and BORDEN, J. H. 1998. Development of pheromone-based mating disruption for control of the eye-spotted bud moth, *Spilonota ocellana. Entomol. Exp. Appl*. 2:101–107.

PHELAN, P. L. and BAKER, T. C. 1990. Information transmission during intra- and interspecific courtship in *Ephestia elutella* and *Cadra figuliella. J. Insect. Behav*. 5:589–602.

PHILLIPS, T. W. 1994. Pheromones of stored-product insects: current status and future prospectives, pp. 479–486, *in* E. Highly, J. E Wright, H. J. Banks, B. R. Champ. (eds.). Proceedings of the 6th international Working Conference on Stored-Product Research, Canberra, Australia.

SAUER, A. E. and KARG, G. 1998. Variables affecting pheromone concentration in vineyards treated for mating disruption of grape vine moth *Lobesia botrana. J. Chem. Ecol*. 24:289–302.

SOWER, L. L. and WHITMER, G. P. 1977. Population growth and mating success of Indian meal moths and almond moths in the presence of synthetic pheromone. *Environ. Entomol*. 6:17–20.

STATEN, R. T., FLINT, H. M., WEDDLE, R. C., HERNANDEZ, E., and YAMAMOTO, A. 1987. Pink boll worm (Lepidoptera: Gelechiidae): Large-scale field trials with a high-rate gossyplure formulation. *J. Econ. Entomol*. 80:1267–1271.

THORPE, K. W., MASTRO, V. C., LEONARD, D. S., LEONHARDT, B. A., MCLANE, W., REARDON, R. C., and TALLEY, S. E. 1999. Comparative efficiancy of two controlled-release gypsy moth mating disruption formulations. *Entomol. Exp. Appl.* 90:267–277.

TREMATERRA, P. and PAVAN, G. 1995. Ultrasound production in the courtship behaviour of *Ephestia cautella* (Walk.), *E. kuehniella* Z. and *Plodia interpunctella* (Hb.) (Lepidoptera: Pyralidae). *J. Stored Prod. Res.* 31:43–48.

VALEUR, P. G. and LÖFSTEDT, C. 1996. Behaviour of male oriental fruit moth, *Grapholita molesta*, in overlapping sex pheromone plumes in a wind tunnel. *Entomol. Exp. Appl*. 79:51–59.

VAN DER PERS, J. N. C., and MINKS, A. K. 1998. A portable electroantennogram sensor for routine measurements of pheromone concentrations in greenhouses. *Entomol. Exp. Appl*. 87:209–215.

ZHU, J. W., RYNE, C., UNELIUS, C. R., VALEUR, P. G., and LÖFSTEDT, C. 1999. Reidentification of the females sex pheromone of the Indian meal moth, *Plodia interpuctella*: Evidence for a four-component pheromone blend. *Entomol. Exp Appl*. 92:137–146.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

EPICUTICULAR HYDROCARBON VARIATION IN *Drosophila mojavensis* CLUSTER SPECIES[1]

WILLIAM J. ETGES[2,*] and LARRY L. JACKSON[3]

[2]*Department of Biological Sciences*
University of Arkansas
Fayetteville, Arkansas 72701

[3]*Chemistry and Biochemistry Department*
Montana State University
Bozeman, Montana 59717

(Received January 1, 2001; accepted June 4, 2001)

Abstract—Epicuticular hydrocarbon variation was investigated among the three species of the *Drosophila mojavensis* cluster (*D. mojavensis*, *D. arizonae*, and *D. navojoa*) within the large *D. repleta* group. Because these hydrocarbons serve as contact pheromones in adult *D. mojavensis*, the chemical characteristics and differences in hydrocarbon profiles in populations of these three sibling species were further investigated. Twenty-seven hydrocarbon components with chain lengths ranging from C_{28} to C_{40}, including *n*-alkanes, methyl-branched alkanes, *n*-alkenes, methyl-branched alkenes, and alkadienes were observed. Hydrocarbon profiles among the three species reared on different cactus hosts were easily aligned with previously identified components in *D. mojavensis*. Male and female *D. navojoa* possessed a 31-methyldotricont-6-ene absent in both *D. arizonae* and *D. mojavensis*, while lacking the 8,24-tritricontadiene present in these two species. *D. navojoa* adults had far less 2-methyloctacosane than these sibling species, but the significance of this difference was obscured by the degree of variation among populations in amounts of this hydrocarbon. Mainland and Baja California populations of *D. mojavensis* were fixed for differences in the amounts 8,24-tritricontadiene, 9,25-pentatricontadiene, and 9,27-heptatricontadiene, consistent with all previous studies. Amounts of 18 of the 27 hydrocarbon components were greater in flies reared on *Opuntia* cactus. Canonical discriminant function analysis resolved all three species into distinct, nonoverlapping groups, suggesting that epicuticular hydrocarbon profiles are species-specific in the *D. mojavensis* cluster. Based on the amounts of interpopulation variation in hydrocarbon profiles in these three species, we hypothesize

[1]This is paper VI in the series "Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*."

*To whom correspondence should be addressed; e-mail: wetges@comp.uark.edu

0098-0331/01/1000-2125$19.50/0

that epicuticular hydrocarbon differences may evolve early during the formation of new species.

Key Words—Species recognition, cuticular hydrocarbons, *Drosophila*, cactus, speciation.

INTRODUCTION

In closely related animal species that no longer share a common fertilization system, species recognition systems may be preserved by strong stabilizing selection (Paterson, 1993) and perhaps enhanced by reinforcement of these barriers if there is continuing gene exchange (Dobzhansky, 1951; Coyne and Orr, 1989; Noor, 1995, 1999). In order to understand more fully the origin of new species, the conditions that cause particular isolating mechanisms to arise must be identified (Masters, 2000), and the progression of changes in courtship cues during the evolution of reproductive isolation across species groups must be understood. Mate recognition in many *Drosophila* species involves a stereotyped series of behavioral cues exchanged between males and females. Courtship is elicited by males and may involve behavioral, acoustic, and chemical cues that females use in the evaluation of prospective mates. In the initial stages of reproductive isolation, how does selection shape divergence in mating systems prior to complete isolation? Do mate recognition systems function within populations in the same ways that they function between populations and species? If sexual selection is unrelated to sexual isolation (Boake et al., 1997; Carson, 2000) and if we are to understand how species originate in general, comparative studies of within- and between-species mating systems may offer insight into the sequential evolution of recognition signals.

A common form of chemical communication in *Drosophila* species involves contact pheromones made of epicuticular hydrocarbons biosynthesized in the pupal stage and early in adult life (Ferveur et al., 1997). Specific components of cuticular hydrocarbons of females elicit male courtship behaviors in *Drosophila melanogaster* (Antony and Jallon, 1982). (*Z*,*Z*)-7,11-Heptacosadiene elicits courtship from *D. melanogaster* males (Antony and Jallon, 1982). (*Z*)-11-Pentacosene (Oguma et al., 1992a) along with (*Z*,*Z*)-5,13-pentacosadiene, (*Z*,*Z*)-5,15-pentacosadiene, and (*Z*,*Z*)-7,15-heptacosadiene elicits courtship from *D. virilis* males (Oguma et al., 1992b). The multimethylene interrupted alkadiene, (*Z*,*Z*)-5,25-hentricontadiene, elicited courtship from male *D. pallidosa* (Nemoto et al., 1994). All of these hydrocarbons have long chains and are thought to act as contact pheromones, probably by stimulating chemoreceptors on the male foreleg or proboscis (Jallon, 1984; Oguma et al., 1992b; Nemoto et al., 1994).

In addition to the exchange of behavioral cues during courtship including courtship songs (Spieth, 1974; Ewing and Miyan, 1986), epicuticular hydrocarbons have been implicated as determinants of mate choice in *D. mojavensis* (Markow

and Toolson, 1990; Toolson et al., 1990; Stennett and Etges, 1997). Hydrocarbon transfer experiments (cf. Coyne et al., 1994) have demonstrated that epicuticular hydrocarbons are involved in mate recognition with mainland and Baja *D. mojavensis* (Etges and Ahrens, 2001). Previous analysis of the epicuticular hydrocarbon profiles of *D. arizonae* and *D. mojavensis* revealed a high degree of similarity between species, some large differences in particular hydrocarbon components among populations of *D. mojavensis*, and significant effects of larval rearing substrates, particularly laboratory food versus cactus, on amounts of epicuticular hydrocarbons (Stennett and Etges, 1997). Detailed analysis of multiple populations of *D. mojavensis* from different parts of the species range revealed consistent geographical differentiation in amounts of C_{33}, C_{35}, and C_{37} alkadiene components, and gender–specific amounts of 16 different hydrocarbons. Further, significant sex $\times$ region interactions for eight of these hydrocarbons showing sexual dimorphism were statistically significant, indicating region-specific male–female hydrocarbon differences (Etges and Ahrens, 2001). In the present study, we characterized the chemical nature of these epicuticular hydrocarbons for all three *D. mojavensis* cluster species so that we can begin to understand the role of these chemical cues in between-species mate recognition.

Phylogeny and Natural History of D. mojavensis *Cluster Species*. The *D. mojavensis* cluster is part of the *D. mulleri* species subgroup inferred from the sharing of chromosomal gene arrangements and phylogenetic analyses of nuclear and mitochondrial gene regions (Wasserman, 1992; Durando et al., 2000). The *mulleri* cluster (*D. aldrichi*, *D. mulleri*, *D. wheeleri*, and *D. nigrodumosa*) is the sister group to the *D. mojavensis* cluster (Durando et al., 2000). Although *D. huaylasi* from Peru is closely related to the *D. mojavensis* cluster and has sometimes been included within it (Durando et al., 2000), we do not include it here because hybridization data, gene arrangements, and differences in male genitalia clearly point to a closer affiliation with the *mulleri* subgroup (Fontdevila et al., 1990). *D. navojoa* is ancestral to both *D. mojavensis* and *D. arizonae*: the latter two species share a common intermediate ancestor (Figure 1). The range of *D. navojoa* extends from the lowlands along the Pacific coast of southern Mexico and north to southern Sonora. *D. arizonae* is widespread from Guatemala and southern Mexico to Arizona and New Mexico, and is sympatric with *D. mojavensis* and *D. navojoa* in southern Sonora. The range of *D. mojavensis* is restricted to the Sonoran desert, Sinaloan thornscrub, and adjacent areas including the Colorado/Mojave deserts in southern California (Heed, 1982). Ecologically, *D. navojoa* is restricted to the more ancestral *Opuntia* breeding habit, similar to most other members of the *mulleri* subgroup, whereas both *D. mojavensis* and *D. arizonae* use a variety of hosts including a number of more derived columnar cacti (Ruiz et al., 1990).

The goals of this study were to characterize the epicuticular hydrocarbons of the three *D. mojavensis* cluster species in order to understand more about

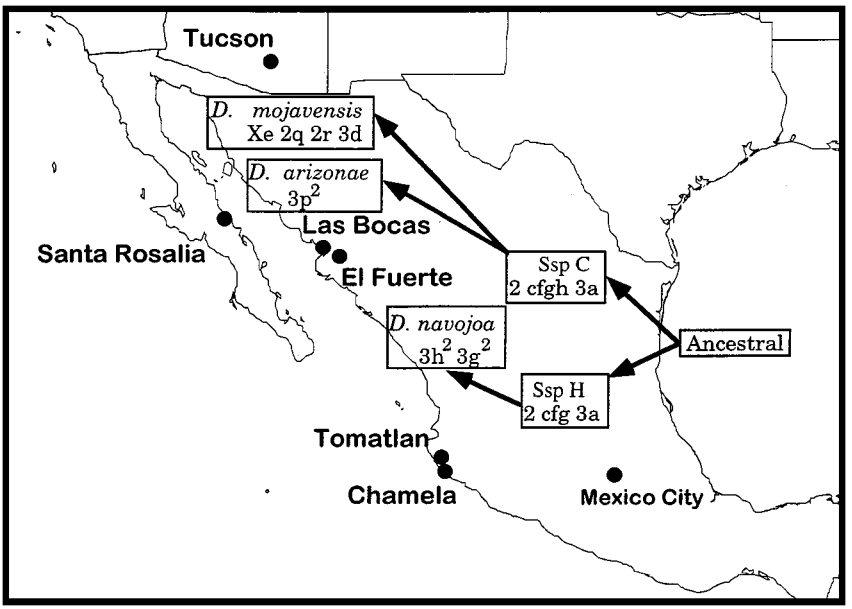


FIG. 1. Map of Mexico and the southwestern United State showing the locations of the populations sampled for this study and a phylogeny of the three *D. mojavensis* cluster species based on chromosomal gene arrangements (Wasserman, 1992).

the magnitude of species-specific mate recognition systems. We assessed variation between two populations of each species reared on fermenting *Opuntia* and *Stenocereus gummosus*, pitaya agria, tissues in order to estimate the degree of intraspecific and substrate-induced variation in hydrocarbon profiles previously documented in *D. mojavensis*. In this study, we used fermenting *Opuntia ficus-indica* and the more chemically specialized agria cactus rearing substrates to characterize further the consequences of this ecological transition of feeding and breeding sites on both expression of adult epicuticular hydrocarbons and components of fitness.

METHODS AND MATERIALS

Origin of Stocks. A stock of *D. arizonae* from Tucson, Arizona, was founded in November 1995 by aspirating approx. 35 adults from the fermenting fruits of *Opuntia ficus-indica*. Another stock originated from seven adults that were baited in a *Stenocereus thurberi–S. alamosensis–Opuntia wilcoxii* forest near Las Bocas, Sonora in April 1996 (Figure 1). Both *D. navojoa* stocks were collected by baiting

and rearing from *Opuntia* pads in March 1997. Eight females and 10 males were collected west of Tomatlan, Jalisco, in a dry forest where *S. standleyi*, *Pachycereus pecten-aboriginum*, *Cephalocereus purpusii*, and an arborescent *Opuntia* species were common. At several locations within the Chamela Biological Station reserve in Jalisco, 34 female and 29 male *D. navojoa* were collected from giant *Opuntia excelsa* plants. The *D. mojavensis* stocks originated from collections made in 1996 in Santa Rosalia, Baja California, and El Fuerte, Sinaloa. The Baja stock was founded from 468 adults that emerged from a *S. gummosus* rot and the Sinaloa stock was initiated with 185 adults from baits and flies that emerged from a *S. thurberi* rot. All flies were reared in the laboratory in large numbers on banana food (Brazner and Etges, 1993) prior to cactus rearing and hydrocarbon analysis.

Chemical Analysis of D. arizonae and D. navojoa Hydrocarbons. The four *D. arizonae* and *D. navojoa* stocks were reared for one generation on banana food in 8-dr shell vials in an incubator programmed on a 14L:10D cycle at 27°C during the day and 17°C at night. All emerged adults were separated by sex and aged for at least 10 days on banana food at room temperature. Epicuticular hydrocarbons were extracted from adults ($N = 549$–902) of each group in Biosil mini-columns. Each column consisted of a Pasteur pipet that contained packed glass wool and Biosil (silica gel, Sigma S-4133) washed several times with HPLC grade hexane. Flies were then added, washed in 8 ml of hexane, and the hydrocarbons were collected in hexane-rinsed vials. After the hexane was evaporated with nitrogen, each sample was sealed and stored at −20°C. Each sample extract was characterized by gas chromatography–mass spectrometry (GC-MS) of the most abundant components composed of methyl-branched hydrocarbons, alkenes, and alkadienes. The samples were analyzed by capillary gas–liquid chromatography by using a Hewlett Packard 5890 GC fitted with a 12-m HP-1 fused silica column. The GC was programmed from 150°C to 300°C at 10°C/min and held at 300°C for 5 min. The temperature of the injector and detector (Hewlett Packard 5971 mass selective detector) was 280°C. The internal standard was 100 ng/fly of octacosane (C_{28}). The unsaturated epicuticular hydrocarbons were derivatized with dimethyl disulfide, and the resulting thiomethyl derivatives were analyzed by GC-MS to identify the positions of the double bonds (Toolson et al., 1990).

Cactus Rearing Experiment. All populations of *D. arizonae*, *D. mojavensis*, and *D. navojoa* were cultured on fermenting cactus tissues in 1997 in order to assess the degree to which rearing substrates affected adult epicuticular hydrocarbon composition. Several hundred adults were collected from each population that had been cultured as described above. Eggs were collected from aged adults and washed in deionized water, 70% ethanol, and again in sterile deionized water. Eggs were counted out in groups of 200, transferred to a 1-cm^2 piece of sterilized filter paper, and placed on fermenting cactus. Cactus cultures were set up in plugged 8-oz bottles with 75 g of aquarium gravel at the bottom covered with a 5.5-cm-diameter piece of filter paper. Bottles were then autoclaved, and after 60 g

of either agria or *O. ficus-indica* tissues were in place, autoclaved again for 10 min. After cooling to room temperature, each culture was inoculated with 0.5 ml of a pectolytic bacterium, *Erwinia cacticida* (Alcorn et al., 1991), and 1.0 ml of a mixture of seven species of yeast common in natural agria rots (Starmer, 1982): *Pichia cactophila*, *P. mexicana*, *P. amethionina* var. *amethionina*, *Cryptococcus cereanus*, *Candida valida*, *C. ingens*, and *C. sonorensis*. Three replicate cultures of each cactus type were started for each of the two populations for each species and cultured in an incubator programmed as above. All unhatched eggs were counted to allow calculation of egg to adult viability. Eclosed adults from each replicate culture were counted daily allowing determination of egg-to-adult development time, separated by sex, and aged on banana food in vials at room temperature.

Aged adults were then transferred to hexane rinsed vials and stored at −20°C until hydrocarbon extracts were prepared by using groups of adults (usually 20–30) as described above. Each hydrocarbon sample was redissolved in hexane (2.5 μl/fly) containing 385 ng of docosane (C_{22}) per microliter as an internal standard. One microliter of each sample was analyzed by capillary gas–liquid chromatography with a Shimadzu G14 fitted with a 30-m DB-1 fused silica column. Injector and detector temperatures were set at 345°C with the injector port in split mode. Running temperatures started at 200°C and increased to 345° at 10°/min, with a hold at 345°C for 7 min (Stennett and Etges, 1997).

Statistical Analyses. Development time was measured in days, and viability was calculated as the number of eclosed adults divided by the number of counted eggs that hatched. Variation in egg to adult development and viability was assessed by ANOVA with PROC GLM in SAS (SAS Institute, 1989). Viability data were arcsin transformed, and development time data were $\log_{10}$ transformed prior to analysis.

Hydrocarbon amounts were estimated by analysis of peak integrations using EZCHROM software (ver. 2.1) provided by Shimadzu. Each sample amount was normalized by the measured amount of the internal standard. Replicate groups of flies were analyzed together. All data were expressed as nanograms per fly of cuticular hydrocarbons and were analyzed with population, rearing substrate, and sex as main effects, and for all interactions between main effects. Population, replicates, and all interactions with population were considered random effects. The TEST command was used in the RANDOM statement to generate the appropriate F ratios and adjusted degrees of freedom by using Satterwaite's approximation (SAS Institute, 1989). Within-species ANOVAs were also evaluated to more closely assess some of the higher order interaction terms. Canonical discriminant function analysis (PROC CANDISC) (SAS Institute, 1989) was performed on the replicate means of each population ignoring sex and cactus differences for all hydrocarbons analyzed. This procedure forms linear combinations of the hydrocarbons with the highest multiple correlation with the populations and maximizes the univariate F ratios. Each canonical variable was obtained by finding the linear combination

least correlated with the previous canonical variable: the first three canonical variables were used to plot the variation in hydrocarbons among populations.

RESULTS

Chemical Descriptions of Hydrocarbons. The major epicuticular hydrocarbons are alkanes, 2-methylalkanes, alkenes, methyl-branched alkenes, and multimethylene interrupted alkadienes (Table 1). The location of the double bonds in the alkenes were at odd-numbered carbons for the hentricontenes (7-hentricontene and 9-hentricontene) but were at even-numbered carbons for the longer-chained alkenes (e.g., 10-tritricontene, 10-tetratricontene, and 14-hexatricontene). Locations of the double bonds in the methyl-branched alkenes were also at even-numbered carbons. The multimethylene interrupted alkadienes had an odd number

TABLE 1. KEY MASS SPECTRA PEAKS IN IDENTIFICATION OF EPICUTICULAR HYDROCARBONS OF *D. arizonae*, *D. mojavensis*, AND *D. navojoa*

		m/*z*		
ECL[a]	Hydrocarbon	Untreated	Dimethyl disulfide derivative	Hydrogenated
28.00	*n*-octacosane	394		
28.65	2-methyloctacosane	365, 393		
30.65	2-methyltricontane	393, 421		
30.78	7-and 9-hentricontene	434	173, 355, 528	
33 Br2	11-and 13-methyldotricontane	168, 322 196, 294		
32.47	31-methyldotricont-8-ene	462	159, 397, 556	421, 449
32.56	31-methyldotricont-6-ene	462	131, 425, 556	421, 449
32.63	8,24-tritricontadiene	460	159, 173, 381, 395	
32.70	7,25-tritricontadiene	460	145, 159, 395, 409	
32.79	10-, 12-, and 14-tritricontene	462	187, 369; 215, 341; 243,313	464
34 diene	8,26-tetratricontadiene	474	159, 409	
34 diene	6,26- and 6,24-tetratricontadiene	474	131, 381, 409	
34 ene	10-, 12-, and 14-tetratricontene	476	187, 383; 215, 355; 243, 327	478
35 ene 1	33-methyltetratricont-10-ene	490	187, 397, 584	449, 477
35 ene 2	33-methyltetratricont-8-ene	490	159, 425, 584	449, 477
34.59	9,25-pentatricontadiene	488	173, 187, 395, 409	
34.66	8,26-pentatricontadiene	488	159, 173, 409, 423	
34.66	7,27-pentatricontadiene	488	145, 159, 423, 437	
37 ene	35-methylhexatricont-10-ene	518	187, 425	520
36.5	9,27-heptatricontadiene	516	173, 187, 423, 437	
36.7	14-, 16-, and12-hexatricontene	518	243, 369; 271, 341; 215, 397	520

[a] Equivalent chain length calculated as in Stennett and Etges (1997).

of carbons to the double bond from one end, and an even number of carbons from the other end to the double bond. In the tetratricontadienes (34 dienes), the double bonds were an even number of carbons from both ends.

Epicuticular Hydrocarbon Differences Among Species. A total of 27 hydrocarbon peaks were scored in each sample with chain lengths ranging from C_{28} to C_{40}. The number of observed peaks and their retention times were similar among the three species with only a few notable qualitative differences. The most ancestral species, *D. navojoa*, possessed 31-methyldotricont-6-ene, which was not observed in the other two species with an equivalent chain length of $C_{32.56}$. 8,24-Tritricontadiene was present in high quantities in *D. arizonae* and mainland *D. mojavensis* but was absent in *D. navojoa*. The Baja California population of *D. mojavensis* population from Santa Rosalia was characterized by the near absence of the $C_{32.63}$, $C_{35.59}$, and $C_{36.5}$ alkadienes that are major peaks in mainland populations, such as El Fuerte, consistent with all previous studies (Stennett and Etges, 1997; Etges and Ahrens, 2001). *D. navojoa* was also characterized by far lower amounts of 2-methyloctacosane ($C_{28.65}$) than *D. arizonae* and *D. mojavensis* (± 1 SD); $35.4 \pm 11.9 < 123.1 \pm 32.4 < 175.3 \pm 71.6$ ng/fly, respectively. This difference was not significant in the mixed model nested ANOVA (Appendix 1) because the mean square error term, populations nested within species, was so large. This was also the case for $C_{32.63}$ (absent in *D. navojoa*), $C_{34.59}$, and $C_{36.5}$, indicating that significant geographic variation in a variety of hydrocarbon components has obscured the levels of statistical significance of the differences between species.

Population Differences. Within-species ANOVAs were performed with populations to consider random effects in order to assess more directly hydrocarbon differences among populations and some of the higher order interaction terms. The degree of geographic variation observed between the Baja California and mainland population of *D. mojavensis* in hydrocarbon amounts (Table 2) was consistent with earlier results (Stennett and Etges, 1997). These same two populations were part of a larger study of epicuticular hydrocarbon variation among six populations from Baja California and five mainland Mexico populations (Etges and Ahrens, 2001). Thus, we can directly compare the magnitude of interpopulation variation in epicuticular hydrocarbons with that of the other two species. Here 15 of the 27 hydrocarbon components varied geographically: these were the same components that contributed to the overall geographic differences between Baja California and mainland populations of *D. mojavensis*. A greater proportion, 22/27, differed between the Tucson, Arizona, and southern Sonoran populations of *D. arizonae*. Just 11 of these components varied among the two populations of *D. navojoa*; only four of these are major peaks: C_{28} alkane, 2-methyloctacosane, 7,25-tricontadiene, and 8,26-pentatricontadiene (Table 2). Such intraspecific variation in *D. navojoa* was surprising given that the two populations studied were only 45 km apart (Figure 1).

Together, these 27 hydrocarbon components significantly discriminated among each of the six populations. The first three canonical variables accounted

TABLE 2. *F* VALUES FROM WITHIN-SPECIES ANOVAS FOR 27 HYDROCARBON COMPONENTS[a]

Hydrocarbon component	C_{RT}		*D. arizonae*	*D. mojavensis*	*D. navojoa*
n-Octacosane	C_{28}	(P)	31.11***	25.62***	60.59***
		(C)	7.15*	1.83	13.77**
2-Methyloctacosane	$C_{28.65}$	(P)	23.00***	178.83***	11.07**
		(C)	6.17*	2.90	0.23
2-Methyltricontane	$C_{30.65}$	(P)	34.92***	0.03	0.0
		(C)	0.30	6.29*	0.12
7- and 9-Hentricontene	$C_{30.78}$	(P)	22.79***	1.36	1.36
		(C)	20.19***	14.96**	1.12
Unknown	C_{33Br1}	(P)	NA	92.30***	NA
		(C)	NA	0.22	NA
11- and 13-Methyldotricontane	C_{33Br2}	(P)	27.07***	74.06***	1.15
		(C)	13.45**	0.77	5.25
31-Methyldotricont-8-ene	$C_{32.47}$	(P)	30.77***	7.34*	0.26
		(C)	14.46**	5.84	2.72
31-Methyldotricont-6-ene	$C_{32.56}$	(P)	NA	NA	1.28
		(C)	NA	NA	0.22
8,24-Tritricontadiene	$C_{32.63}$	(P)	37.28***	70.13***	NA
		(C)	31.91**	7.31*	NA
7,25-Tricontadiene	$C_{32.70}$	(P)	11.26**	0.24	10.05**
		(C)	20.03***	11.12**	0.85
10-, 12-, and 14-Tritricontene	$C_{32.79}$	(P)	39.37***	12.10**	2.91
		(C)	4.77*	23.96***	0.08
8,26-Tetratricontadiene	C_{34} diene	(P)	26.30***	39.14***	6.95*
		(C)	14.46**	24.40***	7.42*
6,26- and	C_{34} alkene	(P)	3.92	0.05	19.98***
6,24-Tetratricontadiene		(C)	24.42***	22.40***	29.37***
10-, 12- and14-Tetratricontene	C_{34} ene	(P)	0.90	19.36***	20.00***
		(C)	13.85**	13.64**	53.58***
33-Methyltetratricont-10-ene	C_{35} alkene1	(P)	3.31	30.82***	0.44
		(C)	2.68	0.26	3.04
33-Methyltetratricont-8-ene	C_{35} alkene 2	(P)	17.46**	1.52	0.19
		(C)	2.10	2.82	6.20*
9,25-Pentatricontadiene	$C_{34.59}$	(P)	44.29***	85.68***	4.66
		(C)	23.66***	19.49***	0.01
8,26-Pentatricontadiene and	$C_{34.66}$	(P)	40.94***	13.23**	11.03*
7,27-Pentatricontadiene		(C)	22.07***	7.17*	0.83
Unknown branched alkene	C_{36a}	(P)	14.28**	12.28*	12.13*
		(C)	21.38***	6.05*	27.44***
Unknown branched alkene	C_{36b}	(P)	8.55*	3.86	25.93***
		(C)	4.37	34.89***	42.30***
35-Methylhexatricont-10-ene	C_{37} alkene	(P)	8.42*	40.17***	11.36**
		(C)	18.10***	3.30	5.95
9,27-Heptatricontadiene	$C_{36.5}$	(P)	48.50***	54.94***	4.93
		(C)	21.34***	11.11**	0.58

TABLE 2. CONTINUED

Hydrocarbon component	C_{RT}		*D. arizonae*	*D. mojavensis*	*D. navojoa*
Unknown alkadiene	$C_{36.6}$	(P)	43.81***	6.42	2.58
		(C)	12.48**	22.51***	0.08
14-, 16-, and 12-Hexatricontene	$C_{36.7}$	(P)	41.35***	4.42	3.31
		(C)	21.38***	9.89**	0.61
Unknown alkene	C_{38}	(P)	16.83**	0.94	15.65**
		(C)	5.42	6.00	13.44***
Unknown	C_{39}	(P)	1.04	4.80	6.63*
		(C)	1.08	7.78**	17.54***
Unknown	C_{40}	(P)	28.43**	0.46	6.67*
		(C)	2.11	6.52	10.34**
Total hydrocarbons		(P)	44.13***	1.10	5.35
		(C)	23.54***	12.50**	0.25

[a] For each component, F values and significance levels are listed for differences between populations (P) over that for differences between cactus hosts (C). Significance of all P values was adjusted using the sequential Bonferroni procedure across species.
C_{RT} refers to the hydrocarbon component's retention time or carbon chain length as described in Stennett and Etges (1997) or in this paper. NA: not applicable because hydrocarbon component is absent for this species. $^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001$; significance tests are based on a stepwise Bonferroni test with initial $P = 0.05$ using the number of P values in each row to correct for the number of simultaneous tests.

for 93.9% of the total hydrocarbon variation (Figure 2). All pairwise squared Mahalanobis distances between populations were significant ($p < 0.0001$), as were the overall multivariate differences among populations (Wilks $\lambda = 0.00012742$, $F = 20.56$, $p < 0.0001$). The first five canonical correlations were all significantly greater than zero ($p < 0.0001$). Thus, significant geographic variation exists in the epicuticular hydrocarbon profiles of the populations of these three species.

Sex Differences. Amounts of 2-methyloctacosane, 2-methyltricontane, the C_{38} alkene, and the C_{40} alkene differed between males and females of all three species (Appendix 1). However, 17 hydrocarbon components differed between males and females in a population-specific manner as indicated by the significant sex × population within-species interaction terms. Such interactions imply that male–female differences in amounts of these hydrocarbon components differ among populations of these three species. These interactions were also observed in the larger analysis of epicuticular hydrocarbon variation between Baja California and mainland populations of *D. mojavensis* (Etges and Ahrens, 2001), so local variation in sexual dimorphism of hydrocarbon profiles is not restricted to this species.

Substrate Differences. Although not statistically significant in all cases ($P < 0.05$; Appendix 1), hydrocarbon amounts generally differed due to cactus-rearing substrates for 18 of the epicuticular hydrocarbons assayed. In every case, flies reared on *Opuntia* cactus had increased amounts of cuticular hydrocarbons as

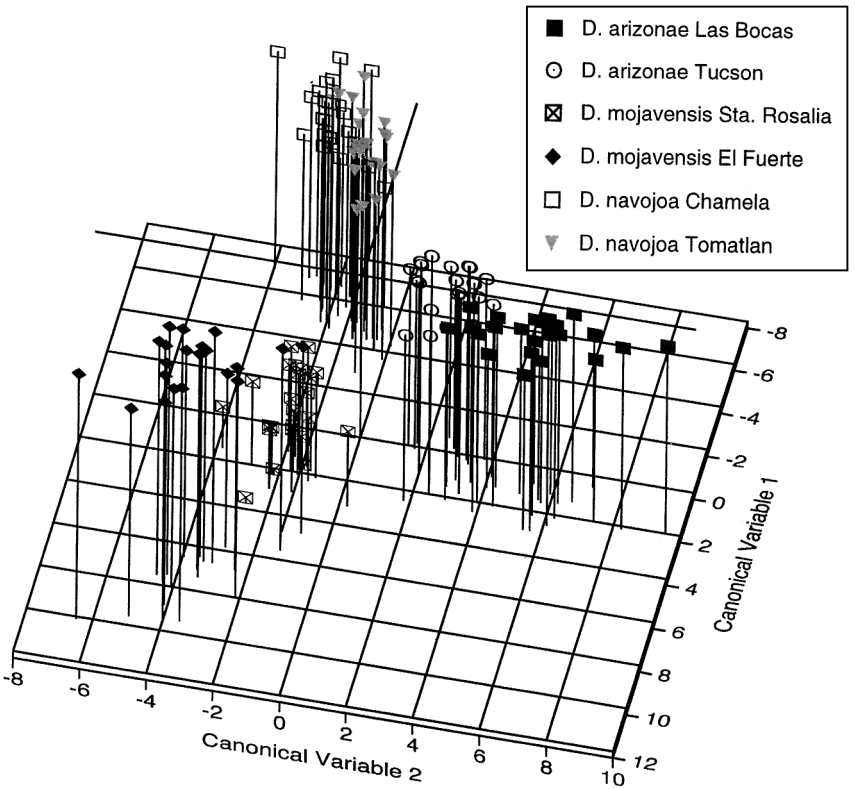


FIG. 2. Three dimensional plot of the populations of *D. mojavensis*, *D. arizonae*, and *D. navojoa* based on the first three canonical discriminant variables (CDVs) formed from the 27 hydrocarbon components observed in this study. CDV 1 accounted for 53.6%, CDV 2 accounted for 26.0%, and CDV 3 accounted for 14.3% of total variance, respectively. Sex and host cactus were ignored to emphasize populations and species differences. All Mahalanobis distances between populations were significant ($P < 0.0001$).

compared to agria-reared flies (Figure 3). Results of the within-species ANOVAs (Table 2) also suggested that the effect of rearing flies on agria versus *Opuntia* was usually statistically significant in one or two of these species for a given hydrocarbon component. Thus, many of the cases of marginal significance in the nested ANOVAs were due to this result. Only the two C_{34} alkenes and one of the C_{36} alkenes differed in amounts between agria and *Opuntia*-reared flies in all three species (Table 2), and the nested ANOVA suggested that 2-methyloctacosane, 2-methyltricontane, and 14-, 16-, and 12-hexatricontene also varied among species (Appendix 1). The 2-methyloctacosane, 7- and 9-hentricontene, 31-methyldotricont-8-ene, and $C_{36.6}$ alkadiene components varied in a species–specific manner as

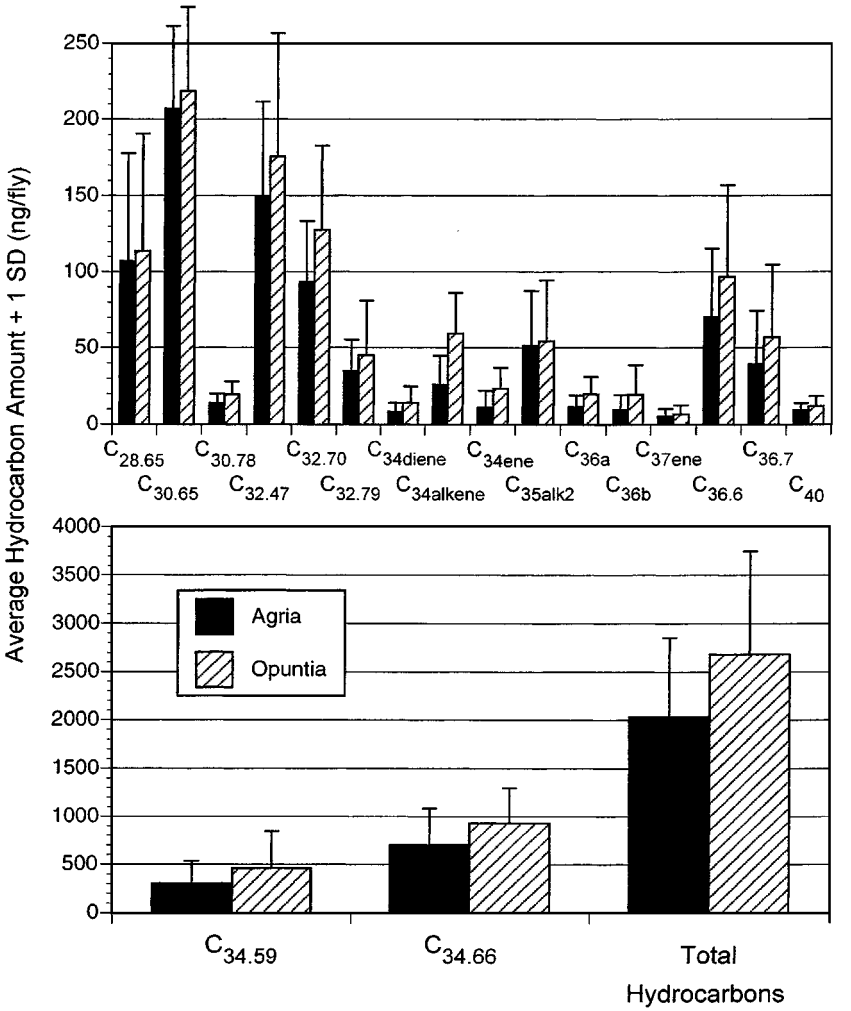


FIG. 3. Averages (+1 SD) of *D. arizonae*, *D. mojavensis*, and *D. navojoa* hydrocarbon amounts for the 18 components that differed between rearing substrates. In all cases, hydrocarbon amounts were greater in flies reared in *Opuntia* than in agria tissues. Individual components are referred to by their equivalent chain lengths or other names (Appendix 1); see Table 2 for chemical names.

shown by the significance of the cactus × species interaction terms. These cactus substrates also influenced the total amounts of hydrocarbons per fly (Figure 3) for *D. arizonae* and *D. mojavensis*, but not for *D. navojoa* (Table 2). Overall, fermenting *Opuntia* cactus tissues must contain more available precursors for synthesizing

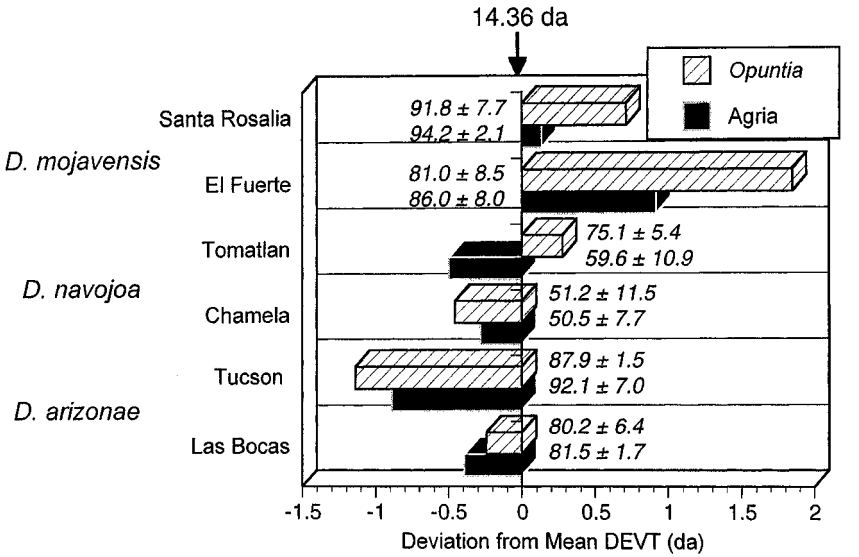


FIG. 4. Deviations in egg to adult development time from the overall mean (indicated above the graph) for the six populations of *D. mojavensis* cluster species in this study illustrating the significant population × cactus interaction term from the ANOVA. Mean egg to adult viability (±1 SD) is indicated in italics adjacent to each type of cactus substrate for each population.

the majority of adult epicuticular hydrocarbons in these three *Drosophila* species than do agria tissues.

Life History Differences. Differences in egg-to-adult development time among populations were expressed in a substrate-specific manner (Figure 4). Although overall differences in development time were only marginally significant among species ($F = 5.42$, $P = 0.10$) in the nested design, females emerged earlier than males in all three species ($F = 94.52$, $P = 0.002$). the interaction between cactus hosts and populations nested within species was also significant ($F = 3.84$, $P = 0.022$). Only *D. mojavensis* populations consistently expressed shorter development times on their primary host plant, agria cactus. The correlation between development time and total hydrocarbons per fly was not significant ($r = 0.52$, $P > 0.05$, $N = 12$), so variation in hydrocarbon amounts was unrelated to the length of preadult development time. Egg to adult viability was lower in the two populations of *D. navojoa* ($F = 39.87$, $P < 0.0001$), in part due to the lower viabilities observed on agria versus *Opuntia*-reared flies (Figure 4). Overall, these life history differences are consistent with the known patterns of host plant use in nature (Ruiz and Heed, 1988) and suggest that any host cactus-induced variation in hydrocarbon amounts should also be expressed in natural populations.

DISCUSSION

The overall chemical similarity of the epicuticular hydrocarbon components among the three *D. mojavensis* cluster species suggests that the biosynthetic pathways for hydrocarbon production and deposition have not yet widely diverged. However, significant quantitative differences in hydrocarbon amounts among populations (Figure 3) indicate that the chemical signatures of these hydrocarbons have evolved within and between species. Whether individual or groups of these epicuticular hydrocarbons serve as within- and/or between-species signaling systems remains to be determined. In *D. mojavensis*, transferring male mainland-specific hydrocarbons to Baja males in "perfuming" experiments significantly enhanced the mating success of these perfumed Baja males with mainland females in comparison with controls. Thus, these cuticular hydrocarbons are part of the mate recognition system in this species (Etges and Ahrens, 2001). Certainly, the role of these chemical cues must be evaluated in the context of other components of the mating systems of these species expressed in an environment-specific manner, including mating behavior (Etges, 1992; Brazner and Etges, 1993; Stennett and Etges, 1997) and courtship songs.

The influences of rearing substrates on adult epicuticular hydrocarbon profiles suggests that an extensive understanding of the ecology and distribution of natural populations is necessary if we are to identify the mechanisms responsible for shaping mate recognition divergence. For a number of *Drosophila* species, preadult rearing environments are significant determinants of both genetic and phenotypic variation in fitness characters (Etges and Heed, 1987; Ruiz and Heed, 1988; Etges and Klassen, 1989; Etges, 1990, 1993; Fanara et al., 1999) as well as mating behavior (Ehrman, 1990; Brazner and Etges, 1993; Kim et al., 1996; Kim and Ehrman, 1998) and epicuticular hydrocarbon variation. Thus, the use of discrete resources in nature, such as fermenting cactus rots, is a key determinant of variation in fitness characters and intraspecific mate recognition systems. The frequency and intensity of courtship interactions should be determined largely by the abundance of adults, the number of different species present, and the male mating propensity at feeding and breeding sites. There can be considerable overlap of species feeding on rots of any of the major host cacti in the Sonoran Desert (Fellows and Heed, 1972), although host plant specificity, resulting from the effects of stem chemistry on larval growth and development (Fogleman and Heed, 1989; Fogleman and Abril, 1990) and interspecific larval competition (Heed and Mangan, 1986), is the general rule.

Since *D. navojoa* is restricted to *Opuntia* cacti and its range only overlaps that of *D. mojavensis* in a small area of southern Sonora and northern Sinaloa (Heed, 1982), these two species probably do not encounter each other frequently in nature. However, Markow and Maveety (1985) documented higher premating

isolation among sympatric populations than allopatric populations of each species and concluded that reproductive character displacement was responsible. Although they included no statistical analysis of their data concerning the significance of the differences in premating isolation between sympatric and allopatric populations, further analysis of their data supports their contention. Their estimates of premating isolation using the Joint I statistic (Stalker, 1942) were greater in sympatric than allopatric populations (two-group comparison; $\chi^2 = 20.56$, 1 *df*, $P < 0.0001$) (Sauer and Williams, 1989). Thus, there is geographic variation in levels of premating isolation between *D. mojavensis* and *D. navojoa*, even though they do not regularly share host plants.

The ecology of *D. mojavensis* is perhaps the best known as it uses different host cacti throughout its range (Heed and Mangan, 1986; Etges et al., 1999). In Baja California, pitaya agria, *S. gummosus*, is the preferred host even though several secondary hosts used elsewhere are sympatric with agria such as organ pipe cactus, *S. thurberi*, and California barrel cactus, *Ferocactus cylindraceous*. In mainland Sonora, Sinaloa, and Arizona, organ pipe cactus is the major host except for where a small patch of agria grows in coastal Sonora and occasional use of sina cactus, *S. alamosensis*, in southern Sonora and coastal Sinaloa (Markow et al., 1983; Ruiz and Heed, 1988). In the Mojave/Colorado deserts of southern California, *D. mojavensis* use California barrel cactus and have been found on Santa Catalina Island near Los Angeles using the fruits and pads of *Opuntia demissa*. The more widespread *D. arizonae* has been reared out of sina, saguaro, *Carnegiea gigantea*, and more rarely from *S. gummosus* in coastal Sonora and the Cape region in Baja California along with *D. mojavensis*. To the north in Arizona and New Mexico outside of agricultural areas, *D. arizonae* breeds in *Opuntia* pads and fruits and feeds on a variety of cacti (Fellows and Heed, 1972; Heed, 1982). In southern Mexico, *D. arizonae* has been collected from fermenting *Myrtillocactus geometrizans* and *S. pruinosus* arms in Chiapas, as well as *Opuntia* pads north of Pachuca, Hidalgo (Etges, unpublished data).

Reproductive character displacement has been described in mainland Mexico and Arizona populations *D. mojavensis* due to sympatry with *D. arizonae* (Wasserman and Koepfer, 1977). Mainland populations of *D. mojavensis* are considered derived from those in Baja California where *D. arizonae* is absent except for a few small demes outside of the desert in the more subtropical thornscrub in the Cape region of Baja California. Johnson (1980) hypothesized that *D. mojavensis* colonized mainland Mexico from Baja California by switching to a secondary host plant, organ pipe cactus, and secondarily became sympatric with *D. arizonae*. Both species occasionally use sina cactus in southern Sonora (Markow et al., 1983; Ruiz and Heed, 1988), and *D. arizonae* has been reared out of agria in low frequencies in coastal Sonora following the summer monsoons, but then disappears through December and January (Etges and Heed, unpublished data). The presence of

D. arizonae on the mainland was hypothesized to have caused a shift in patterns of mate preference in *D. mojavensis*, so that now these populations exhibit behavioral isolation with the more ancestral *D. mojavensis* populations from Baja California (Zouros and d'Entremont, 1980). However, the species range of sina cactus is small relative to the range sizes of both species limiting the overall degree of host plant sharing. Furthermore, rearing *D. mojavensis* on agria cactus reduces premating isolation among populations to nonsignificant levels in comparison to laboratory food and organ pipe cactus (Etges, 1992, 1998; Brazner and Etges, 1993). In similar laboratory trials, rearing *D. mojavensis* and *D. arizonae* on agria cactus also reduced premating isolation from that observed with laboratory-food-reared flies (Yule's $V \pm 1$ SE, $0.811 \pm 0.076 > 0.643 \pm 0.008$, ($\chi^2 = 4.83$, 1 *df*, $P = 0.028$) (Etges, unpublished data), and alters amounts of a number of cuticular hydrocarbon components (Table 2). Thus, the documented sharing of host plants is crucial to the understanding of the evolution of sexual isolation within *D. mojavensis* and between *D. mojavensis* and *D. arizonae*. In the group as a whole, ecological isolation is perhaps the main factor contributing to species isolation.

There have been no studies of sexual isolation between *D. arizonae* and *D. navojoa*, but the degree of genetic differentiation and postmating isolation between them (Ruiz et al., 1990) suggests sexual isolation should be at least as strong as that between *D. mojavensis* and *D. navojoa* (Markow and Maveety, 1985). The role of epicuticular hydrocarbon variation in sexual isolation among *D. mojavensis* cluster species has yet to be studied. Given the degree of geographic variation within *D. mojavensis* cluster species in epicuticular hydrocarbons, it is reasonable to infer that epicuticular hydrocarbons may differentiate prior to species divergence. This is consistent with the broad-scale variation between Baja California and mainland populations of *D. mojavensis* that is responsible for premating isolation between populations (Stennett and Etges, 1997; Etges and Ahrens, 2001). Species-specific mate recognition and sexual isolation may then be more influenced by behavioral and mating song differences (Wasserman and Koepfer, 1977; Byrne, 1999) when species are drawn to the same cacti. Further within-species data concerning mating song variability as well as determination of the role of epicuticular hydrocarbons in species mate recognition is badly needed.

Acknowledgments—Miguel Armella, Chris Babcock, Celeste Durando, Greg Huckins, Conrad Istock, Francisco Molina, and Sandra Perez helped collect wild flies. Mitch Ahrens collected cactus-reared flies in the laboratory and Andre Lechance and Tom Starmer graciously provided yeast stocks. Jim Dunn suggested the nested ANOVA design, and Bill Durham provided us with a GC. Two anonymous reviewers provided helpful advice on the manuscript. We thank the director of the Chamela Biological Station in Jalisco for allowing us to collect there. We are indebted to the Direccion General de Conservacion Ecologico de los Recursos Naturales in Mexico City for issuing cactus collecting permits. This work was funded by NSF BSR-9509032, INT-9724790, and the Universidad Autonoma Metropolitana-Iztapalapa in Mexico City.

APPENDIX 1. NESTED ANOVA RESULTS FOR MAJOR HYDROCARBON COMPONENTS IN TWO POPULATIONS OF *D. mojavensis*, *D. Arizonae*, AND *D. navojoa* REARED ON AGRIA AND *Opuntia* CACTI[a]

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
			A. *n*-Octacosane (C_{28})			B. 2-Methyloctacosane ($C_{28.65}$)		
1. Species	2	66.16	1/2	0.07	NS	466440.30	4.53	NS
2. Population(species)	3	1448.57	2/5	7.62	0.076	154543.83	45.87	0.032
3. Cactus	1	244.14	3/5	3.66	NS	3654.64	71.13	0.003
4. Cactus × species	2	9.98	4/5	0.07	NS	2271.73	22.26	0.015
5. Cactus × population(species)	3	200.23	5/6	1.17	NS	151.94	0.01	NS
6. Replicates	24	1394.31	6/12	12.11	0.0001	12086.19	1.25	NS
7. Sex	1	0.78	7/11	0.55	NS	46229.80	31.28	0.011
8. Sex × species	2	21.51	8/11	7.64	0.066	7184.62	2.43	NS
9. Sex × cactus	1	0.73	9/12	0.15	NS	815.95	2.02	NS
10. Sex × cactus × species	2	8.69	10/12	0.91	NS	1179.00	1.46	NS
11. Sex × population(species)	3	4.21	11/12	0.29	NS	4439.56	3.67	0.015
12. Error	95	455.90				38300.52		
			C. 2-Methyltricontane ($C_{30.65}$)			D. 7- and 9-Hentricontene ($C_{30.78}$)		
1. Species	2	77253.42		2.65	NS	834.04	1.55	NS
2. Population(species)	3	43813.11		33.28	NS	807.81	1.07	NS
3. Cactus	1	5636.83		9.99	0.049	979.90	5.87	0.094
4. Cactus × species	2	3142.43		2.79	NS	318.65	22.26	0.015
5. Cactus × population(species)	3	1685.56		0.43	NS	501.69	2.68	0.069
6. Replicates	24	30855.63		0.72	NS	1518.05	2.72	0.0003
7. Sex	1	62546.87		37.44	0.009	210.31	1.96	NS
8. Sex × species	2	6082.50		1.82	NS	262.92	1.22	NS
9. Sex × cactus	1	654.90		0.37	NS	29.56	1.27	NS
10. Sex × cactus × species	2	11518.45		3.21	0.045	84.64	1.82	NS
11. Sex × population(species)	3	5011.01		0.93	NS	322.95	4.62	0.005
12. Error	95	170412.19				2213.05		

(*Continued*)

APPENDIX 1. CONTINUED

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
		E. 31-Methyldotricont-8-ene ($C_{32.47}$)				F. 31-Methyldotricont-6-ene ($C_{32.56}$)		
1. Species	2	186456.47		2.78	NS	106356.06	438.34	0.0002
2. Population(species)	3	100815.85		3.14	NS	364.02	0.37	NS
3. Cactus	1	23395.77		8.34	0.063	19.47	0.05	NS
4. Cactus × species	2	34078.00		6.07	0.089	40.21	0.05	NS
5. Cactus × population(species)	3	8422.37		0.62	NS	1193.24	4.05	0.018
6. Replicates	24	110327.70		2.95	0.0001	2357.38	0.97	NS
7. Sex	1	71211.10		7.53	0.071	567.10	20.98	0.019
8. Sex × species	2	8506.10		0.45	NS	1168.77	21.67	0.016
9. Sex × cactus	1	1.08		0.00	NS	180.04	1.78	NS
10. Sex × cactus × species	2	3583.57		1.15	NS	185.53	1.84	NS
11. Sex × population(species)	3	28422.40		6.08	0.0008	80.68	0.27	NS
12. Error	95	148019.13				9602.00		
		G. 8,24-Tritricontadiene ($C_{32.63}$)				H. 7,25-Tritricontadiene ($C_{32.70}$)		
1. Species	2	498175.25		6.54	0.081	67692.13	4.64	NS
2. Population(species)	3	114307.36		3.29	NS	21923.18	1.04	NS
3. Cactus	1	32666.90		3.96	NS	38913.29	8.74	0.059
4. Cactus × species	2	33112.95		2.01	NS	15157.24	1.70	NS
5. Cactus × population(species)	3	24789.43		4.19	0.016	13385.06	1.48	NS
6. Replicates	24	48079.49		3.23	0.0001	73626.64	2.94	0.0001
7. Sex	1	6255.15		1.59	NS	5131.89	1.43	NS
8. Sex × species	2	3242.69		0.41	NS	6136.23	0.85	NS
9. Sex × cactus	1	331.25		0.53	NS	283.44	0.27	NS
10. Sex × cactus × species	2	434.06		0.35	NS	1549.09	0.74	NS
11. Sex × population(species)	3	11849.44		6.36	0.0006	10812.35	3.45	0.020
12. Error	95	58970.44				99169.17		

		I. 10-, 12-, and 14-Tritricontene ($C_{32.79}$)			J. 8,26-Tetratricontadiene (C_{34} diene)		
1. Species	2	27818.86	6.56	0.080	4216.17	4.47	NS
2. Population(species)	3	6365.78	0.45	NS	1414.92	1.93	NS
3. Cactus	1	3636.22	9.68	0.053	1093.09	6.20	0.088
4. Cactus × species	2	4403.26	5.86	0.092	537.05	1.52	NS
5. Cactus × population(species)	3	1128.59	1.36	NS	529.67	4.18	0.016
6. Replicates	24	6729.44	1.93	0.013	1030.80	2.96	0.0001
7. Sex	1	17123.73	3.82	NS	2.75	0.03	NS
8. Sex × species	2	17042.86	1.90	NS	7.08	0.04	NS
9. Sex × cactus	1	340.45	2.34	NS	6.58	0.45	NS
10. Sex × cactus × species	2	2508.13	8.63	0.0004	1.11	0.04	NS
11. Sex × population(species)	3	13485.57	30.94	0.0001	246.13	5.65	0.001
12. Error	95	13800.18			1380.69		
		K. 6,26- and 6,24-Tetratricontadiene (C_{34} alkene)			L. 10-, 12-, and 14-Tetretricontene (C_{34} ene)		
1. Species	2	5499.28	2.76	NS	295.35	0.14	NS
2. Population(species)	3	2992.63	0.31	NS	3162.55	1.49	NS
3. Cactus	1	20317.30	11.60	0.042	4837.71	8.50	0.062
4. Cactus × species	2	2390.59	0.68	NS	74.97	0.07	NS
5. Cactus × population(species)	3	5262.46	2.44	0.089	1709.73	2.46	0.087
6. Replicates	24	17549.61	2.77	0.0002	5648.90	3.38	0.0001
7. Sex	1	3414.26	2.00	NS	1551.16	7.52	0.071
8. Sex × species	2	1293.31	0.38	NS	242.45	0.59	NS
9. Sex × cactus	1	334.62	1.27	NS	136.37	1.96	NS
10. Sex × cactus × species	2	1587.81	3.00	0.054	218.56	1.57	NS
11. Sex × population(species)	3	5128.15	6.47	0.0005	619.94	2.97	0.036
12. Error	95	25107.80			6613.49		

(*Continued*)

APPENDIX 1. CONTINUED

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
			M. 33-Methyltetratricont-10-ene (C_{35} alkene 1)			N. 33-Methyltetratricont-8-ene (C_{35} alkene 2)		
1. Species	2	29918.67		5.84	0.092	24751.34	3.66	NS
2. Population(species)	3	7692.63		1.70	NS	10155.61	1.90	NS
3. Cactus	1	97.68		1.05	NS	1445.43	2.98	0.003
4. Cactus × species	2	1125.77		6.09	0.087	14078.26	14.52	0.028
5. Cactus × population(species)	3	276.98		0.49	NS	1452.49	0.34	NS
6. Replicates	24	4542.96		0.82	NS	34574.21	1.47	0.096
7. Sex	1	6152.50		3.74	NS	5770.59	2.53	NS
8. Sex × species	2	2684.38		0.81	NS	1236.94	0.27	NS
9. Sex × cactus	1	686.96		2.98	0.088	4002.40	4.09	0.046
10. Sex × cactus × species	2	1331.97		2.89	0.061	3017.67	1.54	NS
11. Sex × population(species)	3	4948.71		7.16	0.0002	6846.91	2.33	0.079
12. Error	95	21901.10				92872.53		
			O. 9,25-Pentatricontadiene ($C_{34.59}$)			P. 8,26-Pentatricontadiene ($C_{34.66}$) and 7,27-Pentatricontadiene		
1. Species	2	5193134.70		2.33	NS	587654.86	0.18	NS
2. Population(species)	3	3351930.21		4.37	0.070	4848975.72	4.95	0.079
3. Cactus	1	855615.28		5.80	0.095	1691134.64	7.39	0.073
4. Cactus × species	2	533897.68		1.81	NS	499093.03	1.09	NS
5. Cactus × population(species)	3	443598.48		2.90	0.056	687547.88	1.14	NS
6. Replicates	24	1243459.08		2.80	0.0002	4904409.26	2.88	0.0001
7. Sex	1	126983.98		1.00	NS	27083.52	0.16	NS
8. Sex × species	2	47671.09		0.19	NS	39518.40	0.12	NS
9. Sex × cactus	1	9626.20		0.52	NS	6160.70	0.09	NS
10. Sex × cactus × species	2	62454.61		1.69	NS	283728.06	2.00	NS
11. Sex × population(species)	3	380112.01		6.84	0.0003	505519.50	2.38	0.075
12. Error	95	1759166.67				6735046.70		

			Q. Unknown alkene (C_{36} a)			R. Unknown alkene (C_{36} b)	
1. Species	2	1091.40	0.95	NS	11392.48	18.62	0.020
2. Population(species)	3	1727.91	1.20	NS	918.74	0.22	NS
3. Cactus	1	2156.25	5.81	0.095	3506.97	8.76	0.059
4. Cactus × species	2	87.28	0.12	NS	3206.82	4.00	NS
5. Cactus × population(species)	3	1115.42	4.46	0.012	1203.45	2.85	0.057
6. Replicates	24	2025.21	2.02	0.009	3422.58	2.57	0.0006
7. Sex	1	1148.69	7.64	0.07	24.89	0.02	NS
8. Sex × species	2	185.64	0.62	NS	536.90	0.25	NS
9. Sex × Cactus	1	279.75	6.69	0.011	102.06	1.84	NS
10. Sex × Cactus × species	2	252.38	3.02	0.054	728.48	6.56	0.002
11. Sex × population(species)	3	451.94	3.60	0.016	3235.43	19.42	0.0001
12. Error	95	3973.97			5275.30		
			S. 35-Methylhexatricont-10-ene (C_{37} ene)			T. 9,27-Heptatricontadiene ($C_{36.5}$)	
1. Species	2	1689.49	3.08	NS	66717.28	1.30	NS
2. Population(species)	3	822.83	2.38	NS	77250.84	1.11	NS
3. Cactus	1	91.21	23.08	0.017	10210.65	3.29	NS
4. Cactus × species	2	15.50	1.96	NS	7439.22	1.20	NS
5. Cactus × population(species)	3	11.83	0.55	NS	9315.69	7.05	0.0013
6. Replicates	24	171.95	0.95	NS	10516.01	0.81	NS
7. Sex	1	84.65	0.71	NS	20970.68	1.01	NS
8. Sex × species	2	78.95	0.33	NS	23414.31	0.56	NS
9. Sex × cactus	1	0.90	0.12	NS	1265.47	2.34	NS
10. Sex × cactus × species	2	2.41	0.16	NS	4761.07	4.40	0.015
11. Sex × population(species)	3	358.60	15.78	0.0001	62370.22	38.41	0.0001
12. Error	95	719.64			51418.34		
			U. Unknown alkadiene ($C_{36.6}$)			V. 14-, 16-, and 12-Hexatricontene ($C_{36.7}$)	
1. Species	2	75406.38	4.19	NS	90032.95	11.92	0.037
2. Population(species)	3	27042.92	0.44	NS	11339.85	0.63	NS
3. Cactus	1	23554.63	15.66	0.029	10287.00	15.36	0.029
4. Cactus × species	2	17626.42	5.86	0.092	7406.80	5.53	0.098
5. Cactus × population(species)	3	4515.40	1.07	NS	2010.28	0.61	NS
6. Replicates	24	33974.88	1.70	0.038	26522.15	2.22	0.004

(*Continued*)

APPENDIX 1. CONTINUED

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
7. Sex	1	33606.64		1.69	NS	9022.62	1.56	NS
8. Sex × species	2	31350.25		0.79	NS	13946.76	1.20	NS
9. Sex × cactus	1	69.21		0.08	NS	148.10	0.30	NS
10. Sex × cactus × species	2	10801.58		6.47	0.0023	3553.79	3.56	0.032
11. Sex × population(species)	3	59888.07		23.91	0.0001	17415.24	11.65	0.0001
12. Error	95	79311.84				47354.13		
			W. Unknown alkene (C_{38})				X. Unknown (C_{39})	
1. Species	2	1253.94		1.26	NS	126.05	2.97	NS
2. Population(species)	3	1499.74		1.88	NS	63.77	0.88	NS
3. Cactus	1	1039.83		4.65	NS	107.23	5.36	NS
4. Cactus × species	2	19.16		0.04	NS	15.50	0.39	NS
5. Cactus × population(species)	3	671.88		2.49	0.084	60.09	3.33	0.035
6. Replicates	24	2189.03		2.22	0.003	144.04	0.92	NS
7. Sex	1	1689.51		20.40	0.020	0.01	0.00	NS
8. Sex × species	2	1427.34		8.61	0.057	48.33	2.28	NS
9. Sex × cactus	1	138.23		3.37	0.07	24.90	3.82	0.053
10. Sex × cactus × species	2	19.91		0.24	NS	4.59	0.35	NS
11. Sex × population(species)	3	248.68		2.02	NS	31.80	1.63	NS
12. Error	95	3901.38				618.41		
			Y. Unknown (C_{40})				Z. Total hydrocarbons	
1. Species	2	3.14		0.01	NS	13691361.37	1.03	NS
2. Population(species)	3	607.86		4.65	NS	20042887.39	1.73	NS
3. Cactus	1	300.93		9.72	0.052	14287083.41	9.08	0.057
4. Cactus × species	2	21.29		0.34	NS	5836835.07	1.85	NS
5. Cactus × population(species)	3	93.01		1.36	NS	4727332.59	1.59	NS
6. Replicates	24	552.01		1.42	NS	24082067.69	2.57	0.0006
7. Sex	1	696.11		24.22	0.016	4072518.47	1.52	NS
8. Sex × species	2	322.22		5.69	0.096	1902737.72	0.36	NS
9. Sex × cactus	1	108.94		6.74	0.011	54525.83	0.14	NS
10. Sex × cactus × Species	2	12.90		0.40	NS	2057103.82	2.63	0.077
11. Sex × population(species)	3	86.29		1.78	NS	8031515.40	6.85	0.0003
12. Error	95	1534.81				37105155.0		

[a] Approximate mean square ratios for the calculation of *F* values are indicated the first table (A) for C_{28} *n*-alkane. These same mean square ratios were used for each nested ANOVA.

REFERENCES

ALCORN, S. M., ORUM, T. V., STEIGERWALT, A. G., FOSTER, J. L. M., FOGLEMAN, J. C., and BRENNER, D. J. 1991. Taxonomy and pathogenicity of *Erwinia cacticida* sp. nov. *Int. J. Syst. Bacteriol*. 41:197–212.

ANTONY, C. and JALLON, J.-M. 1982. The chemical basis for sex recognition in *Drosophila melanogaster*. *J. Insect Physiol*. 28:873–880.

BOAKE, C. R. B., DEANGELIS, M. P., and ANDREADIS, D. K. 1997. Is sexual selection and species recognition a continuum? Mating behavior of the stalk-eyed fly *Drosophila heteroneura*. *Proc. Natl. Acad. Sci. U.S.A.* 94:12442–12445.

BRAZNER, J. C., and ETGES, W. J. 1993. Pre-mating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. II. Effects of larval substrates on time to copulation, mate choice, and mating propensity. *Evol. Ecol*. 7:605–624.

BYRNE, C. 1999. Behaviour–genetic analysis of lovesongs in desert species of *Drosophila*. PhD dissertation. University of Leicester, Leicester, UK.

CARSON, H. L. 2000. Sexual selection in populations: The facts require a change in the genetic definition of the species, pp. 495–512, *in* R. S. Singh and C. Krimbas (eds.). Evolutionary Genetics: From Molecules to Morphology. Cambridge University Press, New York.

COYNE, J. A., and ORR, H. A. 1989. Patterns of speciation in *Drosophila*. *Evolution* 43:362–381.

COYNE, J. A., CRITTENDEN, A. P., and MAH, K. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science* 265:1461–1464.

DOBZHANSKY, T. 1951. Genetics and the Origin of Species. Columbia University Press, New York.

DURANDO, C. M., BAKER, R. H., ETGES, W. J., HEED, W. B., WASSERMAN, M., and DESALLE, R. 2000. Phylogenetic analysis of the repleta species group of the genus *Drosophila* using multiple sources of characters. *Mol. Phylogenet. Evol*. 16:296–307.

EHRMAN, L. 1990. Developmental isolation and subsequent adult behavior of *Drosophila pseudoobscura*. *Behav. Genet*. 20:609–615.

ETGES, W. J. 1990. Direction of life history evolution in *Drosophila mojavensis*, pp. 37–56, *in* J. S. F. Barker, W. T. Starmer, and R. J. MacIntyre (eds.). Ecological and Evolutionary Genetics of *Drosophila*. Plenum Press, New York.

ETGES, W. J. 1992. Premating isolation is determined by larval substrates in cactophilic *Drosophila mojavensis*. *Evolution* 46:1945–1950.

ETGES, W. J. 1993. Genetics of host-cactus response and life-history evolution among ancestral and derived populations of cactophilic *Drosophila mojavensis*. *Evolution* 47:750–767.

ETGES, W. J. 1998. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. IV. Correlated responses in behavioral isolation to artificial selection on a life history trait. *Am. Nat*. 152:129–144.

ETGES, W. J. and AHRENS, M. A. 2001. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. V. Deep geographic variation in epicuticular hydrocarbons among isolated populations. *Am. Nat*. In press.

ETGES, W. J. and HEED, W. B. 1987. Sensitivity to larval density in populations of *Drosophila mojavensis*: Influences of host plant variation on components of fitness. *Oecologia* 71:375–381.

ETGES, W. J. and KLASSEN, C. S. 1989. Influences of atmospheric ethanol on adult *Drosophila mojavensis*: Altered metabolic rates and increases in fitness among populations. *Physiol. Zool*. 62:170–193.

ETGES, W. J., JOHNSON, W. R., DUNCAN, G. A., HUCKINS, G., and HEED, W. B. 1999. Ecological genetics of cactophilic *Drosophila*, pp. 164–214, *in* R. Robichaux (ed.). Ecology of Sonoran Desert Plants and Plant Communities. University of Arizona Press, Tucson, Arizona.

EWING, A. W. and MIYAN, J. A. 1986. Sexual selection, sexual isolation and the evolution of song in the *Drosophila repleta* group of species. *Anim. Behav*. 34:421–429.

FANARA, J. J., FONTDEVILA, A., and HASSON, E. 1999. Oviposition preference and life history traits in cactophilic *Drosophila koepferae* and *D. buzzatti* in association with their natural hosts. *Evol. Ecol*.13:173–190.

FELLOWS, D. P. and HEED, W. B. 1972. Factors affecting host plant selection in desert-adapted cactophilic *Drosophila*. *Ecology* 53:850–858.

FERVEUR, J.-F., SAVARIT, F., O'KANE, C. J., SUREAU, G., GREENSPAN, R. J., and JALLON, J.-M. 1997. Genetic feminization of pheromones and its behavioral consequences in *Drosophila* males. *Science* 276:1555–1558.

FOGLEMAN, J. C. and ABRIL, J. R. 1990. Ecological and evolutionary importance of host plant chemistry, pp. 121–141, *in* J. S. F. Barker, W. T. Starmer, and R. J. MacIntyre (eds.). Ecological and Evolutionary Genetics of *Drosophila*. Plenum Press, New York.

FOGLEMAN, J. C. and HEED, W. B. 1989. Columnar cacti and desert *Drosophila*: The chemistry of host plant specificity, pp. 1–24, *in* J. O. Schmidt (ed.). Special Biotic Relationships of the Southwest. University of New Mexico Press, Albuquerque, New Mexico.

FONTDEVILA, A., WASSERMAN, M., PLA, C., PILARES, L., ARMENGOL, R. D., SUYO, M. D. P., SANCHEZ, A., VASQUEZ, J., RUIZ, A., and GARCIA, J. L. 1990. Description and evolutionary relationships of two species of the *Drosophila mulleri* cluster (Diptera: Drosophilidae). *Ann. Entomol. Soc. Am.* 83:444–452.

HEED, W. B. 1982. The origin of *Drosophila* in the Sonoran Desert, pp. 65–80, *in* J. S. F. Barker and W. T. Starmer (eds.). Ecological Genetics and Evolution: The Cactus–Yeast–*Drosophila* Model System. Academic Press, Sydney.

HEED, W. B. and MANGAN, R. L. 1986. Community ecology of the Sonoran Desert *Drosophila*, pp. 311–345, *in* M. Ashburner, H. L. Carson, and J. J. N. Thompson (eds.). The Genetics and Biology of *Drosophila*. Academic Press, New York.

JALLON, J.-M. 1984. A few chemical words exchanged during courtship and mating of *Drosophila melanogaster*. *Behav. Genet.* 14:441–478.

JOHNSON, W. R. 1980. Chromosomal polymorphism in natural populations of the desert adapted species, *Drosophila mojavensis*. PhD dissertation. University of Arizona, Tucson Arizona.

KIM, Y. K. and EHRMAN, L. 1998. Developmental isolation and subsequent adult behavior of *Drosophila paulistorum*. IV. Courtship. *Behav. Genet.* 28:57–65.

KIM, Y.-K., KOEPFER, H. R., and EHRMAN, L. 1996. Developmental isolation and subsequent adult behaviour of *Drosophila paulistorum*. III. Alternative rearing. *Behav. Genet*. 26:27–37.

MARKOW, T. A. and MAVEETY, N. 1985. More character displacement for reproductive isolation in the mulleri complex. *Dros. Inf. Serv*. 61:115.

MARKOW, T. A. and TOOLSON, E. C. 1990. Temperature effects on epicuticular hydrocarbons and sexual isolation in *Drosophila mojavensis*, pp. 315–331, *in* J. S. F. Barker, W. T. Starmer, and R. J. MacIntyre (eds.). Ecological and Evolutionary Genetics of *Drosophila*. Plenum Press, New York.

MARKOW, T. A. FOGLEMAN, J. C., and HEED, W. B. 1983. Reproductive isolation in Sonoran Desert *Drosophila*. *Evolution* 37:649–652.

MASTERS, J. C. 2000. The role of selection in speciation, pp. 513–531, *in* R. S. Singh and C. Krimbas (eds.). Evolutionary Genetics: From Molecules to Morphology. Cambridge University Press, New York.

NEMOTO, T., DOI, M., OSHIO, K., MATSUBAYASHI, H., OGUMA, Y., SUZUKI, T., and KUWAHARA, Y. 1994. (*Z*,*Z*)-5,27-Tritriacontadiene: Major sex pheromone of *Drosophila pallidosa* (Diptera: Drosophilidae). *J. Chem. Ecol*. 20:3029–3037.

NOOR, M. A. 1995. Speciation driven by natural selection in *Drosophila*. *Nature* 375:674–675.

NOOR, M. A. F. 1999. Reinforcement and other consequences of sympatry. *Heredity* 83:503–508.

OGUMA, Y., NEMOTO, T., and KUWAHARA, Y. 1992a. (*Z*)-11-Pentacosene is the major sex pheromone component in *Drosophila virilis*. *Chemoecology* 3:60–64.

OGUMA, Y., NEMOTO, T., and KUWAHARA, Y. 1992b. A sex pheromone study of a fruit-fly *Drosophila virilis* Sturtevant (Diptera: Drosophilidae): Additive effect of cuticular alkadienes to the major sex pheromone. *Appl. Entomol. Zool*. 27:499–505.

PATERSON, H. E. H. 1993. Evolution and the Recognition Concept of Species: Collected Writings. Johns Hopkins University, Baltimore, Maryland.

RUIZ, A. and HEED, W. B. 1988. Host-plant specificity in the cactophilic *Drosophila mulleri* species complex. *J. Anim. Ecol*. 57:237–249.

RUIZ, A., HEED, W. B., and WASSERMAN, M. 1990. Evolution of the *mojavensis* cluster of cactophilic *Drosophila* with descriptions of two new species. *J. Hered*. 81:30–42.

SAS INSTITUTE, I. 1989. SAS/STAT User's Guide, Version 6. SAS Institute Inc., Cary, North Carolina.

SAUER, J. R. and WILLIAMS, B. K. 1989. Generalized procedures for testing hypotheses about survival or recovery rates. *J. Wildl. Manage*. 53:137–142.

SPIETH, H. T. 1974. Courtship behavior in *Drosophila*. *Annu. Rev. Entomol*. 19:385–405.

STALKER, H. D. 1942. Sexual isolation studies in the species complex *Drosophila virilis*. *Genetics* 27:238–257.

STARMER, W. T. 1982. Analysis of community structure of yeasts associated with the decaying stems of cactus. I. *Stenocereus gummosus*. *Microb. Ecol*. 8:71–81.

STENNETT, M. D. and ETGES, W. J. 1997. Pre-mating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. III. Epicuticular hydrocarbon variation is determined by use of different host plants in *Drosophila mojavensis* and *Drosophila arizonae*. *J. Chem Ecol*. 23:2803–2824.

TOOLSON, E. C., MARKOW, T. A., JACKSON, L. L., and HOWARD, R. W. 1990. Epicuticular hydrocarbon composition of wild and laboratory-reared *Drosophila mojavensis* Patterson and Crow (Diptera: Drosophilidae). *Ann. Entomol. Soc. Am*. 83:1165–1176.

WASSERMAN, M. 1992. Cytological evolution of the *Drosophila repleta* species group, pp. 455–552, *in* C. B. Krimbas and J. R. Powell (eds.). *Drosophila* Inversion Polymorphism. CRC Press, Boca Raton, Florida.

WASSERMAN, M. and KOEPFER, H. R. 1977. Character displacement for sexual isolation between *Drosophila mojavensis* and *Drosophila arizonensis*. *Evolution* 31:812–823.

ZOUROS, E. and D'ENTREMONT, C. J. 1980. Sexual isolation among populations of *Drosophila mojavensis*: Response to pressure from a related species. *Evolution* 34:421–430.

Journal of Chemical Ecology, Vol. 27, No. 10, October 2001 (© 2001)

CALL FOR PAPERS

SPECIAL ISSUE ON AQUATIC CHEMICAL ECOLOGY

There will be a special issue of the *Journal of Chemical Ecology* devoted entirely to papers dealing with "Aquatic Chemical Ecology". Publication is planned for late summer/early fall 2002. The issue will consist of invited mini-reviews and contributed papers. Those wishing to submit a paper for consideration for this special issue should do so by February 1, 2001.

Please send the original and two copies of the manuscript (including illustrations) and the names and addresses of three potential reviewers to:

John T. Romeo
Journal of Chemical Ecology
Department of Biology
University of South Florida
Tampa, Florida 33620-5200

0098-0331/01/1000-2151$19.50/0

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

SYNTHESIS AND CHARACTERIZATION OF DIEPOXYALKENES DERIVED FROM (3*Z*,6*Z*,9*Z*)-TRIENES: LYMANTRIID SEX PHEROMONES AND THEIR CANDIDATES

HIROYUKI YAMAZAWA,[1] NAOTO NAKAJIMA,[1] SADAO WAKAMURA,[2] NORIO ARAKAKI,[3] MASANOBU YAMAMOTO,[1] and TETSU ANDO[1,*]

[1]*Graduate School of Bio-Applications and Systems Engineering*
Tokyo University of Agriculture and Technology
Koganei, Tokyo 184-8588, Japan

[2]*Laboratory of Insect Behavior*
National Institute of Agrobiological Science
Ohwashi 1-2, Tsukuba, Ibaraki 305-8634, Japan

[3]*Okinawa Prefectural Agricultural Experimental Station*
Sakiyama, Naha, Okinawa 903-0814, Japan

(Received March 6, 2001; accepted July 5, 2001)

Abstract—All stereoisomers of three diepoxyalkenes derived from (3*Z*,6*Z*,9*Z*)-trienes with a C_{21}, C_{19}, or C_{18} straight chain, lymantriid sex pheromones and their candidates, were synthesized by MCPBA oxidation of optically active epoxyalkadienes. Their chromatographic behaviors were examined with GC and LC equipped with achiral and chiral columns. Detailed inspection of the mass spectra of these epoxides indicated the following diagnostic ions for determining the chemical structures: *m/z* 128, 167, M-87 and M-85 for (*Z*)-*cis*-3,4-*cis*-6,7-diepoxy-9-enes; *m/z* 111, M-125 and M-69 for (*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-3-enes; and *m/z* M-125 and M-139 for (*Z*)-*cis*-3,4-*cis*-9,10-diepoxy-6-enes. Mass chromatographic analysis that monitored these fragment ions revealed the existence of a new pheromonal compound with a C_{21} chain in an extract from virgin females of a lymantriid species, *Perina nuda* F. The three diepoxyalkenes were converted into the corresponding DMDS adducts, which showed characteristic ions from fragmentation between the two thiomethyl groups, reflecting the position of an original double bond.

Key Words—*cis*-epoxides, sex pheromones, Lepidoptera, Lymantriidae, mass spectrometry, chiral HPLC, stereochemistry, diepoxyhenicosene, leucomalure.

*To whom correspondence should be addressed. E-mail: antetsu@cc.tuat.ac.jp

0098-0331/01/1100-2153$19.50/0

INTRODUCTION

Sex pheromones have been identified from female moths of more than 500 species. About 75% of them are composed of unsaturated C_{10}–C_{18} straight-chain compounds with a functional group at the terminal position (type I), which are biosynthesized in the pheromone glands from saturated fatty acids. Another 15% consist of unbranched C_{17}–C_{23} (3*Z*,6*Z*,9*Z*)-trienes, (6*Z*,9*Z*)-dienes, and their monoepoxy derivatives (type II), which lack a terminal functional group and are biosynthesized from linolenic acid and linoleic acid. The type II pheromones have been identified from geometrid, noctuid, arctiid, and lymantriid species, which belong to highly evolved families (Arn et al., 1992, 1997; Millar, 2000).

Despite the great number of species in the above families, the structural diversity known for pheromonal compounds in type II is limited. Some females may be able to oxidize monoepoxy derivatives further to diepoxides, potentially generating a greatly increased number of pheromone components. From a (3*Z*,6*Z*,9*Z*)-triene, for example, epoxidation of two double bonds makes three positional isomers of diepoxyalkenes (**1**–**3**) in Figure 1, each of which has four stereoisomers. The first example of a diepoxy compound was reported by Gries et al. (1997), who have identified (*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-3-henicosene (**2a**, leucomalure) from the satin moth, *Leucoma salicis* L. Although the syntheses of all stereoisomers of **2a** were published in our previous paper (Yamamoto et al., 1999a), other compounds with a different chain length and epoxy rings at different positions are also candidates for lymantriid pheromones. Therefore, we systematically synthesized all stereoisomers of the diepoxyalkenes (**1**–**3**) with a C_{21}, C_{19}, or C_{18} chain (Figure 1). This paper presents a convenient method to prepare them and describes their chromatographic behaviors on achiral and chiral columns. Furthermore, these diepoxyalkenes were analyzed by GC-MS without and with derivatization. We report here the diagnostic fragment ions for distinction of the positional isomers, which were applied to the identification of (*Z*)-*cis*-3,4-*cis*-6,7-diepoxy-3-henicosene (**1a**), a minor component of the sex pheromone of *Perina nuda* F. This lymantriid female moth secretes a monoepoxy derivative as a main component of its pheromone (Wakamura et al., unpublished date).

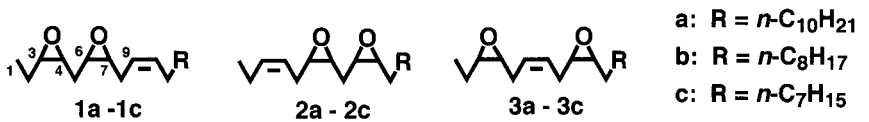


FIG. 1. Chemical structures of diepoxyalkenes with a C_{21}, C_{19}, or C_{18} chain: (*Z*)-*cis*-3,4-*cis*-6,7-diepoxy-9-enes (**1a**–**1c**), (*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-3-enes (**2a**–**2c**), and (*Z*)-*cis*-3,4-*cis*-9,10-diepoxy-6-enes (**3a**–**3c**).

METHODS AND MATERIALS

Chromatography. GC analysis was done on an HP GC System 6890 series with a flame ionization detector and a DB-23 or DB-1 capillary column (0.25 mm ID × 30 m, J & W Scientific). The temperature program was 50°C for 2 min, 10°C/min to 160°C, and finally 4°C/min to 220°C. HPLC involved a JASCO PU-980 liquid chromatograph equipped with an integrator (System Instrument Chromatocorder 21J), an RI detector (Labo System RI-98SCOPE, Tokyo, Japan), and one of the following columns: a Grand pack ODS column (2.0 cm ID × 25 cm, Senshukagaku, Tokyo, Japan) or a Chiralpak AD column (0.46 cm ID × 25 cm, Daicel Chemical Industry Co., Ltd., Tokyo, Japan). Into the former reversed-phase achiral column, a mixture of several milligrams was injected per run by using 5% water in MeOH as a solvent (4.0 ml/min). Into the latter normal phase chiral column, a mixture of less than 0.1 mg was injected per run by using 0.5% 2-propanol in *n*-hexane (0.45 ml/min). A medium pressure liquid chromatographic (MPLC) system was constructed with a solvent pump (JASCO 880- PU), double Lobar columns (Merck Lichroprep Si 60, 1.0 cm ID × 24 cm, 40–63 μm), and an RI detector. About 20 mg of a mixture were injected per run by using 8% THF in *n*-hexane as a solvent (2.0 ml/min).

Instruments. ^{1}H and ^{13}C NMR spectra were recorded on a Jeol Alpha500 Fourier transform spectrometer at 500.2 and 125.7 MHz, respectively, for $CDCl_3$ solutions containing TMS as an internal standard. ^{1}H–^{1}H-COSY and HSQC spectra were also measured with the same spectrometer, using the usual pulse sequences and parameters. Electron impact (EI) GC-MS was performed on a Jeol JMS SX-102A mass spectrometer (magnetic sector type) equipped with an HP-1 (0.25 mm ID × 15 m, Hewlett-Packard Co.) or an HP 5973 mass spectrometer (quadrupole type) equipped with another HP-1 capillary column (0.32 mm ID × 30 m). The column temperature program for the Jeol JMS SX-102A was 50°C for 1 min, 20°C/min to 150°C, then 5°C/min to 220°C, and for HP 5973 it was 120°C for 2 min, then 6°C/min to 270°C. The ionization voltage was 70 eV, and the ion source temperature was 230°C. The specific rotation of each $CHCl_3$ solution was measured on a JASCO DIP-4 polarimeter.

*(*Z*)-*cis*-3,4-*cis*-6,7-Diepoxy-9-enes (**1a**–**1c**) and (*Z*)-*cis*-6,7-*cis*-9,10-Diepoxy-3-enes (**2a**–**2c**).* Synthesis of all stereoisomers of **1a** and **2a** was reported previously (Yamamoto et al., 1999a). In brief, MCPBA oxidation of (6*S*,7*R*)-**4a** produced two pairs of diastereomers of **1a** with a 3*S*,4*R*,6*S*,7*R* and 3*R*,4*S*,6*S*,7*R* configuration (**1a-i** and **1a-iii**) and **2a** with a 6*S*,7*R*,9*S*,10*R* and 6*S*,7*R*,9*R*,10*S* configuration (**2a-i** and **2a-ii**) in a ratio of 2 : 3 : 2 : 3. These four components were separable by MPLC equipped with a Lobar column, and their chemical structures were confirmed by 2D-NMR. From (6*R*,7*S*)-**4a**, other stereoisomers of **1a** with a 3*S*,4*R*,6*R*,7*S* and 3*R*,4*S*,6*R*,7*S* configuration (**1a-ii** and **1a-iv**) and **2a** with a 6*R*,7*S*,9S,10*R* and 6*R*,7*S*,9*R*,10*S* configuration (**2a-iii** and **2a-iv**) were

synthesized. By using the same procedure as for the diepoxy compounds with a C_{21} chain, all stereoisomers of 3,4-6,7-diepoxy-9-enes (**1b** and **1c**) and 6,7-9,10-diepoxy-3-enes (**2b** and **2c**) with a C_{19} or C_{18} chain were prepared by starting from optically active (3*Z*,9*Z*)-*cis*-6,7-epoxy-3,9-nonadecadiene (**4b**) and octadecadiene (**4c**). NMR data of newly synthesized C_{19} and C_{18} diepoxy compounds were analogous to those of the corresponding C_{21} compounds, except for the intensity of the methylene protons at δ1.25 ppm, and the number of methylene carbons around δ29.5 ppm.

*(*Z*)-*cis*-3,4-*cis*-9,10-Diepoxy-6-enes (**3a–3c**).* Optically pure 3*S*,4*R* and 3*R*,4*S* isomers of (6*Z*,9*Z*)-*cis*-3,4-epoxy-6,9-henicosadiene (**5a**) were obtained by chiral HPLC resolution of the racemic mixture (Qin et al., 1997). These enantiomers (5 mg, 16 μmol), separately dissolved in dry CH_2Cl_2 (20 ml), were added to MCPBA (70% pure, 4.0 mg, 23 μmol) and stirred at 0°C for 1 hr. After further stirring at room temperature for 2 hr, the reaction mixtures were washed with 3 N NaOH solutions and dried over Na_2SO_4. Preparative TLC gave a mixed product of two diastereomers of **3a** with 3*S*,4*R*,9*S*,10*R* and 3*S*,4*R*,9*R*,10*S* configurations (**3a-i** and **3a-ii**, 2.6 mg, 50% yield) derived from (3*S*,4*R*)-**5a**, and those with 3*R*,4*S*,9*S*,10*R* and 3*R*,4*S*,9*R*,10*S* configurations (**3a-iii** and **3a-iv**, 2.8 mg, 54% yield) derived from (3*R*,4*S*)-**5a**. Each compound was isolated by chiral HPLC with a Chiralpak AD column. NMR of **3a-i** and **3a-iv:** ^{1}H δ ppm: 0.88 (H-21, t, J = 7 Hz), 1.06 (H-1, t, J = 7.5 Hz), ~1.25 (H-12–20, broad), ~1.55 (H-2 and 11, m), ~2.25 and ~2.4 (H-5 and 8, m), ~2.95 (H-3, 4, 9 and 10, m), 5.60 (H-6 and 7, m); ^{13}C δ ppm: 10.6 (C-1), 14.1 (C-21), 21.1 (C-2), 22.7 (C-20), 26.4, 26.5, and 26.6 (C-5, 8, and 12), 27.8 (C-11), 29.4–29.7 (C-13–18), 31.9 (C-19), 56.22 and 56.35 (C-9 and 10), 57.18 (C-4), 58.30 (C-3), 126.80 and 126.87 (C-6 and 7). NMR of **3a-ii** and **3a-iii**: ^{1}H δ ppm: 0.89 (H-21, t, J = 7 Hz), 1.06 (H-1, t, J = 7.5 Hz), ~1.25 (H-12–20, broad), ~1.55 (H-2 and 11, m), ~2.25 and ~2.35 (H-5 and 8, m), ~2.95 (H-3, 4, 9 and 10, m), 5.61 (H-6 and 7, m); ^{13}C δ ppm: 10.6 (C-1), 14.1 (C-21), 21.1 (C-2), 22.7 (C-20), 26.4, 26.5, and 26.6 (C-5, 8, and 12), 27.8 (C-11), 29.4–29.7 (C-13–18), 31.9 (C-19), 56.30 and 56.43 (C-9 and 10), 57.20 (C-4), 58.32 (C-3), 126.86 and 126.93 (C-6 and 7). $[\alpha]_D$ at 25°C: **3a-i** + 0.4 (c = 0.4), **3a-ii** + 6.0 (c = 1.7), **3a-iii** − 5.7 (c = 2.1), and **3a-iv** − 0.4 (c = 0.6).

Optically pure 9*S*,10*R* and 9*R*,10*S* isomers of (3*Z*,6*Z*)-*cis*-9,10-epoxy-3,6-henicosadiene (**6a**) were also obtained by chiral HPLC resolution of the racemic mixture (Qin et al., 1997). Two other sets of diastereomers were prepared, i.e., **3a-i** and **3a-iii** from (9*S*,10*R*)-**6a** and **3a-ii** and **3a-iv** from (9*R*,10*S*)-**6a**. By the same procedure, all stereoisomers of **3b** and **3c** were synthesized from optically active (6*Z*,9*Z*)-*cis*-3,4-epoxy-6,9-nonadecadiene (**5b**) and octadecadiene (**5c**).

Pheromone Extract. Pheromone glands were removed from 1-day-old virgin females of *P. nuda* around 30 min after lights went on, and soaked in *n*-hexane for 15 min to extract the pheromone. This extract was fractionated on a column with Florisil (200 mg, 100–200 mesh, Floridin Co.). Substances were eluted

successively with 1 ml each of *n*-hexane and 5%, 20%, and 50% ether in *n*-hexane, and an aliquot [3 female equivalents (FE)] of the third fraction, which was recovered with 20% ether in *n*-hexane and included diepoxyalkenes, was analyzed by using a JMS SX-102A GC-MS.

Derivatization with DMDS. Each synthetic diepoxyalkene (10 μg) was dissolved in a mixture of DMDS (10 μl) and diethyl ether (100 μl) including iodine (1.8 mg). The reaction mixture was kept at 40°C for 3 hr and diluted with diethyl ether after cooling. Iodine was removed by shaking with a 10% aqueous $Na_2S_2O_3$ solution, and crude products were analyzed by an HP 5973 GC-MS.

RESULTS

Synthesis of C_{21}, C_{19}, and C_{18} Diepoxyalkenes. MCPBA oxidation of 6,7-epoxy-3,9-dienes (**4**) proceeded without regioselectivity and produced a mixture of 3,4-6,7-diepoxy-9-enes (**1**) and 6,7-9,10-diepoxy-3-enes (**2**). (6*S*,7*R*)-**4a** was converted into four C_{21} diepoxy compounds with a 6*S*,7*R* configuration (**1a-i**, **1a-iii**, **2a-i**, and **2a-ii**), and (6*R*,7*S*)-**4a** was converted into the others with a 6*R*,7*S* configuration (**1a-ii**, **1a-iv**, **2a-iii**, and **2a-iv** as shown in Figure 2A (Yamamoto et al., 1999a). Each compound isolated by a chromatographic technique was identified by the NMR data. The methyl protons at position 1 of **1a** resonated

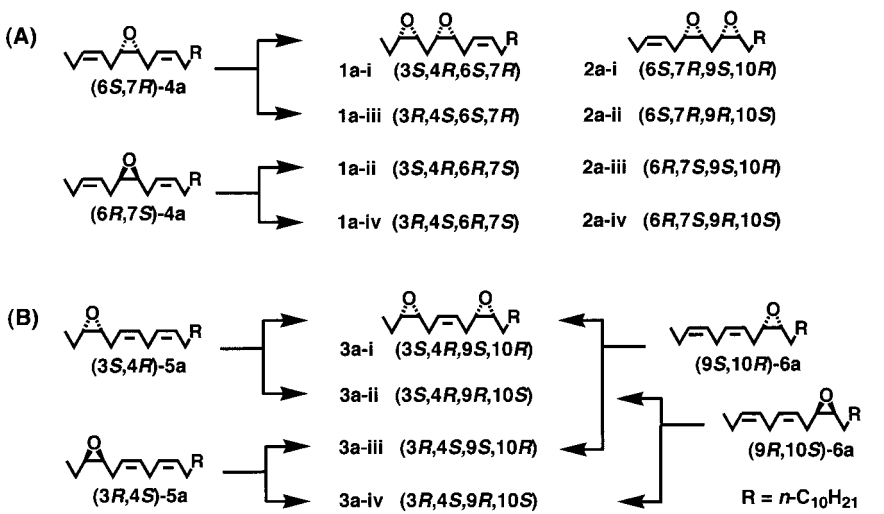


FIG. 2. Conversion of optically active epoxyhenicosadienes (**4a**–**6a**) into diepoxyhenicosenes (**1a**–**3a**): synthesis of all stereoisomers of **1a** and **2a** from *cis*-6,7-epoxy-3,9-henicosadiene (**4a**) (A), and synthesis of all stereoisomers of **3a** form *cis*-3,4-epoxy-6,9-henicosadiene (**5a**) and *cis*-9,10-epoxy-3,6-henicosadiene (**6a**) (B).

at a lower field than those of **2a**, and splitting of the methylene protons between two epoxy rings in stereoisomers with a symmetrical S^*,R^*,R^*,S^* configuration[4] was simpler than those with another S^*,R^*,S^*,R^* configuration (Yamamoto et al., 1999a). All stereoisomers of **1b** and **2b** with a C_{19} chain and **1c** and **2c** with a C_{18} chain were synthesized and characterized in the same manner. Oxidation of 3,4-epoxy-6,9-dienes (**5**) and 9,10-epoxy-3,6-dienes (**6**) happened exclusively at one of two double bonds located far from the epoxy ring of the parent monoepoxides. Stereoisomers of **3** were prepared by selective monooxidation of optically active **5** or **6**. MCPBA oxidation of (3*S*,4*R*)-**5a** produced a mixture of 3*S*,4*R*,9*S*,10*R* and 3*S*,4*R*,9*R*,10*S* isomers of **3a** (**3a-i** and **3a-ii**), and oxidation of (9*S*,10*R*)-**6a** produced another mixture of 3*S*,4*R*,9*S*,10*R* and 3*R*,4*S*,9*S*,10*R* isomers (**3a-i** and **3a-iii**), as shown in Figure 2B. Since these three stereoisomers were separable on a chiral HPLC column (Table 1) and these two sets of the mixtures include **3a-i** as a common component, the absolute configuration of each separated isomer could be assigned. In addition, the 3*R*,4*S*,9*R*,10*S* isomer of **3a** (**3a-iv**) was prepared as a common component of the monooxidation products from (3*R*,4*S*)-**5a** and (9*R*,10*S*)-**6a**. All stereoisomers of 3,4-9,10-diepoxy-6-enes with a C_{19} or C_{18} chain (**3b** and **3c**) were synthesized in the same manner.

Chromatographic Behavior of Diepoxyalkenes. Table 1 shows the retention times of diepoxyhenicosenes (**1a**–**3a**) from GC and LC analyses. On capillary GC columns, the diastereomers of each positional isomer were separable. The isomers with the S^*,R^*,R^*,S^* configuration, orienting two epoxy rings to the opposite face, eluted faster than the isomers with the S^*,R^*,S^*,R^* configuration, orienting two epoxy rings to the same face. A polar DB-23 column was better for the separation of the diastereomers than a nonpolar DB-1 column. However, **1a** and **2a** with the S^*,R^*,R^*,S^* configuration showed the same retention time, and it was not possible to separate all six isomers of the diepoxide on either GC column. GC could not be used to determine unambiguously which isomer was present in all cases. A chiral capillary GC column (Chiraldex A-PH), which resolved racemic 3,4-epoxy-6,9-nonadecadiene **5b** (Ando et al., 1997), did not separate the enantiomers of any diepoxyalkenes synthesized in this study. The diastereomers of **1a** and **2a** were also separated by either normal phase LC by using a Lobar column or reversed-phase LC with an ODS column. Although a diastereomeric mixture of **3a** showed only one peak on the LC, the first half of the elution was enriched with (S^*,R^*,S^*,R^*)-**3a**, and the second half with (S^*,R^*,R^*,S^*)-**3a**. Thus, each diepoxide with the S^*,R^*,S^*,R^* configuration eluted faster than the corresponding diastereomer on the LC, interestingly, in the reverse order of that on the GC. Since **3a** has a markedly short retention time on an ODS column, the achiral HPLC is also useful for separating **3a**

[4] S^*, R^*, R^*, S^* means 3*S*,4*R*,6*R*,7*S* and 3*R*,4*S*,6*S*,7*R* for **1**, 6*S*,7*R*,9*R*,1*S* and 6*R*,7*S*,9*S*,10*R* for **2**, and 3*S*,4*R*,9*R*,10*S* and 3*R*,4*S*,9*S*,10*R* for **3**.

TABLE 1. CHROMATOGRAPHIC BEHAVIOR OF DIEPOXYALKENES (**1a**–**3a**) DERIVED FROM (*Z*3,*Z*6,*Z*9)-3,6,9-HENICOSATRIENE

	Retention time (min)					
	3,4-6,7-Diepoxide (**1a**)		6,7-9,10-Diepoxide (**2a**)		3,4-9,10-Diepoxide (**3a**)	
Chromatography	S^*,R^*,S^*,R^{*a}	S^*,R^*,R^*,S^{*b}	S^*,R^*,S^*,R^{*a}	S^*,R^*,R^*,S^{*b}	S^*,R^*,S^*,R^{*a}	S^*,R^*,R^*,S^{*b}
GC[c]						
DB-23	30.45	29.28	30.66	29.28	30.92	30.56
DB-1	23.58	23.28	23.77	23.28	23.76	23.62
MPLC[d]	35.5	40.7	32.0	38.3	35.0 (Fr.1)[e]	35.0 (Fr. 2)[e]
Achiral HPLC[f]	45.6	48.8	41.5	44.5	39.7 (Fr.1)[e]	39.7 (Fr. 2)[e]
Chiral HPLC[g]	**i**: 21.4	**ii**: 19.3	**i**: 25.6	**ii**: 17.7	**i**: 30.2	**ii**: 29.6
	iv: 23.0 (1.11)	**iii**: 23.5 (1.34)	**iv**: 22.1 (1.23)	**iii**: 24.1 (1.63)	**iv**: 28.1 (1.10)	**iii**: 30.6 (1.10)

[a] S^*,R^*,S^*,R^* means 3*S*,4*R*,6*S*,7*R* and 3*R*,4*S*,6*R*,7*S* isomers of **1a** (**1a-i** and **1a-iv**), 6*S*,7*R*,9*S*,10*R* and 6*R*,7S,9*R*,10*S* isomers of **2a** (**2a-i** and **2a-iv**), and 3*S*,4*R*,9*S*,10*R* and 3*R*,4*S*,9*R*,10*S* isomers of **3a** (**3a-i** and **3a-iv**).

[b] S^*,R^*,R^*,S^* means 3*S*,4*R*,6*R*,7*S* and 3*R*,4*S*,6*S*,7*R* isomers of **1a** (**1a-ii** and **1a-iii**), 6*S*,7*R*,9*R*,10*S* and 6*R*,7*S*,9*S*,10*R* isomers of **2a** (**2a-ii** and **2a-iii**), and 3*S*,4*R*,9*R*,10*S* and 3*R*,4*S*,9*S*,10*R* isomers of **3a** (**3a-ii** and **3a-iii**).

[c] Capillary column (0.25 mm ID × 30 m); temperature program: 50°C for 2 min, 10°C/min to 160°C, and finally 4°C/min to 220°C.

[d] Lobar column: Merck Lichroprep Si 60 (1.0 cm ID × 24 cm, 40–63 μm); solvent: 8% THF in *n*-hexane at a flow rate of 2.0 ml/min.

[e] The first half fraction (Fr. 1) is enriched with (S^*,R^*,S^*,R^*)-**3a**, and the second half fraction (Fr. 2) with (S^*,R^*,R^*,S^*)-**3a**.

[f] Reversed-phase column: Grand pack ODS (2.0 cm ID × 25 cm); solvent: 5% water in MeOH at a flow rate of 4.0 ml/min.

[g] Chiral column: Chiralpak AD (0.46 cm ID × 25 cm); solvent: 0.5% 2-propanol in *n*-hexane at a flow rate of 0.45 ml/min. Values in parentheses mean separation factors (α).

from the other two positional isomers (**1a** and **2a**). Satisfactory resolution of each pair of diepoxyalkene enantiomers was accomplished by chiral HPLC, as indicated by retention times and separation factors (α). Besides the enantiomers, the chiral AD column usefully separated some of the diastereomers and positional isomers of the diepoxyalkenes. The separation profiles of diepoxynonadecenes (**1b**–**3b**) and diepoxyoctadecenes (**1c**–**3c**) were similar to those of **1a**–**3a** listed in Table 1. Without distinction of chain lengths, the elution orders of positional isomers, diastereomers, and enantiomers were the same with all chromatographic methods.

MS Analysis of Diepoxyalkenes. Figure 3 shows the EI mass spectra of three positional isomers of diepoxynonadecene (**1b**–**3b**). In addition to small M^+ and $[M\text{-}18]^+$ ions, each positional isomer showed characteristic fragment ions, i.e., *m/z* 128, 167, 207, and 209 for **1b** (Figure 3A), *m/z* 111, 169, and 225 for **2b** (Figure 3B), and *m/z* 155 and 169 for **3b** (Figure 3C). These ions seemed to reflect the chemical structures, and this was confirmed by a systematic MS analysis of the positional isomers of diepoxyalkenes with a C_{21}, C_{19}, and C_{18} chain. As indicated

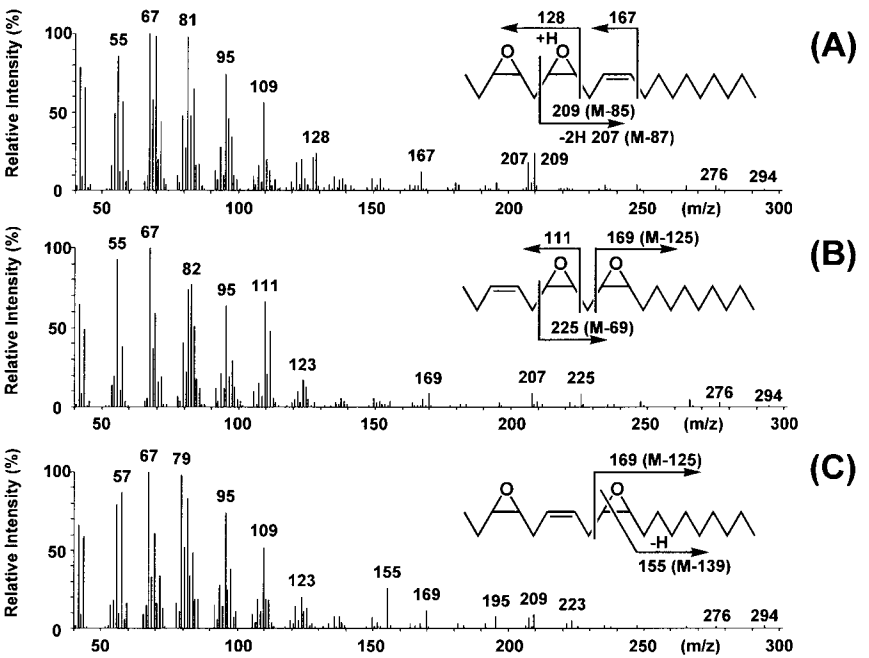


FIG. 3. Mass spectra of three positional isomers of diepoxyalkene: (*Z*)-*cis*-3,4-*cis*-6,7-diepoxy-9-nonadecene (**1b**) (A), (*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-3-nonadecene (**2b**) (B), and (*Z*)-*cis*-3,4-*cis*-9,10-diepoxy-6-nonadecene (**3b**) (C).

in Table 2, the results suggest the following diagnostic ions for determining the position of the two epoxy rings: *m/z* 128, 167, M-87 and M-85 for 3,4-6,7-diepoxy-9-enes (**1**); *m/z* 111, M-125 and M-69 for 6,7-9,10-diepoxy-3-enes (**2**); and *m/z* M-139 and M-125 for 3,4-9,10-diepoxy-6-enes (**3**).

GC-MS Analysis of a Pheromone Extract of P. nuda. Mass chromatograms of a mixture that included three positional isomers of synthetic diepoxyhenicosene, (*S**,*R**,*S**,*R**)-**1a**, (*S**,*R**,*R**,*S**)-**2a**, and (*S**,*R**,*S**,*R**)-**3a**, indicated their coexistence in a ratio of 1 : 1 : 1 and an absence of their diastereomers in the mixture (Figure 4A). The pheromone extract that included diepoxides scarcely showed ion peaks at *m/z* 183 (M-139) and 197 (M-125), which were diagnostic for **2a** and **3a**, at the retention times of these diepoxides. However, diagnostic ions for **1a** at *m/z* 128, 167, 235 (M-87), and 237 (M-85) were observed at two different retention times (Figure 4B). This measurement revealed that *P. nuda* females secrete at least

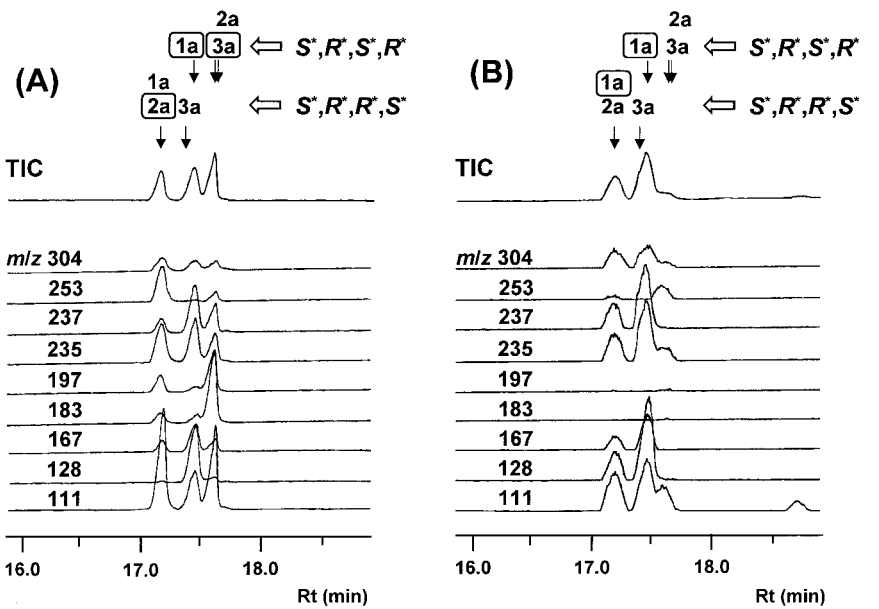


FIG. 4. Mass chromatography of a mixture of three positional isomers of synthetic diepoxyhenicosenes (**1a**–**3a**) (A) and a natural pheromone of *Perina nuda* (B), monitoring their M^+ and diagnostic fragment ions listed in Table 2. GC-MS analysis was achieved with a Jeol JMS SX-102A mass spectrometer equipped with an HP-1 column (0.25 mm ID × 15 m). The column temperature program was 50°C for 1 min, 20°C/min to 150°C, then 5°C/min to 220°C. Synthetic standards [(*S**,*R**,*S**,*R**)-**1a**, (*S**,*R**,*R**,*S**)-**2a**, and (*S**,*R**,*S**,*R**)-**3a**] used in (A) and components estimated for the pheromone [(*S**,*R**,*R**,*S**)-**1a** and (*S**,*R**,*S**,*R**)-**1a**] in (B) are shown with compound numbers in frames.

TABLE 2. GC-MS DATA OF DIEPOXYALKENES (**1–3**) WITH AN *S**,*R**,*S**,*R** CONFIGURATION[a]

		R_t (min)	Base ion *m/z*	M^+ *m/z*	Relative intensity (%) of ions at indicated *m/z*									
					111	128	167	M-139[b]	M-125[c]	M-87[d]	M-85[e]	M-69[f]	M-18[g]	M
cis-3,4-*cis*-6,7-Diepoxy-9-ene														
1a	C_{21}	17.40	69	322	16	32	16	1	1	22	27	0	4	1
1b	C_{19}	14.32	67, 69	294	13	24	12	1	1	18	24	0	3	0.5
1c	C_{18}	12.80	67, 81	280	19	21	11	3	1	18	25	0	3	0.5
cis-6,7-*cis*-9,10-Diepoxy-3-ene														
2a	C_{21}	17.57	67	322	54	1	4	5	9	9	5	10	4	1
2b	C_{19}	14.48	67	294	48	0	5	4	9	9	4	8	3	0.5
2c	C_{18}	12.94	67	280	41	0	3	4	10	9	3	7	2	0.5
cis-3,4-*cis*-9,10-Diepoxy-6-ene														
3a	C_{21}	17.63	67, 79	322	22	1	4	25	12	9	9	1	3	0.2
3b	C_{19}	14.54	67, 79	294	18	1	3	26	12	7	9	1	1	2
3c	C_{18}	13.01	67, 79	280	18	0	3	26	13	6	7	1	2	1

[a] Measured by JMS SX-102A with a HP-1 capillary column. Temperature program: 50°C for 1 min, 20°C/min to 150°C, then 5°C/min to 220°C.
[b] 183 for C_{21}, 155 for C_{19}, and 141 for C_{18}.
[c] 197 for C_{21}, 169 for C_{19}, and 155 for C_{18}.
[d] 235 for C_{21}, 207 for C_{19}, and 193 for C_{18}.
[e] 237 for C_{21}, 209 for C_{19}, and 195 for C_{18}.
[f] 253 for C_{21}, 225 for C_{19}, and 211 for C_{18}.
[g] 304 for C_{21}, 276 for C_{19}, and 262 for C_{18}.

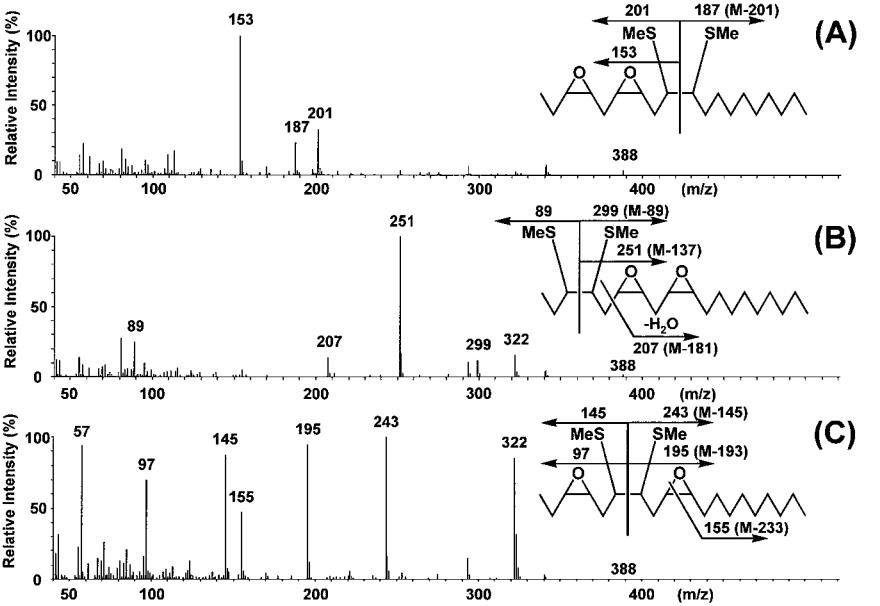


FIG. 5. Mass spectra of DMDS adducts derived from (*Z*)-*cis*-3,4-*cis*-6,7-diepoxy-9- nonadecene (**1b**) (A), (*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-3-nonadecene (**2b**) (B), and (*Z*)-*cis*-3,4-*cis*-9,10-diepoxy-6-nonadecene (**3b**) (C).

two stereoisomers of **1a**. The ratio of (*S**,*R**,*S**,*R**)-**1a** and (*S**,*R**,*R**,*S**)-**1a** was about 3 : 2.

GC-MS Analysis of DMDS Adducts Derived from Diepoxyalkenes. Diepoxyalkenes (**1**–**3**) were converted into DMDS adducts, which showed informative fragment ions reflecting the position of their original double bonds. DMDS adducts derived from **1** produced characteristic ions at *m/z* 201 and M-201 from cleavage of the bond between the 9 and 10 positions. In addition, they showed other significant ions at *m/z* 153, probably derived by further removal of MeSH from the *m/z* 201 ion (Figure 5A). DMDS adducts of **2** produced diagnostic ions at *m/z* 89, M-181, M-137, and M-89 (Figure 5B). The ion at *m/z* M-89 arises from cleavage of the bond between the 3 and 4 positions and might be further degraded to the ion at *m/z* M-137 by removal of MeSH. DMDS adducts of **3** produced abundant ions at *m/z* 97, 145, M-233, M-193, and M-145 (Figure 5C). The ions at *m/z* 145 and M-145 are probably derived from cleavage of the bond between the 6 and 7 positions and might be further degraded to the ions at *m/z* 97 and M-193 by removal of MeSH. The relative intensities of the fragment ions produced from the DMDS adducts with a C_{18}, C_{19}, or C_{21} chain and their retention times are listed in Table 3. The adducts of **1**–**3** with the same chain length eluted separately from a capillary GC column.

TABLE 3. GC-MS DATA OF DMDS ADDUCTS DERIVED FROM DIEPOXYALKENES (**1**–**3**) WITH *S*, *R*, *S*, *R* CONFIGURATION[a]

		R_t (min)	Base ion *m/z*	M^+ *m/z*	Relative intensity (%) of ions at indicated *m/z*									
					89	145	201	M-233[b]	M-201[c]	M-193[d]	M-181[e]	M-89[f]	M-66[g]	M
DMDS of 3,4-6,7-diepoxy-9-ene														
1a	C_{21}	28.10	153	416	2	0	37	2	18	0	0	0	2	1
1b	C_{19}	25.66	153	388	1	0	32	1	20	1	0	0	2	3
1c	C_{18}	24.44	153	374	1	0	31	4	20	1	0	0	2	2
DMDS of 6,7-9,10-diepoxy-3-ene														
2a	C_{21}	28.83	279[h]	416	28	0	0	3	0	0	10	9	14	1
2b	C_{19}	26.25	251[h]	388	24	0	0	3	0	0	12	11	13	1
2c	C_{18}	25.02	237[h]	374	23	0	0	4	0	1	16	12	14	1
DMDS of 3,4-9,10-diepoxy-6-ene														
3a	C_{21}	28.52	57	416	4	74	0	25	0	70	2	0	72	0
3b	C_{19}	26.02	243	388	5	87	0	47	1	95	1	0	86	0.4
3c	C_{18}	24.80	57	374	5	74	1	42	0	87	1	0	75	0.4

[a] Measured by HP 5973 with a HP-1 capillary column. Temperature program: 120°C for 2 min and 6°C/min to 270°C.
[b] 183 for C_{21}, 155 for C_{19}, and 141 for C_{18}.
[c] 215 for C_{21}, 187 for C_{19}, and 173 for C_{18}.
[d] 223 for C_{21}, 195 for C_{19}, and 181 for C_{18}.
[e] 235 for C_{21}, 207 for C_{19}, and 193 for C_{18}.
[f] 327 for C_{21}, 299 for C_{19}, and 285 for C_{18}.
[g] 350 for C_{21}, 322 for C_{19}, and 308 for C_{18}.
[h] M-137.

DISCUSSION

Several pheromone epoxyalkadienes were previously stereoselectively synthesized by using Sharpless asymmetric epoxidation of an allylic alcohol or pig pancreatic lipase-catalyzed asymmetric hydrolysis of a *meso*-diacetoxyepoxybutane (Mori, 1994; Millar, 2000; and references cited therein). For the synthesis of diepoxyalkenes, stereoselective construction of both epoxy rings seemed quite arduous. Therefore, we chose an alternate route in which the second epoxy ring was introduced into epoxyalkadienes without stereoselectivity, and then chromatographic techniques were effectively utilized to isolate each stereoisomer from the produced mixture. First, each stereoisomer of diepoxyalkenes **1** and **2** was prepared by MCPBA oxidation of optically active 6,7-epoxyalkadiene **4**. This oxidation proceeded without regioselectivity and with low stereoselectivity, yielding a mixture of four components (two sets of diastereomers of **1** and **2**). The four components were separable by one of the following types of chromatography: MPLC with a Lobar column, HPLC with an ODS column, or HPLC with a chiral column. Even though a mixture of all possible stereoisomers of **1** and **2** (a total of eight components) is made by the oxidation of racemic **4**, the separation of each component can be accomplished by a combination of these achiral and chiral types of chromatography, as indicated by the data summarized in Table 1. In contrast, MCPBA oxidation of **5** or **6** was regioselective to yield only **3**. When racemic **5** or **6** is treated with MCPBA, a mixture of four stereoisomers of **3** is produced. Each isomer is separable by chiral HPLC from this mixture. If a (3*Z*,6*Z*,9*Z*)-triene is treated with two equivalents of MCPBA, a mixture composed of all stereoisomers of **1–3** (a total of 12 components with the same chain length) will be obtained. Table 1 shows that each component can be separated by two successive HPLC procedures with achiral ODS and chiral AD columns. For the separation of enantiomers of epoxyalkadienes and epoxyalkenes derived from (3*Z*,6*Z*,9*Z*)-trienes and (6*Z*,9*Z*)-dienes, we utilized chiral HPLC (Qin et al., 1997; Pu et al., 1999; Yamamoto et al., 1999b). The work reported here extends the application of the chiral HPLC technique to diepoxyalkenes.

GC-MS is one of the most useful techniques for analyzing insect pheromones because of its high sensitivity, excellent resolution, and rational data that reflect the structures of samples. Pheromone compounds in type II show characteristic mass spectra, indicating the length of their carbon chain and functionality, i.e., the numbers of double bond(s) and epoxy ring(s). For differentiation of the positional isomers of epoxyalkadienes and epoxyalkenes, both Millar and we have previously offered some diagnostic fragment ions based on the spectra of many compounds systematically synthesized (Ando et al., 1993, 1995; Millar, 2000). In this study, we found additional diagnostic ions for diepoxyalkenes and successfully identified a new diepoxyalkene (**1a**) as a minor pheromonal component from a lymantriid species, *P. nuda*, by examining the profile of those ions in a

GC-MS analysis of the pheromone extract. This species is a defoliator of the Malayan banyan in the Ryukyu Islands of Japan. Although the main pheromonal component has been identified as 6,7-epoxyalkadiene (**4a**) with a 6*S*,7*R* configuration, its attractive activity is weak, suggesting one or more minor components that play an important role for male attraction (Wakamura et al., unpublished date). Diastereomers and positional isomers of diepoxyalkenes show similar chromatographic behavior even on a polar capillary GC column. If a natural pheromone is composed of a diepoxyalkene, these fragment ions are useful for the elucidation of chemical structure. Our GC-MS analysis of the *P. nuda* extract excluded the possibility of the existence of other diepoxyalkenes, **2a** and **3a,** in the fraction containing diepoxyalkenes, but it revealed the presence of two diastereomers of **1a**. Chiral HPLC analysis of the extract showed that they have 3*S*,4*R*,6*S*,7*R* and 3*R*,4*S*,6*S*,7*R* configurations, and field tests with synthetic lures indicated strong synergistic activity for male attraction to **4a** (Wakamura et al., unpublished data).

For many pheromones of type I, the positions of double bonds have been successfully determined by GC-MS after making DMDS adducts (Buser et al., 1983). In the case of the type II pheromones, epoxyalkenes were easily converted to DMDS adducts. The adducts of 6,7-epoxy-9-enes and 9,10-epoxy-6-enes provided diagnostic GC-MS data. For example, by this DMDS method, we have confirmed the structure of the natural pheromone secreted by a geometrid female, *Biston robustum* Butler (Yamamoto et al., 2000). This study found that DMDS adducts of **1**–**3** also showed diagnostic fragment ions for determining the original positions of the double bonds. These ions would be useful in elucidating the chemical structures of natural diepoxyalkenes.

REFERENCES

ANDO, T., OHSAWA, H., UENO, T., KISHI, H., OKAMURA, Y., and HASHIMOTO, S. 1993. Hydrocarbons with a homoconjugated polyene system and their monoepoxy derivatives: Sex attractants of geometrid and noctuid moths distributed in Japan. *J. Chem. Ecol.* 19:787–798.

ANDO, T., KISHI, H., AKASHIO, N., QIN, X.-R., SAITO, N., ABE, H., and HASHIMOTO, S. 1995. Sex attractants of geometrid and noctuid moths: Chemical characterization and field test of monoepoxides of 6,9-dienes and related compounds. *J. Chem. Ecol.* 21:299–311.

ANDO, T., OHTANI, K., YAMAMOTO, M., MIYAMOTO, T., QIN, X.-R., and WITJAKSONO. 1997. Sex pheromone of Japanese giant looper, *Ascotis selenaria cretacea*: Identification and field tests. *J. Chem. Ecol.* 23:2413–2423.

ARN, H., TÓTH, M., and PRIESNER, E. 1992. List of sex pheromones of Lepidoptera and related attractants. OILB-SROP/IOBC-WPRS, Secrétariat Général, F-84143 Montfavet, France, 179 pp.

ARN, H., TÓTH, M., and PRIESNER, E. 1997. List of sex pheromones of Lepidoptera and related attractants, supplement 1992–1996. *Technol. Trans. Mating Disrup.* 20:257–293.

BUSER, H.-R., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

GRIES, R., HOLDEN, D., GRIES, G., WIMALARATNE, P. D. C., SLESSOR, K. N., and SAUNDERS, C. 1997. 3*Z*-*cis*-6,7-*cis*-9,10-Di-epoxy-heneicosene: Novel class of Lepidopteran pheromone. *Naturwissenschaften* 84:219–221.

MILLAR, J. G. 2000. Polyene hydrocarbons and epoxides: A second major class of lepidopteran sex attractant pheromones. *Annu. Rev. Entomol.* 45:575–604.

MORI, K. 1994. Synthetic and stereochemical aspects of pheromone chemistry. *Pure Appl. Chem.* 66:1991–1998.

PU, G.-Q., YAMAMOTO, M., TAKEUCHI, Y., YAMAZAWA, H., and ANDO, T. 1999. Resolution of epoxydienes by reversed-phase chiral HPLC and its application to stereochemistry assignment of mulberry looper sex pheromone. *J. Chem. Ecol.* 25:1151–1162.

QIN, X.-R., ANDO, T.,YAMAMOTO, M.,YAMASHITA, M., KUSANO, K., and ABE, H. 1997. Resolution of pheromonal epoxydienes by chiral HPLC, stereochemistry of the separated enantiomers and their field evaluation. *J. Chem. Ecol.* 23:1403–1417.

YAMAMOTO, M., YAMAZAWA, H., NAKAJIMA, N., and ANDO, T. 1999a. A convenient preparation of optically active diepoxyhenicosene (Leucomalure), lymantriid sex pheromone, by chiral HPLC. *Eur. J. Org. Chem.* 1999:1503–1506.

YAMAMOTO, M., TAKEUCHI, Y., OHMASA, Y., YAMAZAWA, H., and ANDO, T. 1999b. Chiral HPLC resolution of monoepoxides derived from 6,9-dienes and its application to stereochemistry assignment of fruit-piercing noctuid pheromone. *Biomed. Chromatogr.* 13:410–417.

YAMAMOTO, M., KISO, M., YAMAZAWA, H., TAKEUCHI, J., and ANDO, T. 2000. Identification of the chiral sex pheromone secreted by the giant geometrid moth, *Biston robustum Butler. J. Chem. Ecol.* 26:2579–2590.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

ALKADIENES AND ALKENES, SEX PHEROMONE COMPONENTS OF THE ALMOND SEED WASP *Eurytoma amygdali*

F. D. KROKOS, M. A. KONSTANTOPOULOU, and B. E. MAZOMENOS*

Chemical Ecology & Natural Products Laboratory
Institute of Biology, NCSR ≪D≫
P.O. Box 60228, Aghia Paraskevi Attikis 153 10, Greece

(Received March 24, 2000; accepted July 4, 2001)

Abstract—Whole body extracts of virgin *Eurytoma amygdali* females were attractive to males in laboratory bioassays. Extracts of various body parts of the female wasp elicited different responses to males, with the thorax extract being the most active. Preparative fractionation of the crude hydrocarbon extract on a silver nitrate impregnated silica gel column (alkanes, alkenes, and alkadienes) revealed that the highest male response was elicited by alkadienes and the lowest by alkenes, with the alkane fraction being inactive. The identification of alkenes and alkadienes was based on gas chromatographic, mass spectrometric, and gas-phase infrared data. Laboratory bioassays suggested that the two alkadienes, (*Z*,*Z*)-6,9-tricosadiene [(*Z*,*Z*)-6,9-$C_{23:2}$], and (*Z*,*Z*)-6,9-pentacosadiene [(*Z*,*Z*)-6,9-$C_{25:2}$], and to a lesser extent alkenes, identified in the female extract of *E. amygdali* were male attractants.

Key Words—*Eurytoma amygdali*, Eurytomidae, Hymenoptera, unsaturated hydrocarbons, (*Z*,*Z*)-6,9-tricosadiene, (*Z*,*Z*)-6,9-pentacosadiene, sex pheromones.

INTRODUCTION

The almond seed wasp, *Eurytoma amygdali* Enderlein (Hymenoptera: Eurytomidae), is a serious pest of almonds in a number of southeastern European countries, the Middle East, and some of the countries of the former Soviet Union (Mentzelos and Atjemis, 1970; Plaut, 1971,1972; Talhouk, 1977; Arambourg et al., 1983; Zerova and Fursov, 1991). The damage caused to the crop can reach up to 90% by mummifying the infested almond. Control measures for this pest are based on cultural practices, such as collection of infested almonds and chemical control by the application of a systemic insecticide against the young larvae.

*To whom correspondence should be addressed.

0098-0331/01/1100-2169$19.50/0

The wasp develops one generation per year, and larvae overwinter inside the infested almonds. The adults emerge in the following April, and the females oviposit in the unripe green almonds leaving one egg. The hatching larva feeds on the developing endosperm.

Male attraction to virgin females has been reported by Pittara and Katsoyannos (1985), who suggested that attraction resulted from chemicals released by the virgin females. Katsoyannos et al. (1992) have used traps baited with virgin females to monitor populations of *E. amygdali*; however, use of this trap setup (living female wasps) is limited and can be applied only by trained personnel. There is little knowledge of the chemicals involved in the mating process of the Eurytomidae family, and so far there is no reference in the literature for the determination of sex pheromones. This study reports on the chemical composition and characterization of whole body extracts and headspace volatiles from male and female *E. amygdali* and the identification of chemicals that are involved in the mating process of this wasp.

METHODS AND MATERIALS

Insects. The insects used were obtained from infested almonds collected from the field in late autumn and stored at 4–6°C. Portions of infested almonds were transferred regularly from the cold room to the laboratory. Adults were separated by sex as soon as they emerged and were kept in cages at 25 ± 2°C and 14 L : 10D photoregime. Wasps were provided with a 10% sucrose solution.

Extraction of Cuticular Lipids. Whole bodies of 2- to 3-day-old females or males were extracted with methylene chloride for 20 min. After exchange of the solvent to hexane, extracts were fractionated on a silica solid-phase extraction (SPE) column (Sep-Pak Plus Silica Cartridges, 690 mg, Waters Chromatography Division, Part No. WAT020520) and eluted with approximately 2 ml hexane (fraction I), followed by an equal volume of methylene chloride (fraction II), and finally methanol (fraction III). The three fractions were concentrated by a gentle stream of nitrogen to the appropriate volume and were used for bioassays or further analysis. The hexane fraction was fully characterized by GC-MS and GC-FTIR. For GC-MS, 1 μl containing ca. 2–3 insect equivalents was injected in duplicate, while for GC-FTIR, 1 μl containing ca. 15 insect equivalents was injected in duplicate.

Solid-Phase Microextraction (SPME). Collection of chemicals from the insect cuticle was also performed by rubbing a SPME fiber (100 μm polydimethylsiloxane, Supelco) over the insect body. Two individuals were used for each collection. The same SPME fiber was also used to collect airborne volatiles by headspace extraction. In this case, four individuals were placed in a 4-ml vial, left for 1 hr, and the fiber was subsequently inserted and exposed to the volatiles for 2 hr.

Hydrocarbon Fractionation and Identification. Cuticular hydrocarbons were separated by the degree of unsaturation on a silver nitrate-impregnated silica gel ($AgNO_3$–SiO_2) column (20% w/w $AgNO_3$ impregnated silica gel, 6.5 g, 1 cm ID $\times$ 8 cm height, gravity flow). The column was eluted stepwise by a series of hexane–diethyl ether mixtures (100 : 0, 95 : 5, and 50 : 50 by volume, each 6 ml). Alkanes were collected in the first fraction, alkenes in the second, and alkadienes in the third . The composition of each fraction was determined by GC-MS. The use of equivalent chain length (ECL) values provided a means of identifying the homolog series of branched methylalkanes. ECL values were calculated from a standard containing a series of *n*-alkanes (C_{14}–C_{31}) at concentrations ranging from 100 to 500 ng/μl each in *n*-hexane. All alkane standards were purchased from Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany) at a purity of 97–99%.

Determination of Double Bonds. Double-bond positions in mono- and diunsaturated compounds were determined by mass spectral analysis (GC-MS) of the epoxide derivatives (Bierl-Leonhard et al., 1980; Hogge et al., 1985). Epoxy derivatives were prepared at room temperature by overnight reaction of an extract containing ca. 25 insect equivalents with 400 μg of *m*-chloroperbenzoic acid (MCPBA, Sigma-Aldrich Chemie) in a 1 : 1 mixture of *n*-heptane and methylene chloride. After completion of the reaction, an equivalent of ca. 5 female insects were analyzed by GC-MS. Epoxy derivatives of synthetic standards of (*Z*)-9-$C_{23:1}$ (Sigma-Aldrich Chemie), (*Z*,*Z*)-6,9-$C_{23:2}$, and (*Z*,*Z*)-6,9-$C_{25:2}$ (both alkadienes were a kind gift from Vioryl S. A, Athens, Greece) were also prepared by reacting 20 μg of each standard with 100 μg of MCPBA under the above same experimental conditions. The geometry (*E* or *Z*) of the double bonds was determined by GC-FTIR.

Gas Chromatography–Mass Spectrometry. Gas chromatography–mass spectrometry analysis was carried out on a Hewlett Packard 5890 Series II gas chromatograph interfaced to a Fisons VG Trio 1000 (Manchester, UK) quadrupole mass spectrometer. Electron impact ionization was used, with an electron energy of 70 eV and a trap current of 200 μA. Extracts were chromatographed on a 60-m $\times$ 0.25-mm $\times$ 0.1-μm film thickness DB-5 column (J & W Scientific). The oven temperature program was set at 50°C for 2 min, then raised to 250°C at 5°C/min, held for 1 min, then 2°C/min to 280°C, and held for 50 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Splitless injections were made (1 μl) at an injector temperature of 250°C and a splitless period of 90 sec. For the SPME analysis, the fiber was desorbed into the injection port for 5 min.

Gas Chromatography–Fourier Transform Infrared Spectroscopy. GC-FTIR analysis was carried out on a Perkin Elmer Autosystem gas chromatograph interfaced to a Perkin Elmer System 2000 (Perkin Elmer Ltd.) fourier transform infrared spectrometer equipped with a liquid-nitrogen-cooled narrow-band (4000–750 cm^{-1}) infrared detector (mercury cadmium telluride). Helium was used

as the carrier gas at (2 ml/min), and the effluent from the GC was mixed with the sweep gas helium (0.5 ml/min; transfer line temperature 280°C) and passed through the IR light pipe with KBr windows. Spectra were obtained at 8 cm^{-1} resolution. Extracts were chromatographed on a 30-m × 0.32-mm × 1-μm film thickness DB-5 column (J & W Scientific). The oven temperature program was 50°C for 2 min, then 5°C/min to 280°C, and held for 60 min. Splitless injections were made (1 μl) at an injector temperature of 250°C and a splitless period of 90 sec.

Laboratory Bioassays. Bioassays were performed in a 50- × 40- × 30-cm screen cage; ten 1- to 3- day-old males were present in the cage during the bioassay. The cage was placed in front of an operating hood, and a fan was used to generate the airstream. The appropriate amount of each sample (2–3 female equivalents) was dissolved in 100 μl of *n*-hexane and was poured on a 2- × 2-cm piece of Whatman No. 1 filter paper. The filter paper was hung 5 cm below the top of the cage and served as the source. The SPME fiber that had been used to collect volatiles released by *E. amygdali* females was also evaluated for male response. The SPME holder was standing on the top of the cage in a place that allowed the exposed fiber to penetrate ca. 5 cm inside the cage.

Each test lasted 10 min. The number of males exhibiting wing raising and a lateral swing of the body or landing on the filter paper was recorded. During each testing period, samples were tested individually with 10-min intervals between tests. The experiments were repeated 10 times (filter paper) and five times (SPME) with different batches of males. Samples were tested in random sequence; the same amount of *n*-hexane alone on a similar filter paper was used as control. Bioassays were conducted 3 to 4 hr after the onset of the photoperiod.

Data were transformed by using arcsine transformation and subjected to analysis of variance (ANOVA). Means were separated by Tukey's studentized range honestly significant difference (HSD).

RESULTS AND DISCUSSION

Extraction Techniques. Although methylene chloride extraction was efficient in removing chemicals from the insects' bodies, it proved complicated and time-consuming when further clean-up of the extract was needed to remove inactive lipid material that interfered with the separation and identification of the compounds. The SPME technique offers a fast, solventless alternative, and it can be applied as an easy-to-use first screening to determine the nature of the volatile compounds (Monnin et al., 1998; Turillazzi et al., 1998; Sledge et al., 2000). Comparative GC-MS analysis of male and female extracts with all three techniques (solvent extraction, SPME rubbing, and headspace SPME) exhibited a similar chromatographic profile with mainly quantitative rather than qualitative differences (Table 1). The SPME technique compares well with the conventional solvent

TABLE 1. RELATIVE QUANTITIES OF CHEMICAL COMPOUNDS IDENTIFIED FROM MALE AND FEMALE *E. amygdali* WHOLE-BODY EXTRACTS AND HEADSPACE VOLATILES COLLECTED ON A SPME FIBER

Compound	ECL	Ident.	Diagnostic ions[a]	Solvent extract		SPME, female
				Male	Female	
Alkanes						
n-Tetradecane	14.00	Std.		−	−	++
n-Nonadecane	19.00	Std.		+	+	−
n-Heneicosane	21.00	Std.		++	+	−
n-Docosane	22.00	Std.		+	tr[b]	−
n-Tricosane	23.00	Std.		++	++	++
3-Methyl tricosane	23.73	MS	56/57, 308/309	+	+	−
n-Tetracosane	24.00	Std.		++	++	++
n-Pentacosane	25.00	Std.		+++	+++	+++
11- + 13-Methyl pentacosane	25.32	MS	168/169, 196/197, 224/225	+	+	+
3-methyl pentacosane	25.73	MS	56/57, 336/337	+	+	−
n-Hexacasosane	26.00	Std.		+	+	++
n-Heptacosane	27.00	Std.		+++	+++	+++
11- + 13-Methyl heptacosane	27.34	MS	168/169, 196/197, 224/225, 252/253	++	++	++
3-Methyl heptacosane	27.75	MS	56/57, 364/365	++	+++	++
n-Nonacosane	29.00	Std.		++	++	+
9- + 11- + 13- + 15-Methyl nonacosane	29.34	MS	140/141, 168/169, 196/197, 224/225, 252/253, 280/281, 308/309	+++	+++	+++
3-Methyl nonacosane	29.75	MS	56/57, 393/394	++	++	tr
Alkenes						
(*Z*)-9-Tricosene	22.61	Std.	155(19)/225(10)	−	++	++
7-Tricosene	22.66	MS	127(23)/253(8)	−	tr	−
(*Z*)-9-Pentacosene	24.60	MS/FTIR	155(22)/253(10)	−	+	+
9-Heptacosene	26.11	MS	155(17)/281(6)	−	tr	−

TABLE 1. CONTINUED

Compound	ECL	Ident.	Diagnostic ions[a]	Solvent extract		SPME, female
				Male	Female	
7-Heptacosene		MS	127(13)/ 309(6)	–	tr	–
(*Z*)-5-Octacosene	27.13	MS/FTIR	99(18)/ 351(4)	–	++	++
(*Z*)-9-Nonacosene	28.62	MS/FTIR	155(23)/ 309(7)	tr	++	+
7-Nonacosene	28.69	MS	127(21)/ 337(5)	–	tr	–
(*Z*)-7-Triacontene	29.02	MS/FTIR	127(21)/ 351(4)	–	tr	–
(*Z*)-5-Triacontene	29.12	MS/FTIR	99(20)/ 379(5)	tr	++	+
(*Z*)-9-Hentriacontene	30.58	MS/FTIR	155(21)/ 337(6)	tr	+	+
7-Dotriacontene	31.01	MS	127(20)/ 379(5)	–	tr	–
5-Dotriacontene	31.10	MS	99(20)/ 407(5)	–	tr	–
Alkadienes						
(*Z*,*Z*)-6,9-Tricosadiene	22.61	Std.		–	++	+
(*Z*,*Z*)-6,9-Pentacosadiene	24.60	Std.		–	++	+
Alcohols						
Hexadecanol		Std.		+++	+	–
Octadecenol		MS		+++	+	–
Terpenoids						
trans-Caryophyllene		Std.		–	–	+

[a] For alkenes, diagnostic ions are referred to the corresponding epoxides and number in parentheses is the relative abundance of each ion.
[b] tr: trace (<0.5%); +: 0.5–1.0%; ++: 1.0–5.0%; +++: >5.0%.

extraction approach and offers the advantage of detecting volatile compounds that could be lost during conventional treatment of a solvent extract.

Cuticular Hydrocarbons. The composition from both sexes is presented in Table 1. The vast majority of compounds identified are long-chain saturated, monounsaturated, and diunsaturated hydrocarbons. The saturated hydrocarbon profile from each sex was qualitatively similar, with some quantitative differences. Each saturated hydrocarbon component was identified based on its equivalent chain length (ECL) and fragmentation pattern when analyzed by GC-MS. Monomethyl alkanes substituted at carbon 3 elute approximately 0.3 carbon units before the corresponding *n*-alkane, and as they fragment on each side of the methyl branch, with the charge retained on the fragment containing the branching methyl group, they give spectra with a strong $(M-29)^+$ ion. A weak ion at $(M-57)^+$ is also observed.

Monomethyl alkanes with a branch on carbons 11–15 have strong *m/z* ion pairs, indicating cleavage internal to the methyl branch. These compounds all elute approximately 0.7 carbon units before the *n*-alkane with the same number of total carbons. The signal intensities for the odd and even fragments in the *m/z* ion couplets for each of the daughter ions are nearly equivalent, indicative of a single methyl branch remaining following a cleavage (Page et al., 1997).

Mass spectra of the epoxide derivatives of alkenes show preferential cleavage *alpha* to the epoxy group (Bierl-Leonhard et al., 1980), producing fragments indicative of the position of the epoxy group and, thus, the position of the double bond in the original compound (Figure 1). Double bonds are located at positions 7 or 9 (major isomer) for the odd-numbered alkenes (C_{23}, C_{25}, C_{27}, C_{29}, and C_{31}) and at positions 5 or 7 for the even-numbered monounsaturated hydrocarbons (C_{28}, C_{30}, and C_{32}). The *E* or *Z* nature of the double bonds for the major compounds present in the alkene fraction was determined by GC-FTIR. The presence (*E* configuration) or absence (*Z* configuration) of an absorption around 970 cm^{-1} was used to determine the configuration of double bonds. Weak but useful bands above 3000 cm^{-1} were used for the assignment of *Z* unsaturated compounds (Doumenq et al., 1989; Leal, 1991; Attygalle et al., 1994). An absorption highly characteristic for the *Z* configuration occurs at 3013–3011 cm^{-1} ($=CH_2$ stretch band), while *E* compounds fail to show any bands in this region. These two absorptions (970 cm^{-1} and 3013–3011 cm^{-1}), in combination, can be used for the unambiguous determination of *Z* or *E* bonds in long-chain unsaturated compounds. All spectra obtained from the alkenes examined showed an absorption at 3013 cm^{-1} and a lack of absorption around 970 cm^{-1}, confirming the *Z* nature of the double bond (Figure 2A). The presence of (*Z*)-9-$C_{23:1}$ in the female extract was also confirmed by comparing chromatographic, mass spectrometric, and IR data with that of a synthetic standard.

The third fraction from the silver nitrate-impregnated silica gel column showed the presence of two alkadienes ($C_{23:2}$ and $C_{25:2}$) when analyzed by GC-MS. Mass spectra for both alkadienes showed an increased ion at *m/z* 222 and *m/z* 250 for $C_{23:2}$ and $C_{25:2}$, respectively (Figure 3). Descoins et al. (1986) reported that 6,9 dienes have a characteristically increased $(M-98)^+$ ion that originates from cleavage between carbons 7 and 8 and subsequent hydrogen transfer. An indication of the position of the double bonds at carbon 6 and carbon 9 was given by epoxidation, under conditions that favor the formation of unsaturated monoepoxides, followed by examination of the mass spectrometric fragmentation pattern. For example, the two peaks that correspond to the unsaturated monoepoxides of $C_{23:2}$ had characteristic ions at *m/z* 153 and 225 (indicative of an epoxide at carbons 9,10) and *m/z* 113 and 265 (indicative of an epoxide at carbons 6,7). The amount of natural alkadienes available did not allow subsequent catalytic hydrogenation of the unsaturated monoepoxides or an unambiguous determination of double-bond positions as has been described by Hogge et al. (1985).

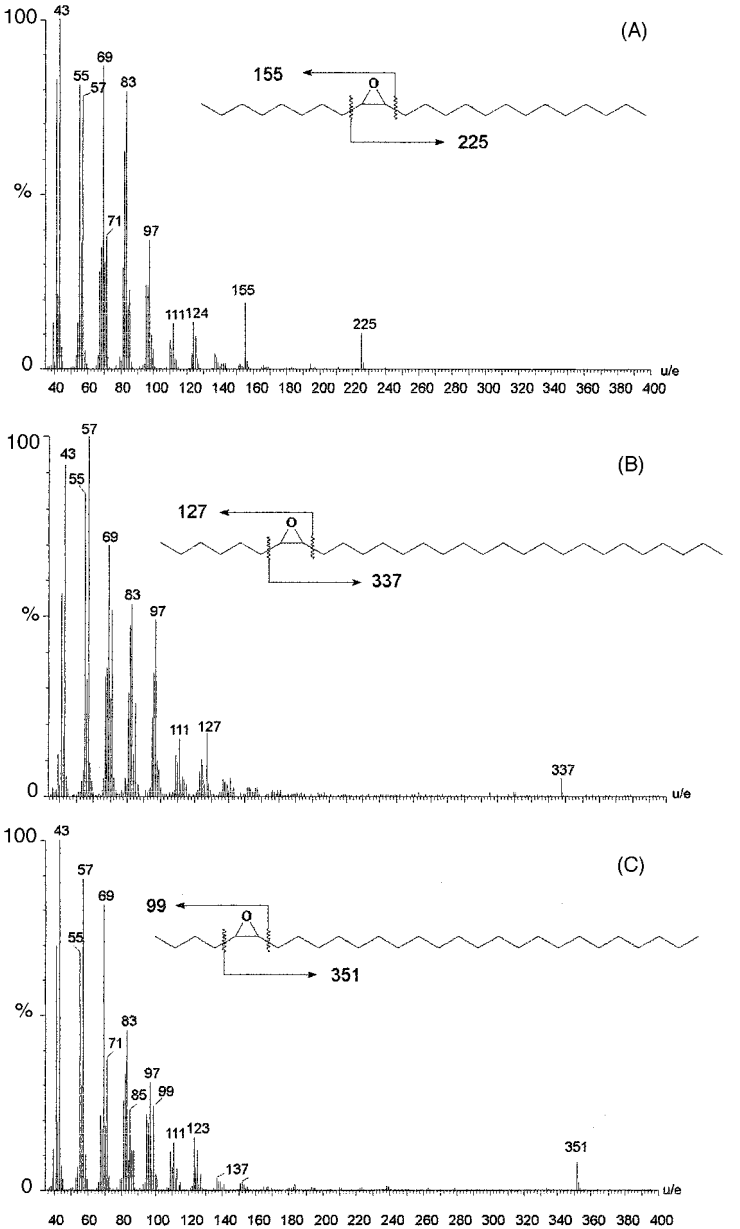


FIG. 1. EI mass spectra of epoxy derivatives of naturally occurring (A) (*Z*)-9-$C_{23:1}$, (B) (*Z*)-7-$C_{29:1}$, and (C) (*Z*)-5-$C_{28:1}$, showing the diagnostic *alpha* cleavage ions at mass 155 and mass 225 for (A), mass 127 and mass 337 for (B), and mass 99 and mass 351 for (C).

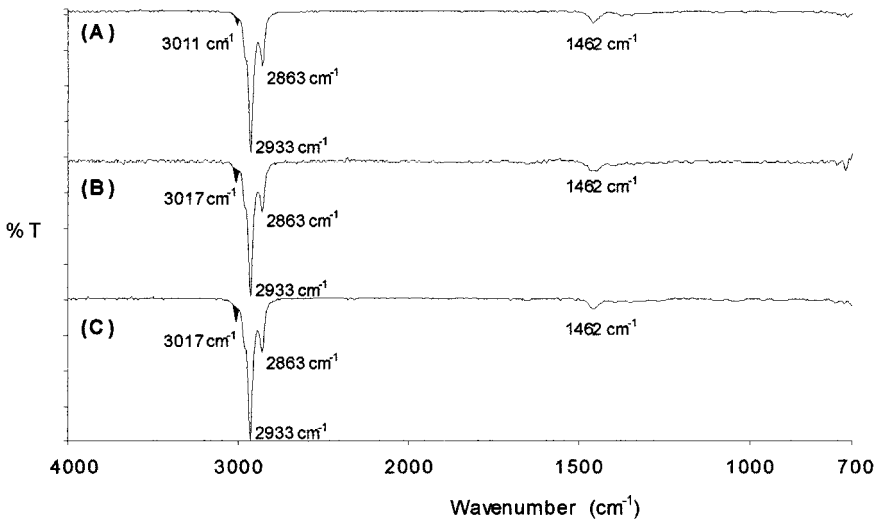


FIG. 2. Gas-phase IR spectra of naturally occurring (A) (*Z*)-9-$C_{23:1}$, (B) (*Z*,*Z*)-6,9-$C_{23:2}$, and (C) synthetic (*Z*,*Z*)-6,9-$C_{23:2}$.

Similar principles for interpreting IR spectra for double-bond configuration of monounsaturated compounds have been applied to diunsaturated compounds as well (Attygalle et al., 1995). The GC-FTIR spectrum of tricosadiene is presented in Figure 2B. The lack of any absorption around 970 cm^{-1} indicates the absence of an *E* bond. Conjugated *E*,*E* polyenes do not show this characteristic *E* absorption.

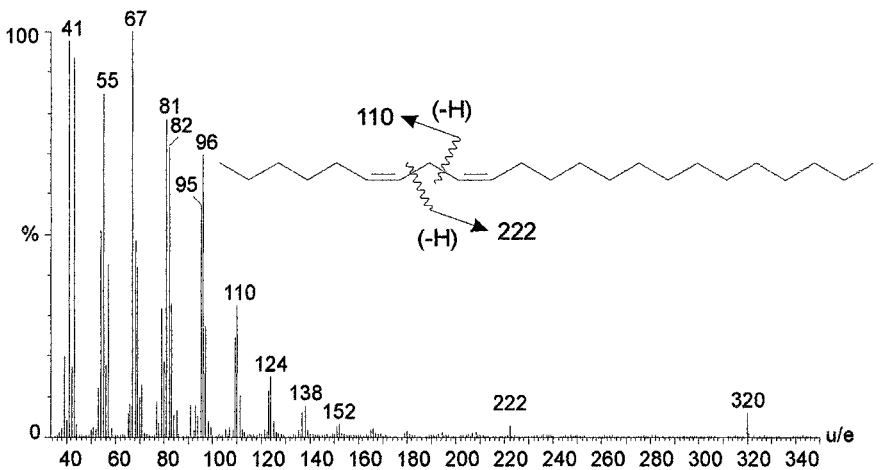


FIG. 3. EI mass spectrum of naturally occurring (*Z*,*Z*)-6,9-$C_{23:2}$.

Instead, a band highly characteristic for (*E,E*)-conjugated diene moieties appears at 983–982 cm-1. In the spectrum of Figure 2B, there is no absorption around 982 cm^{-1}, excluding any possibility for the presence of an *E* bond in tricosadiene. Furthermore, an absorption at 3017 cm^{-1} with significantly enhanced intensity, when compared to the corresponding absorption of the monounsaturated (*Z*)-9 tricosene (Figure 2A,B), supports the presence of two *Z* bonds in the molecule ($A_{3016-3018\,cm^{-1}}/A_{2866-2864\,cm^{-1}} = 0.413$ and 0.198 for $C_{23:2}$ and $C_{23:1}$, respectively). The gas phase IR spectrum of diunsaturated compounds also provides information for the location of the double bonds. Attygale et al. (1995) reported that conjugated *Z,Z* dienes lack a sharp band in the 3036–3011 cm^{-1} area in the GC-IR spectrum and that *Z,Z* dienes whose double bonds are separated by only a single methylene group show the characteristic $=CH_2$ stretch band at 3017 cm^{-1}. The $=CH_2$ stretch band of tricosadiene (Figure 2B) that appears at 3017 cm^{-1} (characteristic of methylene-interrupted double bonds) further supports the mass spectrometric interpretation for the double bonds located at carbons 6 and 9. The structure of (*Z,Z*)-6,9-$C_{23:2}$ and (*Z,Z*)-6,9-$C_{25:2}$ was unambiguously confirmed by comparison of chromatographic, mass spectrometric, and IR data with that of synthetic standards (Figure 2B,C).

Bioassays. Close range laboratory bioassays indicated that whole-body methylene chloride extracts elicited a strong sexual response by males. A high proportion (75%) in the bioassay cage exhibited wing raising and lateral body movement, characteristic mating behavior for Eurytomidae. Close to 40% of the responding males landed on the filter paper baited with the crude extracts. Excited males approached the source mainly by walking on the top of the screen cage. Usually, when the males were close to the source, they tried to copulate, and a number of them jumped on the filter paper (Table 2).

In order to locate the pheromone-producing gland on the female body, extracts from female heads, thoraxes, and abdomens were evaluated for male response. Thorax extracts elicited maximal response, and a high proportion also responded to

TABLE 2. *E. amygdali* MALES RESPODING TO EXTRACTS FROM DIFFERENT PARTS OF VIRGIN FEMALE BODY AND TO HEADSPACE VOLATILES COLLECTED ON SPME FIBER[a]

Source	Dose	% Males wing raising	% Males landing
Crude female extract	2 FE	74.7^{a}	36.8^{a}
Crude female head extract	2 FE	15.3^{c}	7.2^{c}
Crude female thorax extract	2 FE	64.6^{ab}	32.6^{ab}
Crude female abdomen extract	2 FE	53.4^{b}	22.8^{b}
SPME fiber after collection of female volatiles	4 FE	56.2^{b}	25.3^{b}
Control (hexane)	2 μl	1.6^{d}	0^{c}

[a]$N = 10$ except for SPME, where $N = 5$. Means followed by the same letter are not significantly different [Tukey studentized range (HSD) test; ($P > 0.05$)].

abdomen extracts (Table 2). Similar results have been reported for *Bephratelloides pomorum*, another Eurytomidae species (Leal et al., 1997). It is possible that the gland where the pheromone is synthesized is located in the thorax; however, the pheromone components are spread to the abdomen when they are released.

The SPME fiber that was used to collect volatiles was also evaluated for male response just after collection and prior to GC analysis. Males were attracted to the fiber and exhibited a clear sexual behavior. Male attraction to the SPME fiber was lower than to the whole-body extract, indicating that either the quantity of the active chemicals absorbed on the fiber was lower than that obtained in the extract or that the emission rate of substances from the fiber was lower than that of the filter paper (Table 2).

Since the whole-body extract elicited the maximal response from males, subsequent isolation of the active components was based on whole-body extracts. Fractionation dictated the use of a large amount of starting material, and clean-up on a silica SPE cartridge removed coextractable inactive lipid materials. Bioassays revealed that the active components were eluted in fractions I (hexane fraction), while fraction II and III (methylene chloride and methanol) showed low activity. Combination of fraction I with either of the other two fractions did not increase male attractiveness (Table 3).

Bioassays indicated that alkanes were inactive and that alkenes elicited low response (Table 4). A high response, significantly different from the crude female extract, was observed for the third fraction, which contained the two identified alkadienes, (*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$, as well as for the synthetic standard consisting of (*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$ at a ratio of 7 : 3 (the ratio

TABLE 3. *E. amygdali* MALES RESPODING TO INDIVIDUAL AND COMBINED FRACTIONS COLLECTED FROM SPE COLUMNS USED TO PURIFY FEMALE WHOLE-BODY EXTRACTS ($N = 10$)[a]

Source	Dose	% Males	
		wing raising	landing
Crude	2 FE	58.8^{c}	31.4^{c}
Fraction I	2 FE	45.3^{c}	34.1^{c}
Fraction II	2 FE	12.3^{a}	11^{b}
Fraction III	2 FE	11.6^{a}	5.4^{ab}
Fractions I+II	2 FE	39.7^{c}	34.9^{c}
Fractions I+III	2 FE	42.4^{c}	27.5^{c}
Fractions II+III	2 FE	15.6^{b}	3.2^{a}
Fractions I+II+III	2 FE	43.7^{c}	29.7^{c}
Control	2 μl	5.2^{a}	1.6^{a}

[a] Means followed by the same letter are not significantly different [Tukey studentized range (HSD) test; ($P > 0.05$)].

TABLE 4. *E. amygdali* MALES RESPONDING TO DIFFERENT FRACTIONS OF FEMALE WHOLE-BODY EXTRACT AND SYNTHETIC ALKADIENES ($N = 10$)

Source	Dose	% Males	
		wing raising	landing
Crude female extract	2 FE	77.4[a]	40.3[a]
Fraction I from $AgNO_3$–SiO_2 column (alkanes)	2 FE	4.1[d]	0[c]
Fraction II from $AgNO_3$–SiO_2 column (alkenes)	2 FE	17.4[c]	9.0[c]
Fraction III from $AgNO_3$–SiO_2 column (alkadienes)	2 FE	64.3[b]	29.2[b]
Synthetic, (*Z*,*Z*)-6,9-$C_{23:2}$ + (*Z*,*Z*)-6,9-$C_{25:2}$ (7:3)	10 μg	57.6[b]	28.3[b]
Control (hexane)	2 μl	1.4[d]	0[c]

[a] Means followed by the same letter are not significantly different [Tukey studentized range (HSD) test; ($P > 0.05$)].

that had been determined on the female extract after fractionation on the silver nitrate impregnated silica gel column). These findings indicate the importance of these two compounds on the mating behavior of male *E. amygdali*.

The saturated hydrocarbon sex profiles were qualitatively similar with some quantitative differences. The major differences are that alkadienes and, to a lesser extent, alkenes are present only in the female extract (Table 1). The alkadienes have one double bond in the same location as the alkenes (at carbon 9), and whether both originate from the same biosynthetic pathway remains to be established. It has been reported that *Cardiochiles nigriceps* (Hymenoptera: Braconidae) alkadienes, present only in the female extract, are responsible for mediating courtship behavior (Syvertsen et al., 1995). The presence of the two alkadienes only in the female extracts supports our bioassay findings that (*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$ are the chemicals involved in the mating process of *E. amygdali*. Further research is needed for complete identification of the pheromone and to optimize the composition of the blend for its use in practical applications.

Acknowledgments—We are grateful to Dr. N Ragousis (Vioryl S.A, Athens, Greece) who provided us with the synthetic samples of (*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$.

REFERENCES

ARAMBOURG, Y., FAUVEL, F., and CHEVIN, H. 1983. *Eurytoma amygdali* End. et sa présence en France. Un nouveau chalcidien ravageur de l'amandier. *Arboric. Fruti.* 358:27–28.

ATTYGALLE, A. B., SVATOS, A., WILCOX, C., and VOERMAN, S. 1994. Gas-phase infrared spectroscopy for determination of double bond configuration of monounsaturated compounds. *Anal. Chem.* 66:1696–1703.

ATTYGALLE, A. B., SVATOS, A., WILCOX, C., and VOERMAN, S. 1995. Gas-phase infrared spectroscopy for determination of double-bond configuration of some polyunsaturated pheromones and related compounds. *Anal. Chem.* 67:1558–1567.

BIERL-LEONHARD, B. A., DEVILBISS, E. D., and PLIMMER, J. R. 1980. Location of double-bond position in long-chain aldehydes and acetates by mass spectral analysis of epoxide derivatives. *J. Chromatogr. Sci.* 18:364–367.

DESCOINS, C., LALANNECASSOU, B., MALOSSE, C, and MILAT, M. L. 1986. Analysis of the sex-pheromone produced by the virgin female of Moscis-Latipes (Guenee), Noctuidae, Catocalinae, from Guadeloupe (French Antilla). *C.R. Acad. Sci III* 302:509–512.

DOUMENQ, P., GUILIANO, M., and MILLE, G. 1989. GC/FTIR potential for structural analysis of marine origin complex mixtures *J. Environ. Anal. Chem.* 37:235–244.

HOGGE, L. R., UNDERHILL, E. W., and WONG, J. W. 1985. The determination of position and geometry of unsaturation in multi-unsaturated aliphatic compounds by GC/MS. *J. Chromatogr. Sci*. 23:171–175.

KATSOYANNOS, B. I., KOULOUSSIS, N. A., and BASSILIOU, A. 1992. Monitoring populations of the almond seed wasp, *Eyrytoma amygdali*, with sex pheromone traps and other means, and optimal timing of chemical control. *Entomol. Exp. Appl*. 62:9–16.

LEAL, W. S. 1991. (*R,Z*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one, the sex pheromone of the scarab beetle *Anomala cuprea. Naturwissenschaften* 78:521–523.

LEAL, W. S., MOURA, J. I. L., BENTO, J. M. S., VILELA, E. F., and PEREIRA, P. B. 1997. Electrophysiological and behavioral evidence for a sex pheromone in the wasp *Bephratelloides pomorum. J. Chem. Ecol.* 23:1281–1289.

MENTZELOS, J. and ATJEMIS, A. 1970. Studies on the biology and control of *Eurytoma amygdali* in Greece. 1970. *J. Econ. Entomol.* 63:1934–1936.

MONNIN, T., MALOSSE, C., and PEETERS, C. 1998. Solid-phase microextraction and cuticular hydrocarbon differences related to reproductive activity in queenless ant *Dinoponera quadriceps. J. Chem. Ecol.* 24:473–490.

PAGE, M., NELSON, L. J., BLOMQUIST, G. J., and SEYBOLD, S. J. 1997. Cuticular hydrocarbons as chemotaxonomic characters of pine engraver beetles (*Ips* spp.) in the *grandicollis* subgeneric group. *J. Chem. Ecol.* 23:1053–1099.

PITTARA, I. S. and KATSOYANNOS, B. I. 1985. Male attraction to virgin females in the almond seed wasp, *Eurytoma amygdali* Enderlein (Hymenoptera, Eyrytomidae). *Entomol. Hell.* 3:43–46.

PLAUT, H. N. 1971. On the biology of the adult of the almond seed wasp, *Eyrytoma amygdali* End. (Hym., Eyrytomidae) in Israel. *Bull. Entomol. Res.* 61:275–281.

PLAUT, H. N. 1972. On the biology of the immature stages of the almond seed wasp, *Eurytoma amygdali* End. (Hym., Eyrytomidae) in Israel. *Bull. Entomol. Res.* 61:681–687.

SLEDGE, M. F., MONETI, G., PIERACCINI, G., and TURILLAZZI, S. 2000. Use of solid-phase microextraction in the investigation of chemical communication in social wasps. *J. Chromatogr.* 873:73–77.

SYVERTSEN, T. C., JACKSON, L. L., BLOMQUIST, G. L., and VINSON, S. B. 1995. Alkadienes mediating courtship in the parasitoid *Cardiochiles nigriceps* (Hymenoptera:Braconidae). *J. Chem. Ecol.* 21:1971–1989.

TALHOUK, A. S. 1977. Contributions to the knowledge of almond pests in East Mediterranean countries. V. The fruit-feeding insects, *Eurytoma amygdali* End., and *Arnasia lineatella Z. Z. Angew. Entomol.* 83:145–154.

TURILLAZZI, S., SLEDGE, M. F., and MONETI, G. 1998. Use of a simple method for sampling cuticular hydrocarbons from live social wasps. *Ethol. Ecol. Evolt.* 10:293–297.

ZEROVA, M. D. and FURSOV, V. N. 1991. The palearctic species of *Eurytoma* (Hymenoptera: Eurytomidae) developing in stone fruits (Rosaceae: Prunoideae). *Bull. Entomol. Res.* 81:209–219.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

IDENTIFICATION AND CLONING OF A PHEROMONE-BINDING PROTEIN FROM THE ORIENTAL BEETLE, *Exomala orientalis*

GUIHONG PENG[1] and WALTER SOARES LEAL[1,2,*]

[1]*Laboratory of Chemical Prospecting*
National Institute of Sericultural and Entomological Science
1-2 Ohwashi, Tsukuba 305-8634, Japan

[2]*Department of Entomology*
University of California Davis
Davis, California 95616-8586

(Received January 29, 2001; accepted July 18, 2001)

Abstract—We have identified and cloned a pheromone-binding protein (EoriPBP) from the Japanese and American populations of the Oriental beetle, *Exomala orientalis* (Coleoptera: Scarabaeidae). The protein showed more than 90% amino acid identity to the previously identified pheromone-binding proteins from *Popillia japonica* (PjapPBP) and *Anomala osakana* (AosaPBP), as well as to one of the odorant-binding proteins from *Phyllopertha diversa* (PdivOBP1). EoriPBP has 116 amino acids, with a calculated molecular mass of 12,981 Da, pI of 4.3, and six highly conserved cysteine residues. 5′-RACE amplifications led to the characterization of a signal peptide with 19 amino acids. The signal peptide showed high amino acid identity to the signal peptide for AosaPBP. Comparison of the amino acid sequences of the PBPs involved in the detection of similar ligands, i.e., monounsaturated lactones and ketone, suggests that the most variable residues among the PBPs from *E. orientalis*, *P. japonica*, and *A. osakana* are probably the most discriminating residues. As with the pheromone-binding protein from *Bombyx mori*, the residues at positions 61, 64, 71, and 82 in EoriPBP, PajpPBP, and AosaPBP, which are either valine, leucine, isoleucine, or methionine, are likely to be specificity determinants.

Key Words—*Exomala orientalis*, *Popillia japonica*, *Anomala osakana*, odorant-binding protein, signal peptide, Scarabaeidae.

*To whom correspondence should be addressed. E-mail: wsleal@ucdavis.edu

0098-0331/01/1100-2183$19.50/0

INTRODUCTION

Insects depend heavily on their ability to detect chemical cues from the environment. These chemicals are involved in mediating a wide range of behaviors, from communication between conspecific individuals to the recognition of specific features of the environment, such as food sources, oviposition sites, etc. The chemical signals (semiochemicals) are normally buried in a complex mixture of odorants from myriad sources. This has led to the development of a remarkably selective and sensitive olfactory system, which approaches the theoretical limit for a detector. While minimal structural modification of pheromone molecules renders them inactive (Kaissling, 1987), a single molecule of the native ligand is reported to be sufficient to activate pheromone-sensitive olfactory neurons in the antennae of the silkworm moth, *Bombyx mori* (Kaissling and Priesner, 1970).

In order to convey their messages, pheromones and other semiochemicals must reach the dendrites of olfactory receptors neurons, where G protein-coupled olfactory receptors (Clyne et al., 1999; Vosshall et al., 1999, 2000) are supposed to be located. The last part of the journey toward the receptors is protein-assisted, given that dendrites are surrounded by sensillar lymph and pheromones are largely hydropobic in nature. In the perireceptor events (Figure 1), odorant-binding proteins (Vogt and Riddiford, 1981) not only ferry the ligands to their receptors, but also protect the chemical signals from odorant-degrading enzymes (Vogt and Riddiford, 1981; Vogt et al., 1985). Activation of the receptors initiates transduction

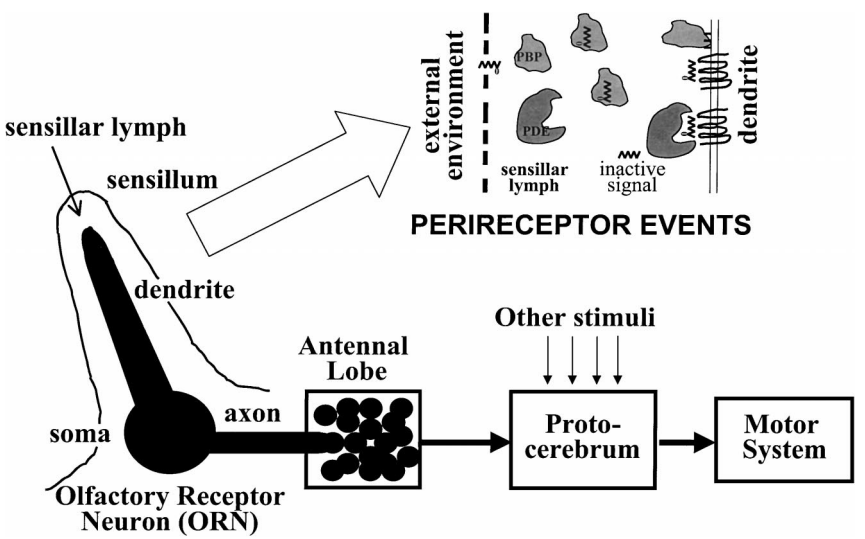


FIG. 1. Schematic view of the overall olfactory processing in insects, highlighting early olfactory processing (perireceptor events).

of the chemical signals into neuronal activities (spikes), which are processed in the antennal lobe, integrated with other stimuli in the central nervous system, and translated into behavior (Figure 1). There is growing evidence in the literature that the inordinated sensitivity and selectivity of the insect olfactory system is achieved by a combination of olfactory-specific proteins, including odorant receptors (Clyne et al., 1999; Vosshall et al., 1999, 2000), odorant-binding proteins (OBPs) (Vogt and Riddiford, 1981; Vogt et al., 1999), and odorant-degrading enzymes (Vogt and Riddiford, 1981; Vogt et al., 1985; Rybczynski et al., 1990; Rogers et al., 1999). Because binding studies have suggested that interactions between odorants and OBPs are specific and selective (Du and Prestwich, 1995), selectivity of the insect olfactory system is achieved at least partly in the early olfactory processing.

We have previously identified odorant-binding proteins from three species of scarab beetles (Coleoptera: Scarabaeidae), i.e., *Popillia japonica*, *Anomala osakana* (Wojtasek et al., 1998), and *Phyllopertha diversa* (Wojtasek et al., 1999), which utilize the enantiomers of japonilure (Tumlinson et al., 1977; Leal, 1996, 1998) and an alkaloid compound (Leal et al., 1997) as their sex pheromones (Figure 2). In order to get a better understanding of the relationship between OBP structures and their ligands, we have identified and cloned a pheromone-binding

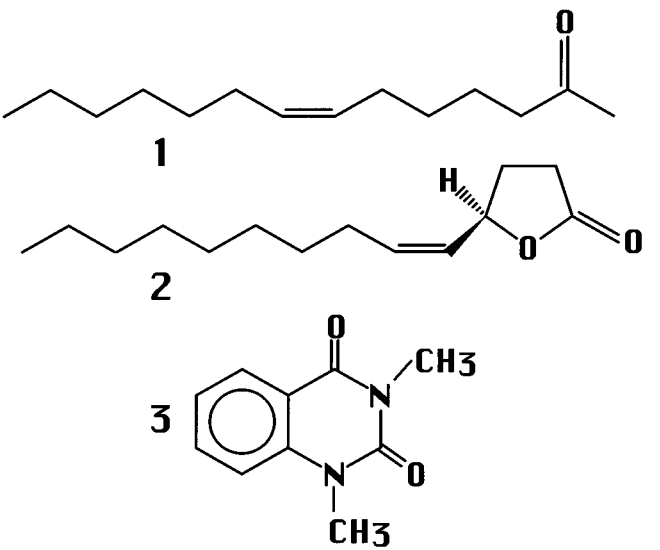


FIG. 2. Structures of some scarab beetle pheromones: **1**, (*Z*)-7-tetradecen-2-one, the sex pheromone of the Oriental beetle (*E. orientalis*); **2**, (*R,Z*)-5-(—)-(1-decenyl)oxacyclopentan-2-one, (*R*)-japonilure, the sex pheromone of the Japanese beetle (*P. japonica*); **3**, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione, the sex pheromone of the pale-brown chafer (*P. diversa*).

protein gene from the Oriental beetle, *Exomala orientalis* (Waterhouse), whose pheromone is an unsaturated ketone (Figure 2) both in the Japanese (Leal, 1993; Leal et al., 1994a) and in American populations (Zhang et al., 1994).

METHODS AND MATERIALS

Analytical Procedures. *N*-terminal amino acid sequences were obtained on a Hewlett-Packard Protein Sequencer model 241, with PTH derivatives separated on a Hewlett-Packard (=Agilent) series 1100 HPLC system. Proteins were analyzed on 15% native polyacrylamide gels run in a Mini-Protean II (Bio-Rad) and stained with Coomassie blue R-250. For sequencing, proteins were separated on native PAGE and transferred by electroblotting to polyvinyl difluoride (PVDF) membranes. Bands were cut off, dried, loaded on a column, and the proteins sequenced. DNA sequences were obtained with an automated ABI PRISM Model 377 sequencer (Applied Biosystems). DNA sequences were analyzed using MacVector (Oxford Scientific).

Insects. Adults collected in traps baited with the synthetic pheromone in Tsukuba were kept in culture dishes at 25°C, 75% relative humidity, and 14L:10D photoperiod and provided with saturated sucrose solution on cotton. In the United States, beetles were collected in Rhode Island with pheromone traps, frozen, and shipped to Davis, California.

Protein Extracts. Beetles were anesthetized on ice, antennae and legs were collected, frozen in liquid nitrogen, and lyophilized. Homogenization was performed in ice-cold glass homogenizers in 10 mM Tris HCl, pH 8. Homogenized samples were centrifuged twice at 12,000 rpm, 4°C, for 5 min. The supernatants were concentrated on a SpeedVac.

Molecular Cloning. Total RNA was extracted from 50 antennae of *E. orientalis* males by using TRIzol Reagent (Gibco BRL), and mRNA was purified with the PolyATtract (Promega) kit. The first cDNA strand was synthesized by using the SuperScript preamplification system (Gibco BRL). The following degenerated primer was designed based on the *N*-terminal protein sequences of the mature protein, 5′-ATGWSNGARGARATGGARGAR-3′, and used in polymerase chain reaction (PCR) with oligo-dT 15 plus GAG; the last codon makes subsequent PCR more specific. PCR reactions were carried out in a MiniCycler (model 150, MJ Research) by using Taq DNA Polymerase (Roche), with annealing at 50°C. Optimized PCR products were ligated into the pCR2. 1/TA cloning vector (Invitrogen), and transformed into OneShot Top 10F′ competent cell (Invitrogen). Positive insertions were identified by screening positive colonies by PCR with M13 primers. Sequencing of plasmid DNA from several colonies was performed following extraction with the QIAprep Spin Miniprep Kit (Qiagen). Signal peptide sequences were

obtained by using 5′-RACE System for Rapid Amplification of cDNA Ends (Gibco BRL) with the following primers: 5′-GTGGGTTTGATAAACATT-3′ (GSP1), 5′-CATGGATTCGCTCCAGGTTT-3′ (GSP2), and 5′-CCATCATCGCCAACGATT-3′ (nested GSP); GSP, gene specific primers.

RESULTS AND DISCUSSION

Analysis of protein extracts from antennal and control tissues (legs) of the Japanese population of the Oriental beetle by native polyacrylamide gel electrophoresis (PAGE) showed one antenna-specific protein (Figure 3) migrating at the bottom of the gel, with a mobility similar to those of OBPs identified to date. The profile of bands on native gels obtained from male and female antennal extracts and visualized by Coomassie blue were indistinguishable (data not shown). The lack of sex specificity in the antenna-specific proteins from scarab beetles, which has been also observed in other scarab species (Wojtasek et al., 1998, 1999), is consistent with the fact that both sexes detect the female-produced sex pheromones (Leal et al., 1994a,b, 1996). We have also analyzed extracts from antennal and control tissues of the American population of the Oriental beetle by native PAGE. Beetles were collected in Rhode Island, frozen, and sent to Davis, California. Although the overall profile was similar to that obtained with the Japanese population, there was a faint band migrating just below the major antenna-specific band (Figure 4). Preliminary sequencing data suggested that, as with previous cases (Wojtasek et al., 1999), the band is due to an isoform of the major protein. The fact that the minor band is not detected in females may be related to the quality of the sample. Females from a laboratory colony were frozen and immediately sent

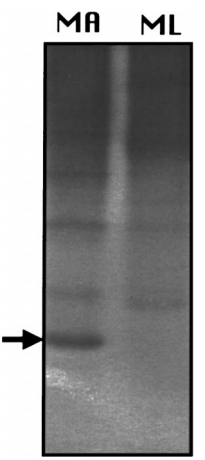


FIG. 3. Soluble protein from the Japanese population of *E. orientalis* separated on 15% native PAGE. MA, male antennae (15 antenna-equivalents); ML, male leg (1 leg equivalent). Arrow indicates an antennae-specific band.

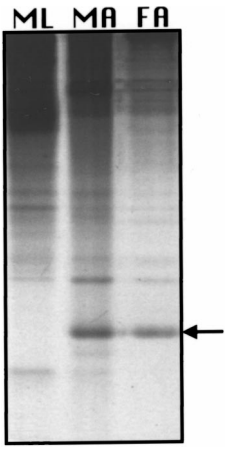


FIG. 4. Soluble protein from the American population of *E. orientalis* separated on 15% native PAGE. MA, male antennae (15 antenna-equivalents); FA, female antennae (18 antenna-equivalents); ML, male leg (2 legs equivalents). Arrow indicates an antenna-specific band.

to Davis, California, whereas males were collected in traps baited with synthetic pheromone and contained beetles that died before the traps were emptied.

We have transferred the antenna-specific band from the native gel to PVDF membrane and obtained its *N*-terminal sequence. Based on the sequence of the first 27 amino acids (MSEEMEELAKQLHDDXVGQTGVDEAHI), the antenna-specific protein from *E. orientalis* showed remarkable similarity to the primary sequence of the PBP from *P. japonica*, PajpPBP (Wojtasek et al., 1998). Given that the undetectable amino acid at position 16 might be due to a cysteine (Leal et al., 1999), only 1 of the 27 amino acids was different from those of PjapPBP sequence.

In order to compare the whole primary sequences of the OBPs from scarab beetles, we cloned the gene encoding the antenna-specific protein in *E. orientalis*. cDNA, derived from the RNA isolated from antennal tissues, was used as template. Polymerase chain reaction (PCR) was carried out by using a degenerate primer based on the *N*-terminal sequence and an oligo-dT to anneal to the poly(A) tail. PCR reactions resulted in amplification of one band of approximately 460 bp. The cDNA encoded a protein with 116 amino acids, with a calculated molecular mass of 12,981Da (considering the formation of three disulfide bonds), and pI of 4.3. Furthermore, the sequence showed six highly conserved cysteine residues at positions 16, 44, 48, 86, 95, and 104 (Figure 5). The occurrence of six cysteines is a hallmark of odorant-binding proteins. They play a pivotal role in 3D structures of PBPs (Leal et al., 1999; Sandler et al., 2000) by forming rigid α-helix links. The amino acid identity between the newly identified protein from *E. orientalis* and PjapPBP, PdivOPB1, and AosaPBP was 93, 91, and 90%, respectively (Figure 5). Because the protein is tissue-specific and shows similarity to previously identified pheromone-binding proteins from scarab beetles,

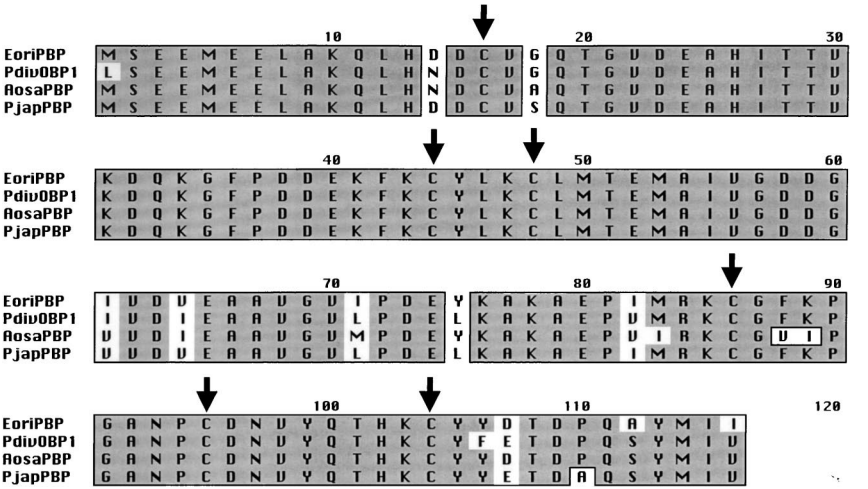


FIG. 5. Sequence alignments of PBPs from *E. orientalis*, *P. diversa*, *A. osakana*, and *P. japonica* using the ClustalW algorithm (MacVector, Oxford Scientific). Arrows indicated the six conserved cysteine residues. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB040144 (EoriPBP), AB040986 (signal peptide for EoriPBP), and AB040985 (signal peptide for AosaPBP).

we suggest that the antenna-specific protein from *E. orientalis* is a pheromone-binding protein.

Pheromone-binding proteins are synthesized in auxilliary cells (trichogen and tormogen cells) (Vogt et al., 1989; Steinbrecht et al., 1992) and secreted into the sensillar lymph surrounding the ORN dendrites and, as such, they have signal peptides. Because we amplified the cDNA with a primer based on the *N*-terminal sequence, the cloned cDNA encoded only the mature protein. In order to obtain the full length cDNA, including the signal peptide, we carried out 5′-rapid amplification of cDNA end (5′-RACE), which leads to an extension of approximately 100 bp. The sequence analysis showed that the signal peptide for EoriPBP has 19 amino acids (MLKLVVLLTLGIYVPAVMS), follows the (-3,-1) rule (von Heijne, 1986), and shows a hydrophobic profile (Figure 6). 5′-RACE with *Anomala osakana* cDNA led to the identification of the signal peptide for AosaPBP, which differs from the signal peptide for EoriPBP only in two positions. In AosaPBP, the amino acids at positions −1 and −11 are Cys and Ser, respectively, as opposed to Ser and Thr in EoriPBP.

Prediction of the secondary structure of EoriPBP by the Chou-Fasman method indicated that the protein is helical-rich (42%), as has been experimentally demonstrated with the pheromone-binding protein from *B. mori*. In BmPBP, the same

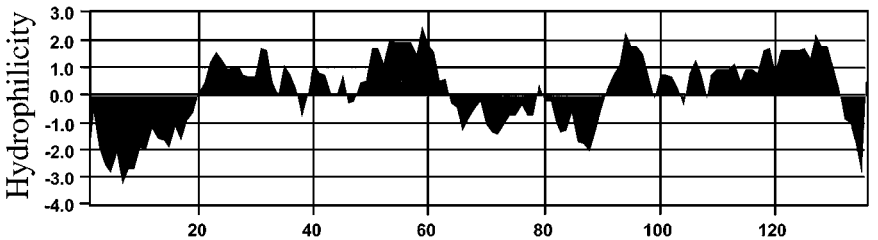


FIG. 6. Hydrophilicity plot of EoriPBP by the von Haijne algorithm (MacVector, Oxford Scientific) indicating the hydophobic nature of the signal peptide (first 19 amino acids).

method predicts 31%, but the crystal structure showed a much higher helical component (60%). Although biased on the low helical component, the method suggests that EoriPBP is possibly part of a family of helix-rich proteins.

In *P. diversa*, two odorant-binding proteins have been previously identified and cloned, but their cognate ligands have never been clearly assigned. On the other hand, binding data support that PjapPBP and AosaPBP are involved in detection of sex pheromones and behavioral antagonists in *P. japonica* and *A. osakana*. Based on the sensitivity of insect olfactory systems to pheromones, one may speculate that the more abundant of the OBPs in *P. diversa*, i.e., PdivOBP2, is involved in the transport and protection of the sex pheromone. However, *P. diversa* has highly sensitive olfactory receptor neurons tuned to green leaf volatile compounds (Hansson et al., 1999; Nikonov et al., 2001). By contrast, the PBPs identified from *P. japonica*, *A. osakana* (Wojtasek et al., 1998), and *E. orientalis* (this paper) have known ligands. Both the lactone pheromones and the unsaturated ketone (Figure 2) have a polar head (functional group) and a hydrophobic tail with unsaturation. The structures of the pheromones for *P. japonica* and *A. osakana* differ in their absolute configurations, whereas the pheromone for *E. orientalis* differs in the functional group and position of the unsaturation. In BmPBP, the double bonds are sandwiched by two of the five phenylalanines, i.e., Phe12 and Phe118, and they were considered to form a general hydrophobic surface for binding and are not specificity determinants for the double bonds in bombykol (Sandler et al., 2000). EoriPBP has only three phenylalanines, i.e., Phe36, Phe42, and Phe88, but Phe88 is unlikely to be participating in the hydrophobic binding site because both AosaPBP and PjapPBP bind the same ligands, the enantiomers of japonilure, and yet AosaPBP has a Val88, as opposed to Phe88. On the other hand, of the residues involved in binding bombykol in BmPBP, the most variable ones are Met61, Leu62, lle91, and Val114, which are likely to be specificity determinants and are near the ends of the pheromone. These residues are either valine, leucine, isoleucine, or methionine, and this variation could be a way to alter the binding pocket (Sandler et al., 2000). Despite the low number of sequences

known from scarab PBPs, the same picture is emerging with four positions, i.e., 61, 64, 71, and 82, which are either valine, leucine, isoleucine, or methionine. In conclusion, despite the high similarity in the amino acid sequences of EoriPBP, PjapPBP, and AosaPBP, the least conserved residues may be involved in binding specificity.

Acknowledgments—The authors would like to thank Dr. Steven R. Alm, University of Rhode Island, for providing frozen beetles of the US population, and Dr. Karl-Ernst Kaissling for his critique of an earlier draft of the manuscript. This project was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (BRAIN) granted to W.S.L. G.P. was supported by the BRAIN fund. Work in the United States was made possible through direct financial support from the department, college, and chancellor's office at UC Davis. We appreciate the helpful discussion of the members of our laboratory.

REFERENCES

CLYNE, P. J., WARR, C. G., FREEMAN, M. R., LESSING, D., KIM, J., and CARLSON, J. R. 1999. A novel family of divergent seven-transmembrane proteins: Candidate odorant receptors in Drosophila. *Neuron* 22:327–338.

DU, G. and PRESTWICH, G. D. 1995. Protein structure encodes the ligand binding specificity in pheromone binding proteins. *Biochemistry* 34:8726–8732.

HANSSON, B. S., LARSSON, M. C., and LEAL, W. S. 1999. Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiol. Entomol*. 24:121–126

KAISSLING, K.-E. 1987. R. H. Wright lectures on insect olfaction, pp. 28–32, *in* K. Colbow (ed.). Lectures on Insect Olfaction. Simon Fraser University, Barnaby, British Columbia.

KAISSLING, K. E. and PRIESNER, E. 1970. Die Riechschwelle des Seidenspinners. *Naturwissenschaften* 57:23–28.

LEAL, W. S. 1993. (*Z*)- and (*E*)-Tetradec-7-en-2-one, a new type of sex pheromone from the Oriental beetle. *Naturwissenschaften* 80:86–87.

LEAL, W. S. 1996. Chemical communication in scarab beetles: Reciprocal behavioral agonist-antagonist activities of chiral pheromones. *Proc. Natl. Acad. Sci. USA* 93:12112–12115.

LEAL, W. S. 1998. Chemical ecology of phytophagous scarab beetles. *Annu. Rev. Entomol*. 43:39–61.

LEAL, W. S., HASEGAWA, M., SAWADA, M., and ONO, M. 1994a. Sex pheromone of oriental beetle, *Exomala orientalis*: Identification and field evaluation. *J. Chem. Ecol*. 20:1705–1718.

LEAL, W. S., KAWAMURA, F., and ONO, M. 1994b. The scarab beetle *Anomala albopilosa sakishimana* utilizes the same sex pheromone blend as a closely related and geographically isolated species, *Anomala cuprea. J. Chem. Ecol*. 20:1667–1676.

LEAL, W. S., YADAVA, C. P. S., and VIJAYVERGIA, J. N. 1996. Aggregation of the scarab beetle *Holotrichia consanguinea* in response to female-released pheromone suggests secondary function hypothesis for semiochemical. *J. Chem. Ecol*. 22:1557–1566.

LEAL, W. S., ZARBIN, P. H. G., WOJTASEK, H., KUWAHARA, S., HASEGAWA, M., and UEDA, Y. 1997. Medicinal alkaloid as a sex pheromone. *Nature* 385:213.

LEAL, W. S., NIKONOVA, L., and GUIHONG, P. 1999. Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. *FEBS Lett*. 464:85–90.

NIKONOV, A., VALIYAVEETTIL, and LEAL, W. S. 2001. A photoaffinity-labeled green leaf volatile compound "tricks" highly selective and sensitive insect olfactory receptor neurons. *Chem. Senses* 26:49–54.

ROGERS, M. E., JANI, M. K., and VOGT, R. G. 1999. An olfactory-specific glutathione-*S*-transferase in the sphinx moth *Manduca sexta*. *J. Exp. Biol*. 202:1625–1637.

Rybczynski, R. G. V. R. and Lerner, M. R. 1990. Antennal-specific pheromone-degrading aldehyde oxidases from the moths *Antheraea polyphemus* and *Bombyx mori*. *J. Biol. Chem*. 265:19712–19715.

SANDLER, B. H., NIKONOVA, L., LEAL, W. S., and CLARDY, J. 2000. Sexual attraction in the silkworm moth: Structure of the pheromone-binding protein-bombykol complex. *Chem. Biol*. 7:143–151.

STEINBRECHT, R. A., OZAKI, M., and ZEIGELBERGER, G. 1992. Immunocytochemical localization of pheromone-binding protein in moth antennae. *Cell Tissue Res*. 270:287–302.

TUMLINSON, J. H., KLEIN, M. G., DOOLITTLE, R. E., LADD, T. L., and PROVEAUX, A. T. 1977. Identification of the female Japanese beetle sex pheromone: Inhibition of male response by an enantiomer. *Science* 197:789–792.

VOGT, R. G. and RIDDIFORD, L. M. 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293:161–163.

VOGT, R. G., RIDDIFORD, L. M., and PRESTWICH, G. D. 1985. Kinetic properties of a sex pheromone-degrading enzyme: The sensillar esterase of *Antheraea polyphemus*. *Proc. Natl. Acad. Sci. USA* 82:8827–8831.

VOGT, R. G., KÖHNE, A. C., DUBNAU, J. T., and PRESTWICH, G. D. 1989. Expression of pheromone binding proteins during antennal development in the gypsy moth *Lymantria dispar*. *J. Neurosci*. 9:3332–3346.

VOGT, R. G., CALLAHAN, F. E., ROGERS, M. E., and DICKENS, J. C. 1999. Odorant binding protein diversity and distribution among insect oredrs, as indicated by LAP, an OBP-related protein from the true bug *Lygus lineolaris* (Hemiptera, Heteroptera), *Chem. Senses* 24:481–495.

VON HEIJNE, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res*. 14:4683–4690.

VOSSHALL, L. B., AMREIN, H., MOROZOV, P. S., RZHETSKY, A., and AXEL, R. 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* 96:725–736.

VOSSHALL, L. B., WONG, A. L., and AXEL, R. 2000. An olfactory sensory map in the fly brain. *Cell* 102:147–159.

WOJTASEK, H., HANSSON, B. S., and LEAL, W. S. 1998. Attracted or repelled: A matter of two neurons, one pheromone binding protein, and a chiral center. *Biochem. Biophys. Res. Commun*. 250:217–222.

WOJTASEK, H., PICIMBON, J.-F., and LEAL, W. S. 1999. Identification and cloning of odorant binding proteins from the scarab beetle *Phyllopertha diversa*. *Biochem. Biophys. Res. Commun*. 263:832–837.

ZHANG, A., FACUNDO, H. T., ROBBINS, P. S., LINN JR, C. E., HANULA, J. L., VILLANI, M. G., and ROELOFS, W. L. 1994. Identification and synthesis of female sex pheromone of the Oriental beetle, *Anomala orientalis* (Coleoptera: Scarabaeidae). *J. Chem. Ecol*. 20:2415–2417.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

PHEROMONE TRAILING BEHAVIOR OF THE BROWN TREE SNAKE, *Boiga irregularis*

MICHAEL J. GREENE,* SHANTEL L. STARK, and ROBERT T. MASON

Department of Zoology
Oregon State University
3029 Cordley Hall, Corvallis, Oregon 97331

(Received February 9, 2001; accepted July 15, 2001)

Abstract—The ability of snakes to follow pheromone trails has significant consequences for survival and reproduction. Of particular importance is the ability of snakes to locate conspecifics during the breeding season via the detection of pheromone trails. In this study, the ability of male brown tree snakes (*Boiga irregularis*), a tropical, rear-fanged colubrid, to follow pheromone trails produced by reproductively active conspecifics was tested in the laboratory by using a Y maze. Males displayed a trailing response to both female and male pheromone trails over blank controls. As males of this species display ritualized combat behavior, these responses likely represent both direct and indirect mechanisms, respectively, for the location of potential mates in the wild. Males did not, however, discriminate between male and female trails when given a choice on the Y maze.

Key Words—Trailing behavior, Y maze, mate location, brown tree snake, *Boiga irregularis*, reptile pheromones, Reptilia, Colubridae, male combat behavior, invasive pest species.

INTRODUCTION

Many species of snakes have demonstrated the ability to locate conspecifics during the breeding season by following pheromone trails passively laid on the substrate as they move through the environment (e.g., Ford, 1982; Ford and Schofield, 1984; Plummer and Mills, 1996; LeMaster et al., 2001). Trailing behavior is also used by snakes in other behavioral contexts, including migration to and from winter hibernacula, in aggregation, and in prey location (reviewed by Ford, 1986;

*To whom correspondence should be addressed. Present address: Department of Biological Sciences 371 Serra Mall, Stanford University, Stanford, California 94305-5020. E-mail: greene@ants.stanford.edu

0098-0331/01/1100-2193$19.50/0

Mason, 1992; Mason et al., 1998). The majority of studies concerning snake trailing behavior have been conducted with northern temperate species, with particular emphasis on the garter snakes of the genus *Thamnophis* (Ford, 1986).

The purpose of this study was to characterize the pheromone-mediated trailing behavior of the brown tree snake, *Boiga irregularis*. The brown tree snake is a rear-fanged, tropical colubrid native to Australia, Papua-New Guinea, and the Solomon Islands (Rodda et al., 1997). The species can reach snout-to-vent lengths of up to 3 m, weighs up to 2 kg, and possesses a relatively thin body typical of arboreal snakes (Rodda et al., 1997). Brown tree snakes are generalist predators that actively forage for a diet consisting of mammals, reptiles, amphibians, and birds (Savidge, 1988). Although primarily arboreal, it spends a significant proportion of its time on the ground and typically forages within the lower 3 m of vegetation (Cogger, 1992). The snake is an introduced pest species in the Pacific, notably on the island of Guam, where it is responsible for the extinctions of several native forest bird species and has caused considerable economic damage (Rodda et al., 1997).

Brown tree snakes display reproductive behaviors similar to other colubrid snake species (Greene and Mason, 2000). Males of this species display stereotyped courtship behaviors that are triggered by a sex pheromone located in female skin lipids (Greene and Mason, 1998). This pheromone has been isolated and initially characterized as a suite of nonvolatile, nonpolar skin lipid molecules (Greene and Mason, 1998). Male brown tree snakes also display ritualized combat behavior in response to other reproductively active males (Greene and Mason, 2000). In snakes, ritualized combat serves as a competition among males to gain access to females during the breeding season (Andrén, 1986; Schuett and Gillingham, 1989). This behavior appears to be stimulated by a pheromone located in male brown tree snake skin lipids (Greene and Mason, 2000).

The brown tree snake does not form large breeding aggregations, although small nonbreeding winter aggregations have been reported in its native and introduced range (Pendleton, 1947; Covacevich and Limpus, 1973). Therefore, it is likely that brown tree snakes, like other snakes, utilize pheromone trails to locate spatially distributed conspecifics during the breeding season. In this study, the ability of the brown tree snake to trail reproductively active conspecifics was tested by using a Y maze in the laboratory.

METHODS AND MATERIALS

Husbandry. The animals used were collected on Guam and housed under laboratory conditions for six years prior to being used in the experiment (Greene et al., 1997). Seven female and 10 male snakes, individually housed in Plexiglas cages designed specifically for arboreal reptiles, were used (Mason et al., 1991). Males in the colony had a mean (±SD) snout-to-vent length (SVL) of 162.2 ±

22.1 cm and a mean mass of 647.5 ± 265.7 g. Females had a mean SVL of 137.9 ± 9.9 cm and a mean mass of 385.7 ± 81.5 g. Snake cages contained branches for climbing and hide boxes attached to the roofs of the cages, in which the snakes spent the majority of their time. Room temperature cycled daily from 24°C to 30°C, and relative humidity was maintained at approximately 80% by using a room humidifier and by daily spraying of the inside of the cages with water. Light (12L : 12D) was provided by overhead fluorescent lights. Snakes were fed thawed mice or chicks every three weeks, and water was available *ad libitum* for drinking and soaking. All snakes in the captive colony were mature adults in breeding condition during the study.

Y-maze Design. To test the ability of the snakes to trail conspecifics, a Y maze was constructed of clear polyvinyl chloride tubing (PVC) with an internal diameter of 5.2 cm (Figure 1). The maze consisted of an initial stretch of tubing 96 cm long that connected to a Y junction separated by a 45° angle into two arms, each also 96 cm long. The maze was elevated at an angle of 25° from the floor to entice the snakes into the initial stretch, as they are excellent climbers and prefer to move to the highest point possible when given a choice. All hide boxes used in housing the snakes could be attached to the initial stretch by using PVC adapters. Empty, clean hide boxes were attached to the ends of the arms, also by using PVC adapters, for collection of the snakes at the conclusion of a trial. The initial stretch, Y junction, and the arms could be taken apart for cleaning between trials.

This maze design allowed for minimal handling of the snakes, thus reducing the display of defensive behaviors in this relatively aggressive species. In addition, the design allowed for easy cleaning and reassembly as well as for containment of the snakes in the maze. Importantly, by having a relatively small internal tube diameter, conspecific pheromone trails were laid down in a seminatural manner, as trail-producing snakes deposited odors on a horizontal surface (bottom of tube) and

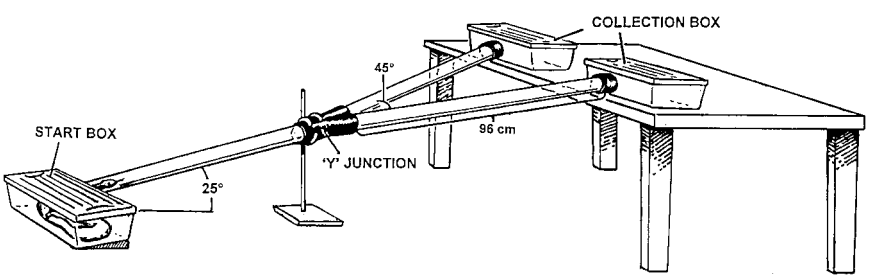


FIG. 1. Diagram of the Y-maze system used to conduct trailing experiments in this study. The Y maze was constructed of 5.2-cm-diameter, clear PVC piping. The initial stretch and arms were each 96 cm long, and the Y junction separated the maze by 45°. The maze was elevated from the floor at a 25° angle. Hide boxes were attached to the maze at the start of initial stretch and at the ends of the arms.

vertical surfaces (sides of the maze) (Ford and Low, 1983). Brown tree snakes, like other snakes species, are highly thigmotactic, preferring to travel along edges as opposed to open areas. The maze design, by having rounded edges and a small internal diameter, reduced concerns of thigmotaxis conflicting with trailing behavior (Costanzo, 1989).

General Experimental Conditions. To characterize the ability of males to trail conspecifics, four experiments were completed: (1) males trailing female pheromone trail versus blank arm ($N = 10$ trials), (2) males trailing male pheromone trail versus blank arm ($N = 9$ trials), (3) males trailing male pheromone trail versus female pheromone trail ($N = 10$ trials), and (4) blank control ($N = 10$ trials, no pheromone trails applied to either arm). Males were tested only once during each experiment. All snakes were in breeding condition when used; courtship and ritualized male combat were exhibited by all snakes during the period of time in which the study was conducted. The trailing ability of females was not tested, as females were generally inactive during this time period and would not exit the start box at the beginning of most trials.

All trials were conducted during the scotophase between 2100 and 0200 hr, when the snakes were normally active and the snake room was dark. Lighting was provided at a minimal level by a red 7 W incandescent bulb placed 2 m behind the maze so that the arms were lit evenly.

Trails were produced by allowing a randomly chosen snake of the desired sex to pass once completely through the initial stretch, Y junction, and one arm of the maze. The side of the Y junction leading to the control arm was blocked with a piece of clean cloth so that no lipids were inadvertently added to the control side. The arm treated with a conspecific pheromone trail was randomly chosen in each case. In experiment 3, in which males were given a choice between male and female pheromone trails, female trails were always applied to the maze immediately before the male trail. Both male and female pheromone trails were present in the initial stretch of the maze and the Y junction, while the arms contained only male or female trails. In experiment 3, a random male and female were chosen to produce the trail for each trial; however, males were never tested against their own trail.

To begin a trial, a hide box containing a snake was removed from its home cage and attached to the initial stretch of the maze. The snake was allowed to enter the maze of its own accord. We defined "trailing" as occurring when a snake's head entered the collection box attached to arms of the maze. During trials, an investigator, blind to the treatment, observed while hidden behind a blind.

A trial was terminated and conducted on another night if a snake exhibited defensive behavior, such as striking and tail-lashing or if a snake did not enter the maze from its hide box within 30 min after the start of the trial. Additionally, trials in which snakes did not display the trail contact response (Brown and MacLean, 1983), a set of behaviors diagnostic of snake pheromone trailing, were not used in the final statistical analyses.

TABLE 1. RESULTS OF EXPERIMENTS TESTING ABILITY OF MALE BROWN TREE SNAKES TO TRAIL CONSPECIFICS

Experiment	Condition	*N*	Trailed	Did not trail	*P* (1-tailed binomial)
1	Males trailing females	10	8	2	0.055
2	Males trailing males	9	8	1	0.020
3	Males trailing females versus males (trailed = male chose female arm)	10	5	5	1.000
4	Males trailing blank maze	10	5	5	1.000

Statistical Analyses. Statistical significance in the trailing response was determined by using a one-tailed binomial test (Sokal and Rohlf, 1995).

RESULTS

In experiment 1, eight of 10 males entered the female-treated arm of the Y maze versus the blank control arm (Table 1; $P = 0.055$). In experiment 2, eight of nine males entered the male treated arm, while only one of nine males entered the blank control arm (Table 1; $P = 0.02$). Males demonstrated no preference for female pheromone trails over male pheromone trails (experiment 3) when given a choice on the Y-maze (Table 1; $P = 1.000$). When tested on a Y maze in which both arms were left blank (experiment 4), males chose the arms of the maze equally (Table 1; $P = 1.000$).

Head-jerking behavior [a behavior only displayed in the context of courtship and male ritualized combat in this species (Greene and Mason, 2000)] was displayed in two trials (by two different males) when males were following female trails in experiment 1 and was observed in two trials (by two different males) when males were following male pheromone trails in experiment 2. Snout-probing behavior, in which the male would press his snout to the pheromone trail, was observed in most trials. While snout-probing, males would often drag their snouts backwards along the pheromone trail. In addition, all individuals that trailed displayed behaviors associated with trailing in other species, including tongue-flicks directed to the trail and periodic pauses with side to side movements of the head (Brown and MacLean, 1983).

DISCUSSION

The ability of male brown tree snakes to follow female pheromone trails most likely represents a mechanism to locate potential mates during the breeding season. Similar responses have been reported in several other species, mostly in colubrids

of the genus *Thamnophis* (Ford, 1986; Mason, 1992; Mason et al., 1998). However, few reports exist of trailing behavior in other snake taxa, including tropical species and those that display combat behavior such as the brown tree snake (Ford, 1986; Mason, 1992).

Male brown tree snakes might follow male pheromone trails in order to locate males that are near or are courting females. By initiating combat, the trailing male may be able to displace the other male and gain access to the local female, as has been suggested for other species (Shine et al., 1981). There would be little cost to this strategy, as combat would be unlikely to ensue without the presence of a female. Field reports of the courtship and combat behavior of the Swedish adder (*Vipera berus*) have described the ability of males of this species to trail both conspecific males and females (Andrén, 1986). Upon contacting and tongue-flicking the integument of a female, courtship will ensue. Conversely, upon contacting another male, males will initiate combat if a female is present. The winner of the combat bout gains access to the local female by establishing a temporary territory that it will defend from other males (Andrén, 1986).

It would be expected that, given a choice between a conspecific male and female trail, males would prefer the female trail. This choice would, at least in a natural situation, lead directly to a female and would allow the male to avoid agonistic interactions with other males. However, in this study, males did not display a significant trailing response to either of the sexes, choosing the male trail as often as the female trail. Behavioral observations and bioassays have shown that male brown tree snakes can discriminate between males and females via pheromones in other contexts, such as courtship, making it unlikely that there are no chemical differences between sexes (Greene and Mason, 1998, 2000). This apparent lack of behavioral discrimination may be an artifact of how the male and female trails were laid down in the maze. In the initial stretch of the maze, both male and female pheromone trails were present together; these trails then diverged at the Y junction. However, the male trail was always laid down on top of the female trail, which may be significant to trailing males, as has been found in vertebrate taxa, such as scent-marking rodents (e.g., Johnston et al., 1997; Ferkin, 1999). Further, under natural conditions, a similar situation might signal to a trailing male that a male and female pair are nearby. If so, following either a male or female pheromone trail may lead to the courting pair where the trailing male would have the opportunity to initiate combat and could, therefore, displace the local male. Also in this situation, the trailing male might attempt to revert to vision in order to locate the movement of the courting pair at short distances, as other species are known to do in the field (Hawley and Aleksiuk, 1975; Andrén, 1986). Unfortunately, because our captive colony of brown tree snakes bred year-round, we were not able to test non-reproductively-active snakes to determine if this particular behavioral response is simply a species recognition response.

It is has been hypothesized that male trailing behavior in response to females is triggered by the female sex pheromone (Mason, 1992). Snake sex pheromones are located in the skin lipids of females and are thought to be composed primarily of long-chain, high-molecular-weight molecules of relatively low volatility (Mason et al. 1989, 1990; Murata et al., 1991; Greene and Mason, 1998). Sex pheromones and other skin lipids are passively deposited on the ground as females pass through the environment, allowing males to trail females upon detection of the skin lipid trail with their vomeronasal organs. In this study, male snakes were observed displaying courtship and combat behaviors (head-nodding in both cases) in response to female and male pheromone trails, respectively. Display of these behaviors by trailing males suggests that the female sex pheromone and the male combat pheromone play a significant role in mediating trailing behavior.

Currently, brown tree snake control methods on Guam are limited to detection by trained dogs and capture by large trapping regimes. However, more attention has been given to using brown tree snake semiochemicals as repellents and attractants to aid in control of this particularly detrimental pest species (Brown Tree Snake Control Committee, 1996; Mason, 1998; Mason and Greene, 2001). Pheromone attractants would be particularly useful in increasing the efficacy of traps in the field by increasing the distances over which trap lures work. Currently, live prey is used to lure snakes into traps, a method that operates over relatively short distances. The results of this study indicate that pheromones located on the skin of male and female brown tree snakes may be effective attractants to males during the breeding season if used in a larger integrated management plan.

Acknowledgments—The authors would like to thank M. P. LeMaster for his thoughtful comments on the manuscript. This work was supported by a grant-in-aid award from the Sigma Xi Scientific Research Society to M.J.G. and by grants from the US Fish & Wildlife Service (USDI 14-16-0009-1577) and the National Science Foundation (INT-9114567), as well as a National Science Foundation National Young Investigator Award (IBN-9357245) to R.T.M. This research was conducted under the authority of US Fish & Wildlife Permits No. PRT-769753 and Oregon State University Institutional Animal Care and Use Committee Protocol No. LAR 932.

REFERENCES

ANDRÉN, C. 1986. Courtship, mating and agonistic behaviour in a free-living population of adders, *Vipera berus* (L.). *Amphibia-Reptilia* 7:353–383.

BROWN, W. S. and MACLEAN, F. M. 1983. Conspecific scent-trailing by newborn timber rattlesnakes, *Crotalus horridus*. *Herpetologica* 39:430–436.

BROWN TREE SNAKE CONTROL COMMITTEE. 1996. Brown tree snake control plan. Aquatic Nuisance Species Task Force-Washington, DC.

COGGER, H. G. 1992. Reptiles and Amphibians of Australia. Comstock/Cornell, Ithaca, New York.

COSTANZO, J. 1989. Conspecific scent trailing by garter snakes (*Thamnophis sirtalis*) during autumn: Further evidence for use of pheromones in den location. *J. Chem. Ecol.* 15:2531–2538.

COVACEVICH, J. and LIMPUS, C. 1973. Two large winter aggregations of three species of tree-climbing snakes in southeastern Queensland. *Herpetofauna* 6:16–21.

FERKIN, M. H. 1999. Scent over-marking and adjacent-marking as competitive tacttics used during chemical communication in voles, pp. 239–246, *in* R. E. Johnston, D. Müller-Schwarze, and P. W. Sorenson (eds.). Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Publishers, New York.

FORD, N. B. 1982. Species specificity of sex pheromone trails of sympatric and allopatric garter snakes. *Copeia* 1982:10–13.

FORD, N. B. 1986. The role of pheromone trails in the sociobiology of snakes, pp. 261–278, *in* D. Duvall, D. Müller-Schwarze, and R. M. Silverstein (eds.). Chemical Signals in Vertebrates, Vol. 4. Plenum Publishing, New York.

FORD, N. B. and LOW, J. R. 1983. Sex pheromone source location by snakes: A mechanism for detection of direction in nonvolatile trails. *J. Chem. Ecol.* 10:1193–1199.

FORD, N. B. and SCHOFIELD, C. W. 1984. Species and sexual specificity of sex pheromone trails in the plains garter snake, *Thamnophis radix. Herpetologica* 40:51–55.

GREENE, M. J. and MASON, R. T. 1998. Chemical mediation of reproduction in the brown tree snake, *Boiga irregularis. Ecoscience* 5:405–409.

GREENE, M. J. and MASON, R. T. 2000. The courtship, mating and combat behavior of the brown tree snake, *Boiga irregularis. Herpetologica* 56:166–175.

GREENE, M. J., NICHOLS, D. K., HOYT, R. F., JR, and MASON, R. T. 1997. The brown tree snake (*Boiga irregularis*) as a laboratory animal. *Lab. Anim.* 26:28–31.

HAWLEY, A. W. and ALEKSIUK, M. 1975. Thermal regulation of spring mating behavior in the red-sided garter snake (*Thamnophis sirtalis parietalis*). *Can. J. Zool.* 53:769–776.

JOHNSTON, R. E., SOROKIN, E. S., and FERKIN, M. H. 1997. Female voles discriminate males' over-markes and prefer top-scent males. *Anim. Behav.* 54:679–690.

LEMASTER, M. P., MOORE, I. T., and MASON, R. T. 2001. Conspecific trailing behaviour of red-sided garter snakes, *Thamnophis sirtalis parietalis*, in the natural environment. *Anim. Behav.* 61:827–833.

MASON, R. T. 1992. Reptilian pheromones, pp. 114–228, *in* C. Gans and D. Crews (eds.). Biology of the Reptilia, Vol. 18. The University of Chicago Press, Chicago, Illinois.

MASON, R. T. 1998. Integrated pest management: The case for pheromonal control of habe and brown tree snake, pp. 196–205, *in* G. H. Rodda, Y. Sawai, D. Chiszar, and H. Tanaka (eds.). Snakes and Man: Controlling Pest Species for Conservation and Human Health. Cornell University Press, Ithaca, New York.

MASON, R. T. and GREENE, M. G. 2001. Invading pest species and the threat to biodiversity: Pheromonal control of Guam brown tree snakes, *Boiga irregularis, in* A. Marchlewska-Koj, J. Lepri, and D. Müller-Schwarze (eds.). Chemical Signals in Vertebrates, IX. Kluwer Academic/Plenum Publishers, in press, New York.

MASON, R. T., FALES, H. M., JONES, T. H., PANNELL, L. K., CHINN, J. W., and CREWS, D. 1989. Sex pheromones in snakes. *Science* 245:290–293.

MASON, R. T., FALES, H. M., JONES, T. H., PANNELL, L. K., and CREWS, D. 1990. Characterization, synthesis and behavioral response to the sex pheromone of the red-sided garter snake (*Thamnophis sirtalis parietalis*). *J. Chem. Ecol.* 16:2353–2369.

MASON, R. T., HOYT, R. F., PANNELL, L. K., WELLNER, E. F., and DEMETER, B. 1991. Cage design and configuration for arboreal reptiles. *Lab. Anim. Sci.* 41:84–86.

MASON, R. T., CHIVERS, D., MATHIS, A., and BLAUSTEIN, A. R. 1998. Bioassays with amphibians and reptiles, pp. 271–325, *in* J. Millar and K. Haynes (eds.). Methods in Chemical Ecology, Vol. 2, Bioassay Methods. Chapman & Hall, New York.

MURATA, Y., PANNELL, L. K., YEH, H., JONES, T. H., FALES, H. M., and MASON, R. T. 1991. New ketodienes from the cuticular lipids of the Guam brown tree snake, *Boiga irregularis*. *J. Nat. Prod.* 54:233–240.

PENDLETON, R. C. 1947. A snake "den" tree on Guadalcanal island. *Herpetologica* 3:189–190.

PLUMMER, M. V. and MILLS, N. E. 1996. Observations on trailing and mating behaviors in hognose snakes (*Heterodon platyrhinos*). *J. Herpetol*. 30:80–82.

RODDA, G. H., FRITTS, T. H., and CHISZAR, D. 1997. Disappearance of Guam's wildlife. *BioScience* 47:565–574.

SAVIDGE, J. A. 1988. Food habits of *Boiga irregularis*, an introduced predator on Guam. *J. Herpetol*. 22:275–282.

SCHUETT, G. W. and GILLINGHAM, J. C. 1989. Male–male agonistic behavior of the copperhead, *Agkistrodon contortrix*. *Amphibia-Reptilia* 10:243–266.

SHINE, R., GRIGG, G. C., SHINE, T. G., and HARLOW, P. 1981. Mating and male combat in Australian blacksnakes, *Pseudachis porphyriacus*. *J. Herpetol.* 15:101–107.

SOKAL, R. R. and ROHIF, F. J. 1995. Biometry. W. H. Freeman & Co., New York.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

IDENTIFICATION AND SYNTHESIS OF A KAIROMONE MEDIATING HOST LOCATION BY TWO PARASITOID SPECIES OF THE CASSAVA MEALYBUG *Phenacoccus herreni*

PAUL-ANDRE CALATAYUD,[1,*] JACQUES AUGER,[2] ERIC THIBOUT,[2] SEVERINE ROUSSET,[2] ANA MILENA CAICEDO,[1] SABINE CALATAYUD,[1] HOLGER BUSCHMANN,[3] JOSETTE GUILLAUD,[4] NICOLE MANDON,[2] and ANTHONY C. BELLOTTI[5]

[1]*IRD (formerly ORSTOM)/CIAT, Cassava Entomology, CIAT*
AA 6713, Cali, Colombia

[2]*School of Sciences*
François Rabelais University, IRBI, UMR CNRS
6035 Parc de Grandmont, F-37200 Tours, France

[3]*Department of Agroecology in the Tropics and Subtropics*
University of Hohenheim
Fruwirthstrasse 12, D-70593 Stuttgart, Germany

[4]*INSA/INRA, Laboratory of Applied Biology, INSA*
20, Avenue A. Einstein
F-69621 Villeurbanne Cedex, France

[5]*CIAT, Cassava Entomology, CIAT*
AA 6713, Cali, Colombia

(Received January 26, 2001; accepted July 10, 2001)

Abstract—Two encyrtid species, *Acerophagus coccois* and *Aenasius vexans*, parasitoids of the cassava mealybug *Phenacoccus herreni* use a contact kairomone from the body surface of their host as a host-location stimulant. The kairomone was synthesized and identified as *O*-caffeoylserine based on a combination of chromatographic methods. The synthetic compound was determined to be active.

Key Words—*O*-Caffeoylserine, *Acerophagus coccois*, *Aenasius vexans*, Hymenoptera, Encyrtidae, Homoptera, Pseudococcidae.

*To whom correspondence should be addressed. E-mail: P.CALATAYUD@CGIAR.ORG

0098-0331/01/1100-2203$19.50/0

INTRODUCTION

The cassava mealybug, *Phenacoccus herreni* William & Cock (Homoptera: Pseudococcidae), is an important pest of cassava in South America (Bellotti et al., 1983; Noronha, 1990). Two encyrtid parasitoids, *Acerophagus coccois* Smith and *Aenasius vexans* Kerrich (Hymenoptera: Encyrtidae), are being studied to control *P. herreni* populations at the International Center for Tropical Agriculture (CIAT) in Cali, Colombia. *A. coccois* is considered a generalist because it parasitizes different mealybug species among the Pseudococcidae family. *A. vexans* is a specialist, parasitizing only *P. herreni* (Dorn et al., 2001).

During host selection, parasitoids use a variety of cues to assess the quality of their hosts, such as shape, surface structure, and both external and internal semiochemicals (waxy secretions and honeydew) (Lewis and Martin, 1990; Vinson, 1991; Godfray, 1994). The structure and quantity of these semiochemicals influence host acceptance by parasitoids and vary according to species, age or stage of development, size, condition, and diet of the parasitoid host (Vinson, 1991; Powell, 1992; Takabayashi and Takahashi, 1993; Röse et al., 1997). One important class of these subtances used by parasitic Hymenoptera to identify their hosts is kairomones (Lewis and Martin, 1990). For example, in the California red scale *Aonidiella aurantii* Maskell (Hemiptera: Diaspididae), a kairomone isolated from their cover and identified as *O*-caffeoyltyrosine, the ester of caffeic acid and tyrosine, mediates host recognition and induces oviposition by the parasitic wasp *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) (Hare et al., 1993; Millar and Hare, 1993).

In cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Homoptera: Pseudococcidae), a serious pest of cassava in Africa (Neuenschwander et al., 1990; Le Rü et al., 1991), preliminary analyses indicated the presence of a compound composed of a serine and a caffeic acid moiety, probably corresponding to *O*-caffeoylserine (Calatayud, unpublished data). This compound was also detected in *P. herreni*; however, it could not be identified in the mealybug because it was always hydrolyzed during the purification process. It was, therefore, necessary to synthesize the ester and then verify whether the synthetic compound was similar to the compound assumed to be *O*-caffeoylserine. Because this putative compound is similar to the contact kairomone *O*-caffeoyltyrosine, important in another parasitoid–homopteran interaction as reported by Hare et al. (1993) and Millar and Hare (1993), we also hypothesized that this substance would act as a contact kairomone for the cassava mealybug parasitoids. Therefore, the objective of this study was to confirm the identification of *O*-caffeoylserine in *P. herreni* after its synthesis and to study its influence on host-location behavior of *A. coccois* and *A. vexans*.

METHODS AND MATERIALS

Insect Rearing. The cassava mealybug, *P. herreni*, was reared at CIAT on 30 to 40-cm potted cassava plants (cv. CMC 40) in a glasshouse at 27–33°C and 12L : 12D photoperiod. Adult females, the stage most parasitized by *A. coccois* and *A. vexans* (Seligmann, 1998), were collected from the colony for chemical analysis and bioassay.

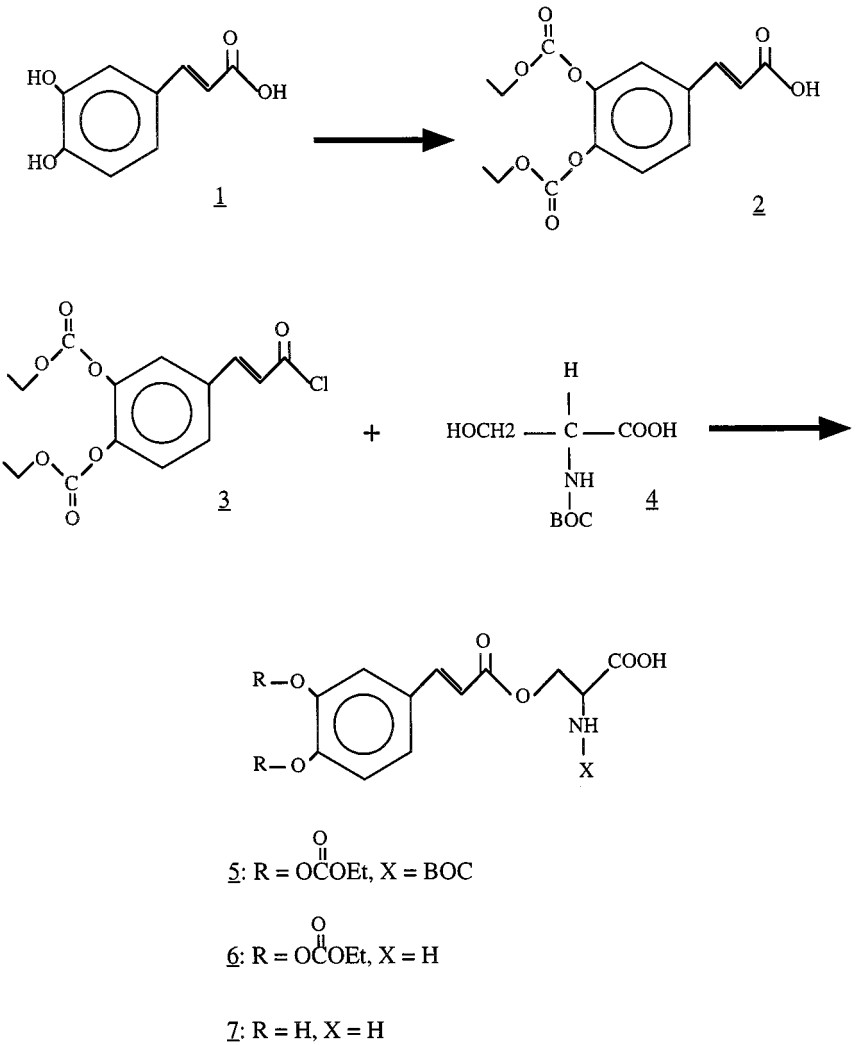
Both parasitoids species, *A. coccois* and *A. vexans*, were reared continuously on mealybug-infested cassava plants (cv. CMC 40) as described above for the cassava mealybug. Emerging adults (both males and females) were placed for 48 hr in transparent polystyrene tubes (15 × 1.5 cm) containing honey droplets as a food source. The wasps were sexed, and females were selected for bioassay.

Synthesis of Kairomone (*Figure 1*). The method used to synthesize *O*-caffeoylserine was that described by Millar and Hare (1993), for synthesizing *O*-caffeoyltyrosine. Caffeic acid (**1**) (10 g, 55 mmol) was added to ethyl chloroformate to produce 13.17 g (73.9% yield) of 3,4-diethoxycarbonyl-caffeic acid (**2**). ^{1}H NMR ($CDCl_3$): ∂ 7.72 (d, 1H, $J = 15.9$ Hz, C<u>H</u>COOH), 7.24–7.46 (3H, m aromatic), 6.42 (d, 1H, $J = 15.9$ Hz, olefinic H), 4.28 (2 overlapped quartets, 4H, $J = 7.1$ Hz, CH_2s), 1.33 (2 overlapped triplets, $J = 7.1$ Hz, methyls).

The acid (**2**) (13.17 g, 40.6 mmol) was stirred with 36.5 ml of thionyl chloride to obtain 10.23 g (73.7% yield) of 3,4-diethoxycarbonyl-caffeoyl chloride (**3**). ^{1}H NMR ($CDCl_3$): ∂ 7.73 (d, 1H, $J = 15.6$ Hz, olefinic H), 7.23–7.48 (3H, m aromatic), 6.56 (d, 1H, $J = 15.6$ Hz, olefinic H), 4.30 (overlapped quartets, 4H, $J = 7.1$ Hz, CH_2s), 1.36 (overlapped triplets, 6 H, $J = 7.1$ Hz, methyls). Then 1.71 g (5 mmol) of the chloride (**3**) was coupled with the *N-t*-BOC-(L)-serine (**4**) (1.03 g, 5 mmol; Sigma Chemical Co., St. Louis, Missouri) to produce 2.39 g (quantitative) of the compound (**5**). ^{1}H NMR ($CDCl_3$): ∂ 7.70 (d, 1H, $J = 16.2$ Hz, olefinic H), 7.22–7.58 (3H, m aromatic), 6.40 (d, 1H, $J = 15.9$ Hz, olefinic H), 5.62 (br. d, 1H, NH), 4.50 (m, 1H, NH-C<u>H</u>), 4.26 (br. quartet, 4H, C<u>H</u>$_2$s), 3.75 (m, 2H, -O-C<u>H</u>$_2$-CH-), 1.27–1.39 (m, 9H, *t*-BOC methyls, 6H, methyls).

After stirring the compound (**5**) (752 mg) with a mixture of methylene chloride and trifluoroacetic acid, the resulting dicarbonate (**6**) was treated with methanolic ammonium hydroxide. *O*-Caffeoylserine (**7**) was purified by TLC using a migration solvent of *n*-butanol–water–AcOH (4 : 1 : 1) for a total yield of 328 mg (87%). Synthesized *O*-caffeoylserine was used to confirm the presence of the compound in *P. herreni* and bioassayed at several concentrations to determine its biological activity on *A. coccois* and *A. vexans*.

To validate the synthetic method, *O*-caffeoyltyrosine was also synthesized, substituting the *N-t*-BOC-(L)-serine with *N-t*-BOC-(L)-tyrosine. The chloride (**3**) (1.71 g, 5 mmol) was coupled with the *N-t*-BOC-(L)-tyrosine (1.41 g, 5 mmol; Sigma) to produce 2.41 g (quantitative) of the compound with the following NMR



5: R = OCOEt, X = BOC

6: R = OCOEt, X = H

7: R = H, X = H

FIG. 1. Scheme of the various molecules obtained during synthesis of *O*-caffeoylserine.

data. ^{1}H NMR ($CDCl_3$): ∂ 7.75 (d, 1H, $J = 15.9$ Hz, olefinic H), 6.95–7.49 (6H, m aromatic), 6.53 (d, 1H, $J = 15.9$ Hz, olefinic H), 5.00 (br. d, 1H, NH), 4.58 (m, 1H, NH-CH), 4.30 (br. quartet, 4H, CH_2s), 3.11 (m, 2H, benzyl CH_2), 1.39 (br. s, 9H, *t*-BOC methyls), 1.34 (t, 6H, $J = 6.5$ Hz, methyls). From 536 mg of this compound, *O*-caffeoyltyrosine was obtained for a total yield of 245 mg (81%). Proton NMR spectra were obtained on an Advance DPX 200 Bruker NMR spectrometer at 200 MHz.

Isolation and Identification of O-Caffeoylserine in Cassava Mealybug. Adult females (2.4 g) were crushed in 5 ml of 70% ethanol. After 8 hr at 10°C, the extract was centrifuged at 21,000 *g* for 3 min, and the aqueous supernatant was washed twice with chloroform to remove lipids. The lyophilisate of the supernatant was suspended in 500 μl of 70% ethanol and banded on to a reverse-phase TLC plate (RP-18, F_{254s}, pre-coated sheet 20 × 20 cm, Merck), developed with acetonitrile–water (50 : 50), and visualized using UV at 254 nm. Two bands with R_f values of 0.75 and 0.69 (shown to contain the putative *O*-caffeoylserine by autoanalysis) were recovered by scraping the plate and extraction in 70% ethanol. After 8 hr at 10°C and centrifugation in 21,000 *g* for 3 min, the supernatants were lyophilized. The lyophilisate was suspended in 70% ethanol and submitted to the same TLC purification as above, but developed in *n*-butanol–2-propanol–water (60 : 20 : 20). Three bands with R_f = 0.81, 0.73, and 0.67 (shown to contain the putative *O*-caffeoylserine by autoanalysis) were recovered as above, let stand at 10°C for 8 hr, centrifuged, and lyophilized. A mixture of compounds including the putative *O*-caffeoylserine remained in the extract. It was not possible to repeat this TLC purification because the putative *O*-caffeoylserine disappeared, probably due to hydrolysis.

The compound was first identified by LC-MS from a portion of the extract. LC-MS experiments were performed on a Finnigan TSQ 700 under atmospheric pressure chemical ionization (APCI) as described elsewhere (Renukappa et al., 1999). In this case, a reversed-phase column (Grom-Sil 120 ODS-5, 250 × 4.4 mm, 5 μm, Grom, Germany) was used. Separation was achieved by using 0.1% aqueous trifluoroacetic acid in a linear gradient of 5–100% with acetonitrile over 35 min and a constant flow of 0.1ml/min.

In the second part, known quantities of the synthetic *O*-caffeoylserine were analyzed, alone or added to the remaining extract, by HPLC and autoanalysis. Samples were analyzed by RP18-HPLC by using the Pico-Tag method from Waters after amino acid derivatization with phenyl isothiocyanate (PITC). The compounds were identified by their retention time and quantified by their UV absorption at 240 nm.

Samples were submitted also to ion-exchange chromatography on an automatic amino acid analyzer (Beckmann 6300). Amino acids were detected by ninhydrin reaction, identified by their retention time and wavelength ratio, and quantified by their absorption at 570 nm.

Determination of O-*Caffeoylserine as Host-Location Kairomone.* To verify that *O*-caffeoylserine is present on the body surface of *P. herreni*, 500 adult females (representing about 500 mg of total fresh weight) without prior maceration were soaked in 500 μl of aqueous 70% ethanol for a final proportion equivalent to 1 μl of extract per individual. Insects without wounds were used, and the suspension was not stirred to limit leaching of internal insect constituents. The suspension was left at 10°C for five days. An aliquot of 100 μl was collected

each day and dried for autoanalysis of amino acids. This experiment was run in triplicate.

To confirm the function of *O*-caffeoylserine as a contact kairomone, 100 mealybugs were soaked in 100 μl of 70% aqueous ethanol at 10°C for three days, pipetted, and used for bioassay. The same experiment was done with pure hexane and bioassayed as a negative control. Because the ethanolic suspension also contained free amino acids, a solution duplicating the composition and concentration of these compounds was also bioassayed. The natural host (i.e., adult female of *P. herreni*) was bioassayed as the positive control. Synthetic *O*-caffeoylserine was bioassayed at several concentrations to confirm its function of as a host-location kairomone for mealybug parasitoids.

Bioassays. Wasps initially investigate mealybugs by palpating with their antennae as they walk from one edge of the mealybug body to the other. They then turn ≈90° and repeat this behavior one or more times. This behavior, previously observed in several Aphelinidae species, has been termed "drumming and turning" by Luck et al. (1982). After drumming and turning, *A. vexans* and *A. coccois* insert their ovipositors inside the host body in most cases, indicating that this behavioral event characterizes their host location. Therefore, the number of drummings and turns was recorded for each wasp observed in the following experiments. The bioassay was conducted on mealybugs or on treated cotton balls, which mimic the mealybug body. For cotton balls, 1 μl of test solution was pipetted onto cotton balls approx. 2.0 mm diameter. One insect or treated cotton ball was placed inside a single glass vial bioassay arena (5 mm diam. × 10 mm high), and for treated cotton balls the solvent was allowed to evaporate. One gravid female was added, and the vial was sealed with a cotton plug. Wasp behavior was observed for 15 min with a 6× dissecting microscope. Bioassays were conducted in the same greenhouse where the parasitoids were reared, between 1000 and 1500 hr at 25–35°C. The natural host (adult females of *P. herreni*) and several solutions were bioassayed as described above.

Five replicate trials of five wasps were bioassayed, with new insects or fresh cotton balls prepared for each trial. Wasps were used only once. The proportion of total drummings and turns on each mealybug or cotton ball was calculated and analyzed by ANOVA after applying $\sqrt{(X+1)}$ transformation. Fisher's (protected least significant difference PLSD) test was used following ANOVA to compare means. Statistical tests were performed with Statview software (Abacus Concept).

RESULTS

Identification of O-*Caffeoylserine in Cassava Mealybug.* LC-MS analysis of *P. herreni* extracts revealed a compound with a molecular weight of 268.3 $(M+H)^+$,

close to that calculated from the formula of *O*-caffeoylserine, corresponding to $C_{12}H_{13}NO_6$, which is 267.2.

To confirm the identity of *O*-caffeoylserine in *P. herreni* extracts, the compound was synthesized according to the method of Millar and Hare (1993), which was used to synthesize another ester, *O*-caffeoyltyrosine. The proton NMRs (CD_3OD) of the two synthetic esters were readily interpreted.

For the *O*-caffeoylserine, we obtained: 7.43 (d, 1H, $J = 15.9$ Hz, olefinic H), 6.78–7.06 (3H, m aromatic), 6.29 (d, 1H, $J = 15.8$ Hz, olefinic H), 5.09 (br s, 1H), 4.10 (m, 1H), 3.79 (q, $J = 11.1$ Hz, 2H). Except for protons lost in exchange in CD_3OD, these NMR data corresponded to those obtained by Lin et al. (1999), confirming the synthesis of this ester.

For the *O*-caffeoyltyrosine, we obtained: 7.77 (d, 1H, $J = 15.9$ Hz, olefinic H β to carbonyl), 7.41 (d, 2H, $J = 8.54$ Hz, aromatic H, tyrosine), 7.04–7.19 (4H, m from aromatic H of tyrosine and caffeic acid), 6.84 (d, 1H, $J = 8.14$ Hz, meta H, caffeic acid), 6.50 (d, 1H, $J = 15.83$ Hz, olefinic H α to carbonyl), 3.82 (dd, 1H, $J = 9.1$, 4.24 Hz, $C\underline{H}NH_2$), 3.35 (dd, 1H, $J = 14.12$, 4.10 Hz, benzylic H), 3.04 (dd, 1H, $J = 14.5$, 9.1 Hz, benzylic H). These NMR data corresponded to those obtained by Millar and Hare (1993), validating the synthesis method.

The retention time of synthetic *O*-caffeoylserine on a reverse-phase HPLC column matched that of the peak at 18.9 min from the *P. herreni* extract. This peak increased in area when the *P. herreni* extract was augmented with 0.4 nmol of synthetic *O*-caffeoylserine (Figure 2).

The retention time of synthetic *O*-caffeoylserine on an ion-exchange column also matched that of the compound isolated from *P. herreni* (chromatograms not shown). This result, together with RP-HPLC data, confirms the identifiy *O*-caffeoylserine in *P. herreni* extracts.

Determination of O-*Caffeoylserine as Host-Location Kairomone.* When mealybugs were soaked in 70% ethanol without prior maceration and submitted to autoanalysis, the *O*-caffeoylserine amounted to 0.026 nmol/mg (approx. 0.03 nmol/mg) or 5.2% (in moles) (Table 1). Compared to the other nitrogenous compounds analyzed, this was not major. Alanine and glutamic acid were much better represented among the compounds analyzed, at 28.4 and 17.1% (in moles), respectively. Furthermore, this concentration of *O*-caffeoylserine was the maximum possible when mealybugs were soaked in 70% aqueous ethanol. In fact, this concentration varied over time (Figure 3) from a minimum of 0.011 ± 0.002 nmol/mg (mean $\pm$ SE, $N = 3$) on day 1 to a maximum of 0.032 ± 0.002 nmol/mg on day 3 and declining to 0.027 ± 0.004 nmol/mg on day 5, probably due to hydrolysis.

The fresh weight of an adult female is about 1 mg on CMC 40. According to the extraction proportion, 1 μl of extract was obtained per individual or per 1 mg of fresh weight. Therefore, 0.03 nmol/mg is equivalent to 0.03 nmol/μl. Synthetic *O*-caffeoylserine was bioassayed based on this concentration (at 0.03 nmol/μl and 10 times higher or 2, 4, or 8 times lower).

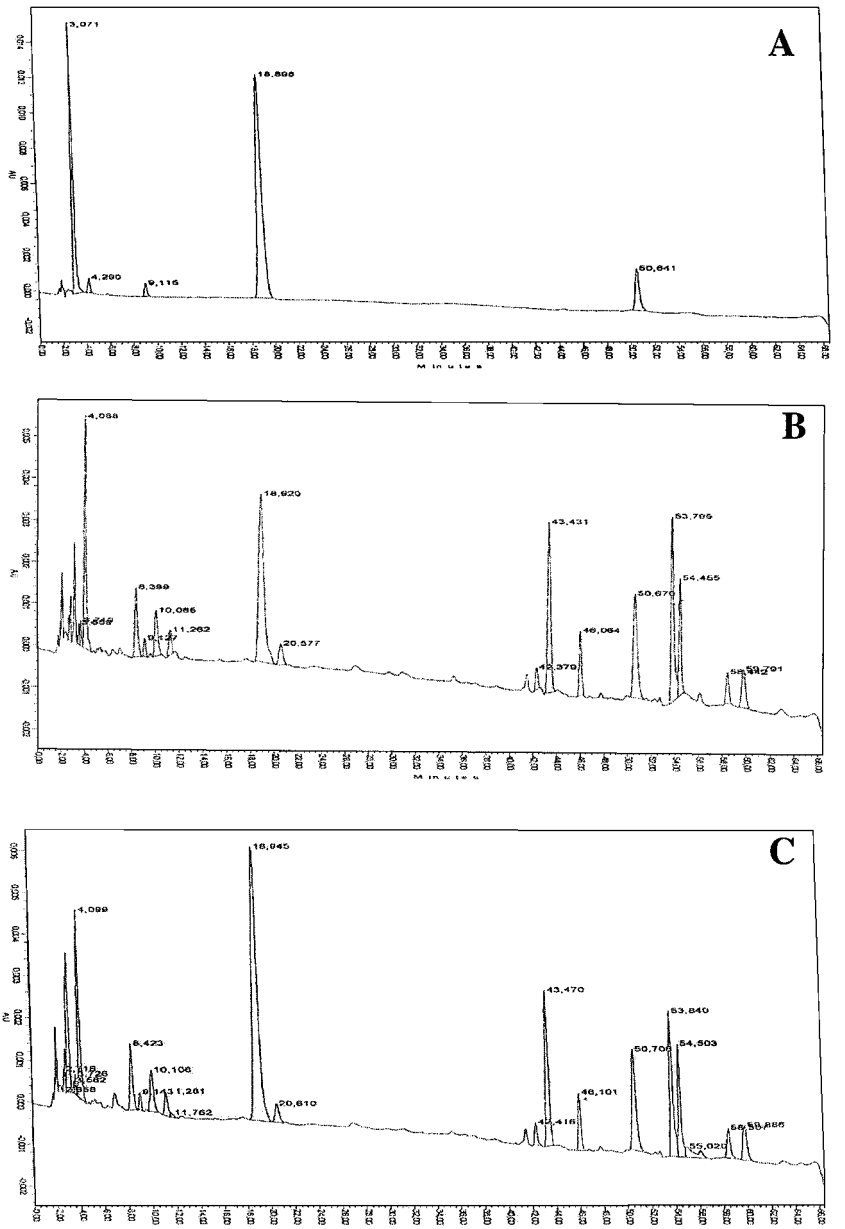


FIG. 2. HPLC chromatograms of (A) the synthetic *O*-caffeoylserine alone (at 2 nmol/10 μl); (B) an extract of *P. herreni* showing the unknown peak at 18.9 min; and (C) the same extract analyzed in (B) augmented with 0.4 nmol of synthetic *O*-caffeoylserine.

TABLE 1. CONTENT AND COMPOSITION OF FREE NITROGENOUS COMPOUNDS IN MEALYBUGS

Free nitrogenous compounds	Concentration (pmol/1 mg)	% (mol)
Asp	30 ± 5	5.9 ± 0.05
Thr	14 ± 2	2.9 ± 0.86
Ser	20 ± 5	3.9 ± 0.25
Asn	8 ± 2	1.5 ± 0.05
Glu	86 ± 25	17.1 ± 1.80
Gln	13 ± 4	2.6 ± 0.40
Pro	24 ± 5	4.7 ± 0.10
Gly	34 ± 7	6.7 ± 0.11
Ala	143 ± 14	28.4 ± 2.41
Val	19 ± 2	3.8 ± 0.30
Met	9 ± 1	1.8 ± 0.10
Ile	7 ± 1	1.4 ± 0.09
Leu	8 ± 1	1.6 ± 0.03
Tyr	13 ± 2	2.7 ± 0.20
Phe	2 ± 1	0.3 ± 0.03
O-Caffeoylserine	26 ± 7	5.2 ± 0.36
Try	22 ± 6	4.4 ± 0.30
Orn	2 ± 1	0.3 ± 0.07
Lys	4 ± 2	0.7 ± 0.02
His	11 ± 4	2.1 ± 0.44
Arg	11 ± 4	2.0 ± 0.45

[a] Values are (mean ± SE, $N = 3$).

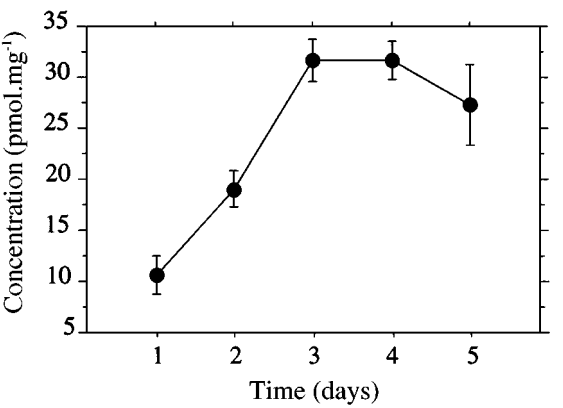


FIG. 3. Concentration of *O*-caffeoylserine in extracts from mealybugs (mean ± SE, $N = 3$) soaked without prior maceration in 70% ethanol as a function of time at 10°C.

For both parasitoid species, the extracts from mealybugs soaked in 70% ethanol were significantly more stimulating for host location than those in hexane (Figure 4), but no oviposition probing was observed. Moreover, these ethanolic extracts showed similar host-location stimulations when *P. herreni* was bioassayed. No stimulation was noted with ethanol alone, suggesting that the mediation of host location was in fact due to compound(s) present in ethanolic extracts of

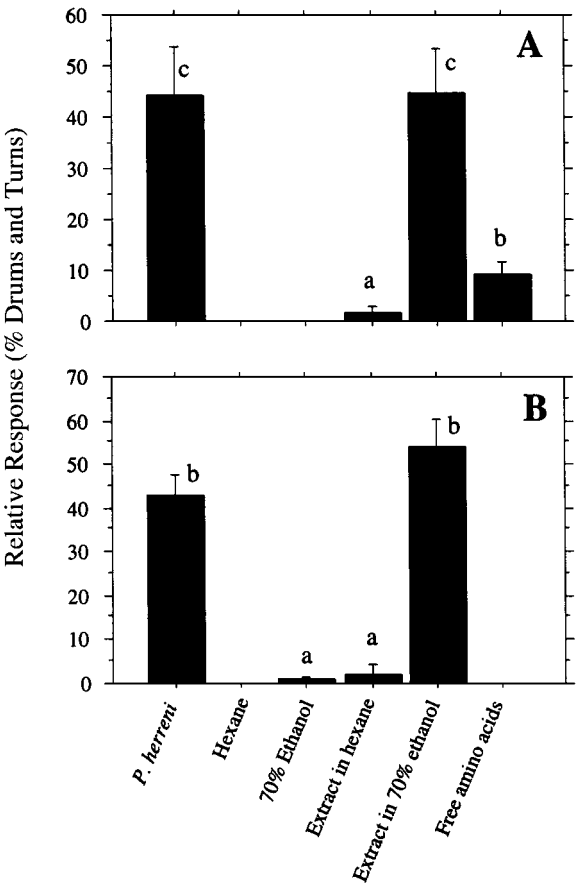


FIG. 4. Percent drums and turns observed for *A. coccois* (A) and *A. vexans* (B) on *P. herreni* and on cotton balls treated with solvent alone (hexane or 70% aqueous ethanol), extracts from mealybugs suspended without prior maceration in hexane or in 70% ethanol, or a mixture of free amino acids using the same concentration and composition as in Table 1. Bars indicate the mean (±SE; five trials with five wasps each). Means with the same letter are not significantly different at 5% level (Fisher's PLSD test following the ANOVA, $P < 0.05$). No statistical comparison was possible for means = 0%.

P. herreni and well solubilized in 70% aqueous ethanol. A mixture of free amino acids, similar in concentration and composition to the one obtained in Table 1, showed no stimulating effects as compared to the extracts of *P. herreni* in ethanol, indicating the presence of other compound(s) in the extracts, different from free amino acids, that have a stimulatory function for host location by *A. coccois* and *A. vexans*.

Among the concentrations in *O*-caffeoylserine tested, 0.03 nmol/μl was highly attractive for *A. coccois* (Figure 5A). Nevertheless, no difference was found between 0.03 and 0.015 nmol/μl. After removing the carbonate-protecting groups

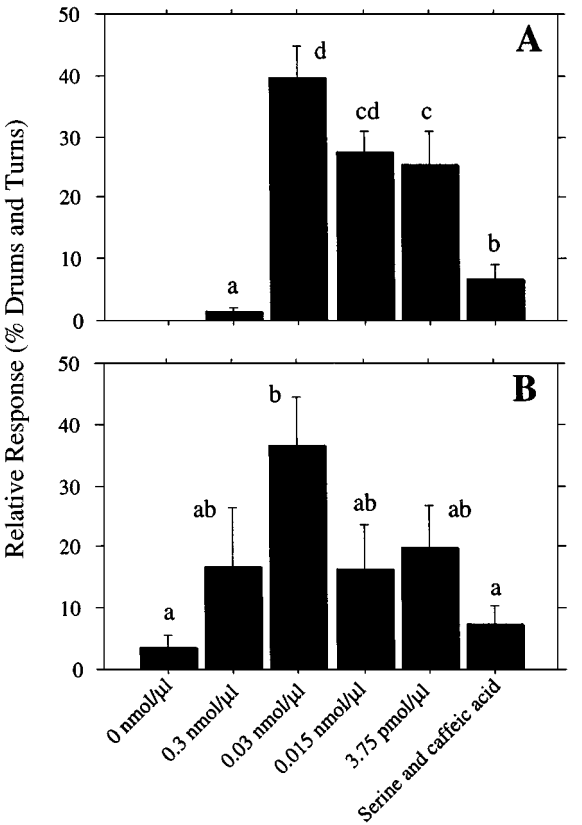


FIG. 5. Percent of drums and turns observed for *A. coccois* (A) and *A. vexans* (B) on cotton balls treated with synthetic *O*-caffeoylserine at several concentrations or with mixture of serine and caffeic acid at 0.03 nmol/μl each compound. Bars indicate the mean ($\pm$SE; five trials with five wasps each). Means with the same letter are not significantly different at 5% level (Fisher's PLSD test following the ANOVA, $P < 0.05$, except for *A. vexans* (B), $P > 0.05$ for ANOVA). No statistical comparison was possible for means = 0%.

(ultimate phase in Figure 1), using Millar and Hare's (1993) synthesis method, there is inevitably a partial ester cleavage, resulting in the presence of free serine and caffeic acid in the synthetic *O*-caffeoylserine solution. Therefore, a mixture of these compounds was also bioassayed at 0.03 nmol/μl, i.e., at the same concentration of *O*-caffeoylserine where the wasps responded better (Figure 5). It appeared that the *A. coccois* female wasps were less stimulated by the hydrolysis products of the *O*-caffeoylserine (caffeic acid and serine) confirming that the ester, *O*-caffeoylserine, is the biologically active compound. Moreover, for this parasitoid species, the ester also induced oviposition probing because 24% (i.e., 6 of the 25) of the wasps bioassayed at 0.03 nmol/μl of *O*-caffeoylserine probed the cotton ball with their ovipositor. Oviposition probing was not observed with the other concentrations of *O*-caffeoylserine tested.

Synthetic *O*-caffeoylserine at 0.03 nmol/μl elicited more drumming and turning of *A. vexans*, but differences were not observed among the concentrations tested (Figure 5B, $P = 0.0547$ for ANOVA), indicating no dosage-dependent response in this parasitoid species. Nevertheless, *O*-caffeoylserine did induce a stimulating effect in this parasitoid species at 0.03 nmol/μl because the wasps exhibited significantly higher drummings and turns than when the solvent alone or the mixture of serine and caffeic acid were bioassayed.

DISCUSSION

The identification of *O*-caffeoylserine in *P. herreni* was confirmed. To our knowledge, this represents the first report of *O*-caffeoylserine isolated from biological material, although this ester was recently synthesized by Lin et al. (1999). Without prior maceration, *O*-caffeoylserine amounted to approx. 0.03 nmol/mg of mealybug fresh weight, representing 5.2% (in moles) of the nitrogenous compounds analyzed (Table 1). Although it can be assumed that the procedure used (i.e., soaking the mealybugs in 70% ethanol for three days at 10°C) is sufficient to leach out a number of internal insect constituents, some arguments support the fact that the method tends to dissolve surface rather than internal constituents and that *O*-caffeoylserine should, in fact, amount to 0.03 nmol/mg on the body surface of *P. herreni* females in the adult developmental stage. First, when mealybugs were crushed, *O*-caffeoylserine amounted to about 9.5 nmol/mg of mealybug fresh weight, i.e., approx. 916 times higher, being much more concentrated than that obtained without maceration. Furthermore, when whole insects were soaked in ethanol, the concentration of *O*-caffeoylserine reached a maximum at three days and was three times higher than on day 1 (Figure 3). If the procedure were efficient in leaching out internal insect constituents, the concentration would increase much more than three times based on the internal concentration of *O*-caffeoylserine mentioned above. Figure 5 shows that both parasitoid species

responded better to 0.03 nmol/μl of *O*-caffeoylserine. This concentration, equivalent to 0.03 nmol/mg of mealybug fresh weight, is similar to that found when mealybugs were soaked in 70% ethanol for three days at 10°C. Given the fact that higher biological activity was obtained at this concentration and that it is the maximum that can be extracted by soaking unmacerated insects in ethanol, we suggest that *O*-caffeoylserine amounts 0.03 nmol/mg on the body surface of adult *P. herreni* females.

The presence of free amino acids in the sample supports the idea that some internal constituents can be leached out as well (Table 1). However, this is more likely due to honeydew secretions containing free amino acids that could stick to the body surface of neighboring mealybugs, given their proximity under mass-rearing conditions. *O*-Caffeoylserine was not found in the honeydew secretion of *P. herreni*.

Using several concentrations of synthetic *O*-caffeoylserine, it was confirmed that this compound influences host-location behavior of two parasitoid species of the cassava mealybug: *A. coccois* and *A. vexans*. Perhaps because of its generalist nature, this was more clearly shown with *A. coccois* due to the fact that the dosage-dependent response was well observed, with higher biological activity at 0.03 nmol/μl (Figure 5A). Although no such response was observed for *A. vexans*, *O*-caffeoylserine also was stimulatory at 0.03 nmol/μl (Figure 5B). In conclusion, for both parasitoid species, 0.03 nmol/μl is the concentration of *O*-caffeoylserine that induces more drummings and turns of wasps (approx. 40% of relative response among the concentrations bioassayed, Figure 5). This concentration, equivalent to 0.03 nmol/mg, is similar, to what can be extracted from the body surface of mealybugs, induces significant drummings and turns (approx. 40% of relative response as compared to the natural host, Figure 4), and indicates that synthetic *O*-caffeoylserine is as active as the chemical isolated from the body surface. All these results are consistent with *O*-caffeoylserine being a contact kairomone that mediates host-location behavior by the two cassava mealybug parasitoids.

Except for *A. coccois* at 0.03 nmol/μl of *O*-caffeoylserine, oviposition probing was not observed with any of the extracts bioassayed, regardless of parasitoid species. It may be that the female wasp behavior for oviposition probing is influenced by other factors such as color and texture of the mealybug body. In fact, in preliminary experiments we observed that for both parasitoid species, texture influenced wasp behavior for host location more than color (Calatayud, unpublished data). Some wasps inserted their ovipositor inside the untreated cotton plug, whereas oviposition probing was not observed with the treated cotton ball inside the glass vial. This was probably due to the fact that the cotton plug was more compact than the cotton ball, suggesting that a good combination between the concentration of *O*-caffeoylserine and texture could enhance oviposition probings. Unfortunately, increasing the pressed texture of the cotton ball was not possible given its size.

O-Caffeoylserine is not provided directly by the host plant; it is either synthesized by the insect itself or with the help of symbiotic bacteria. In fact, it was detected in adult mealybugs reared on holidic diets at 14.9 nmol/mg fresh weight, a higher level than in plant-reared mealybugs (about 9.5 nmol/mg), but not for extracts of host plant (*Manihot esculenta*) leaves. Nevertheless, enzymes commonly involved in the synthesis of phenolic compounds in plants such as phenylalanine or tyrosine ammonia-lyase were not evidenced in *P. herreni* (Calatayud, unpublished data).

The physiological role of *O*-caffeoylserine in the cassava mealybug is not clear. The caffeic acid moiety, as suggested by Hare et al. (1993) for *O*-caffeoyltyrosine, could be a precursor of the quinone compound involved in cuticle sclerotization and tanning. The serine moiety could be a precursor of the bristle (serine polymer) in the ovisacs. The fact that this phenolic compound is present in higher concentration in eggs (at about 26.4 nmol/mg) than in adult females suggests that it may also have a protective function (e.g., antibacterial or antiviral activity) in the insect. All these hypotheses need to be demonstrated explicitly.

Acknowledgments—We thank Juan Christian Hamon for his technical help and Trudy Brekelbaum for her English corrections. This research was supported by a special project at CIAT, co-financed by IRD, and a donation to CIAT from the French Ministry of Foreign Affairs.

REFERENCES

BELLOTTI, A. C., REYES, J. A., VARELA, A. M., and CASTILLO, J. 1983. El piojo harinoso (*Phenacoccus* sp.) de la yuca; una de las plagas agrícolas más importantes del mundo. 27 pp. Centro Internacional de Agricultura Tropical, Cali, Colombia.

DORN, B., MATTIACCI, L., BELLOTTI, A. C., and DORN, S. 2001. Host specificity and comparative foraging behaviour of *Aenasius vexans* and *Acerophagus coccois*, two endoparasitoids of the cassava mealybug. *Entomol. Exp. Appl.* In press.

GODFRAY, H. C. J. 1994. Parasitoids: Behavioral and Evolutionary Ecology. Princeton University Press, Princeton, New Jersey.

HARE, J. D., MILLAR, J. G., and LUCK, R. F. 1993. A caffeic acid ester mediates host recognition by a parasitic wasp. *Naturwissenschaften* 80:92–94.

LE RÜ, B., IZIQUEL, Y., BIASSANGAMA, B., and KIYINDOU, A. 1991. Variations d'abondance et facteurs de régulation de la cochenille farineuse du manioc *Phenacoccus manihoti* (Hom., Pseudococcidae) cinq ans après l'introduction d'*Epidinocarsis lopezi* (Hym., Encyrtidae) parasitoide néotropical au Congo en 1982. *Entomophaga* 36:499–511.

LEWIS, W. J. and MARTIN, W. R., JR. 1990. Semiochemicals for use with parasitoids: Status and future. *J. Chem. Ecol.* 16:3067–3089.

LIN, Z., NEAMATI, N., ZHAO, H., KIRYU, Y., TURPIN, J. A., ABERHAM, C., STREBEL, K., KOHN, K., WITVROUW, M., PANNECOUQUE, C., DEBYSER, Z., DE CLERCQ, E., RICE, W. G., POMMIER, Y., and BURKE, T. R., JR. 1999. Chicoric acid analogues as HIV-1 integrase inhibitors. *J. Med. Chem.* 42:1401–1414.

LUCK, R. F., PODOLER, H., and KFIR, R. 1982. Host selection and egg allocation behaviour by *A. melinus* and *A. lingnanensis*: Comparison of two facultatively gregarious parasitoids. *Ecol. Entomol.* 7:397–408.

MILLAR, J. G. and HARE, J. D. 1993. Identification and synthesis of a kairomone inducing oviposition by parasitoid *Aphytis melinus* from California red scale covers. *J. Chem. Ecol.* 19:1721–1736.

NEUENSCHWADER, P., HAMMOND, W.N.O., AJUONU, O., GADO, A., ECHENDU, N., BOKONON-GANTA, A. H., ALLOMASSO, R., and OKON, I. 1990. Biological control of the cassava mealybug, *Phenacoccus manihoti* (Hom., Pseudococcidae) by *Epidinocarsis lopezi* (Hym., Encyrtidae) in West Africa, as influenced by climate and soil. *Agric. Ecosyst. Environ.* 32:39–55.

NORONHA, A. C. 1990. Insectos e acaros que atacam a cultura da mandioca. Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Centro Nacional de Pesquisa de Mandioca e Fruticultura (CNPMF), Cruz das Almas-BA, Brasil. 27 pp.

POWELL, W. 1992. Synomones and kairomones from plants and honeydew in susceptible and resistant varieties: Their importance to natural enemies. *in* Proceedings of the XIX International Congress of Entomology, 1992, Beijing, China.

RENUKAPPA, T., ROOS, G., KLAIBER, I., VOGLER, B., and KRAUS, W. 1999. Application of high-performance liquid chromatography coupled to nuclear magnetic resonance spectrometry, mass spectrometry and bioassay for the determination of active saponins from *Bacopa monniera* Wettst. *J. Chromatogr. A* 847:109–116.

SELIGMANN, C. D. 1998. Influencia de la nutrición del piojo harinoso *Phenacoccus herreni* de la yuca *Manihot esculenta* en el desarrollo de sus parasitoides *Acerophagus coccois, Aenasius vexans, Apoanagyrus diversicornis.* MS thesis. University of Los Andes, Bogotá, Colombia, 109 pp.

RÖSE, U. S. R., ALBORN, H. T., MAKRANCZY, G., LEWIS, W. J., and TUMLINSON, J. H. 1997. Host recognition by the specialist endoparasitoid *Microplitis croceipes*: Role of host and plant related volatiles. *J. Insect Behav.* 10:313–329.

TAKABAYASHI, J. and TAKAHASHI, S. 1993. Role of the scale wax of *Ceroplastes ceriferus* Anderson (Hemiptera: Coccidae) as a defence against the parasitic wasp *Anicetus ceroplastis* Ishii (Hymenoptera: Encyrtidae). *J. Insect Behav.* 6:107–115.

VINSON, S.B. 1991. Chemical signals used by parasitoids. *Redia* 74:15–42.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

VOLATILES FROM POTENTIAL HOSTS OF *Rhopalicus tutela* A BARK BEETLE PARASITOID

EVA M. PETTERSSON[1]

Chemical Ecology Department
Göteborg University
Box 461, SE-405 30 Göteborg, Sweden

(Received January 23, 2001; accepted July 5, 2001)

Abstract—Host location cues for parasitic wasps that attack bark beetle larvae concealed under the bark of spruce trees were analyzed by collecting odor samples from entrance holes into the bark beetle galleries, isolated larvae, and pupal chambers with or without bark beetle larvae. Odor samples were collected by dynamic headspace adsorptions on Porapak Q or static adsorptions by using solid-phase microextraction (SPME) with Carbowax–divinylbenzene as the adsorbing phase. Samples were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) and GC-mass spectrometry (GC-MS). The antennae of *Rhopalicus tutela* females responded primarily to oxygenated monoterpenes that are typical for damaged host trees. These compounds are attractive to bark beetle parasitoids in long-range host location, suggesting that they are used in both long- and short-range host location. No differences could be detected between samples collected from pupal chambers with or without mature larvae. Larvae outside pupal chambers emitted low quantities of the same compounds present in empty pupal chambers. The data support the hypothesis that volatiles used by host foraging parasitoids arise from the interaction between introduced microorganisms and the bark and/or vascular tissue of the host tree rather than from the bark beetle larvae.

Key Words—*Rhopalicus tutela*, Hymenoptera, Pteromalidae, host location, oxygenated monoterpenes, *Ips typographus*, Coleoptera, Scolytidae, bark beetle.

INTRODUCTION

By definition, parasitoids are insects that spend their immature stages feeding on the tissues of other arthropods that are eventually killed. Parasitoids occurs in several insect orders, although the vast majority are hymenopterans (e.g., Quicke,

[1]Present address: Max-Planck Institute for Chemical Ecology, Carl Zeiss Promenade 10, D-07745 Jena, Germany. E-mail: Pettersson@ice.mpg.de

0098-0331/01/1100-2219$19.50/0

1997). The chalcid parasitoid *Rhopalicus tutela* (Walker) (Hymenoptera: Pteromalidae) oviposits on coniferous bark beetles, like the aggressive *Ips typographus* L. (Coleoptera: Scolytidae). In Sweden, *I. typographus* kills large numbers of its principal host Norway spruce (*Picea abies* L. Karst.) during outbreaks (Krol and Bakke, 1986; Leufvén and Nehls, 1986; Krokene and Solheim, 1996). As for most hymenopteran parasitoids attacking bark beetles, a mature larva in a pupal chamber in the bark of the host tree is the preferred host stage (Berisford, 1980). *Rhopalicus tutela* oviposits through the bark, thus detecting the larvae without any obvious visual cues.

Several hypotheses have been proposed for close-range host location mechanisms of parasitoids ovipositing through the bark. Vibrational cues do not seem to be necessary, since oviposition by *R. tutela* on *I. typographus* was not disrupted by killing or removing host larvae (Mills et al., 1991). This is further supported by similar results from the braconid parasitoid *Coeloides brunneri* (Richerson and Borden, 1972). Infrared radiation (heat) also does not seem to be a necessary cue for *Coeloides bostrychorum* and *R. tutela* in locating their bark beetle hosts (Mills et al., 1991; Pettersson et al., 2001b). Because volatiles derived from bark beetle-infested spruce logs containing susceptible hosts elicit both antennal activity and long-range attraction to parasitoids (Pettersson et al., 2000, 2001a, b), the same chemicals may be important in short-range host location.

During host foraging, hymenopteran parasitoids commonly use semiochemicals emitted by the host itself, sources indirectly associated with the presence of the host (e.g., microorganisms), or the host habitat (Madden 1968; Vinson, 1986; Godfray, 1994; Rutledge, 1996). Successful parasitism depends on appropriate habitat identification, i.e., long-range host location (host habitat location), followed by short-range host location that includes host location, host acceptance, host suitability assessment, and host regulation (Vinson et al., 1998). Parasitoids may use different semiochemicals emitted from differnt sources at various stages of the host-selection process. In general, host habitat cues are most important at long distances, while host insect cues are more important at short distances (Weseloh, 1981).

The objectives of this study were to indentify volatile cues of potential use in short-range host location by bark beetle parasitoids, locate the source emitting these compounds, and evaluate the potential of solid-phase microextraction (SPME) as a sampling technique in gas chromatographic-electroantennographic detection (GC-EAD) analysis of volatile compounds from bark beetle infested spruce trees.

METHODS AND MATERIALS

Experimental Insects. Logs cut from five approximately 90-year-old spruce trees infested with bark beetle larvae (*I. typographus*) parasitized by *Rhopalicus*

tutela and *Roptrocerus xylophagorum* were collected in southern Sweden in the province of Småland (58°50′N, 16°20′E). Cut ends were sealed with paraffin. The logs were wrapped in paper sacs and stored at 3°C. When parasitoids or bark beetles were needed, logs were brought to a temperate (20°C) rearing room. Emerged parasitoids and bark beetles were collected daily and kept separated in humidified flasks at 8°C. Parasitoids used in experiments had not had any previous contact with susceptible bark beetle hosts (in experienced parasitoids). In plastic cages, controlled bark beetle attacks were initiated on spruce logs (ca. 20 cm diam., 100 cm long) with the cut ends sealed with paraffin to prevent desiccation. Mature larvae in pupal chambers (the preferred host stage of the parasitoid) were abundant 22–29 days after attack (at ca. 18°C, laboratory conditions).

Sampling of Volatile Compounds. Volatile compounds were collected from entrance holes into the bark beetle galleries, larvae were separated from their pupal chambers (25 individuals/sample in a 40-ml glass container), and pupal chambers, with or without mature larvae, were exposed by removing thin layers of the bark. Sampling was performed by using dynamic headspace aeration on a Porapak Q adsorbent or passive diffusion by SPME (Pawliszyn, 1997). Biologically active compounds were isolated by coupled GC-EAD and identified by GC-mass spectrometry (GC-MS).

Dynamic headspace Porapak-samples were collected from individual sample sites on logs into small Teflon cylinders (ID 4 mm, 5 mm high) directly connected to the Porapak Q adsorbent (75 mg, mesh 80/100, Alltech) packed into Teflon tubes (3 mm ID, 4 cm long). Air was drawn onto the adsorbents for 12 hr (80 ml/min) by a pump. Adsorption for 2–3 hr resulted in samples that had to be concentrated ($>$30 times) to be useful in GC-EAD analysis. Adsorption samples were extracted with diethyl ether (Puriss p.a., Fluka) to a volume of 300 μl. One microliter of each sample was injected on the GC-EAD, approximately corresponding to the amount of volatiles released during 2.4 min of sampling [(12 hr $\times$ 60 min)/300 μl]. Samples used in GC-MS analysis (identification and quantification) were spiked with an internal standard (heptyl acetate, 5 ng/μl eluted sample).

SPME samples were collected during 45 min. Sampling for a longer time did not yield stronger samples, and sampling for shorter durations captured lower quantities of monoterpenes. Carbowax-divinylbenzene, 65 μm (Supelco, Inc.), was chosen as the fiber adsorption phase, as it is similar to the GC-column phase used (Cp-Wax 58 CB), which is suitable for oxygenated (polar) compounds. The fiber was conditioned in the GC injector (230°C) for 1 min before sampling. Sample sites on logs were covered by a small Teflon cylinder (10 mm ID, 15 mm high) equipped with two small openings that enabled simultaneous sampling by two fibers from one site (Figure 1), giving identical samples for both GC-MS and GC-EAD analyses. An external standard (5 ng heptyl acetate) was used for quantification of the GC-MS samples.

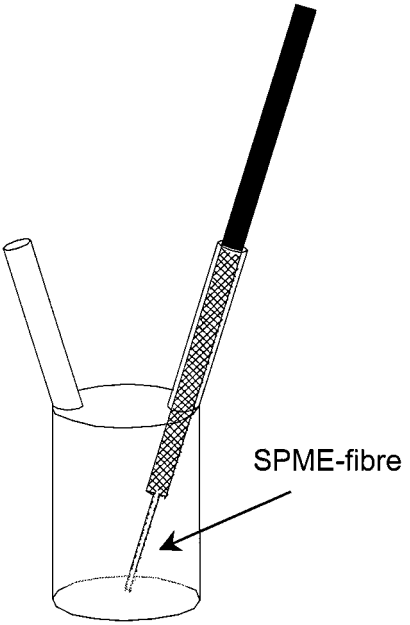


FIG. 1. Teflon cylinder (10 mm ID, 15 mm high) used for SPME sampling with two holes allowing simultaneous sampling by two SPME fibers (one shown in the right hole). Illustration by Claes Johansson.

Chemical Analysis. Eight samples were analyzed for each volatile collection technique. A Hewlett-Packard 5890 series II GC and 5972 MSD was equipped with a fused silica column (25 m $\times$ 0.25 mm) coated with polyethylene glycol nitroterephthalic acid ester (Cp-Wax 58 CB, film thickness 0.2 μm, Chrompack). The carrier gas was helium with a constant flow of 27 cm/sec. One microliter of a Porapak Q sample was injected by using a HP 7673 autosampler. Injector temperature was 210°C, and the splitter was off for 0.5 min; thereafter, it was split with a purge flow of 30 ml/min. SPME samples were injected manually with the injector temperature set at 230°C and with the fiber left in the injector for 20 sec. The GC oven temperature program was: 30°C for 2 min, 10°/min to 230°C, and isothermal for 8 min. The GC-MS transfer line temperature was 240°C. Compounds were identified by mass spectral matches to library spectra (NBS75K, obtained by authentic standards analyzed on the same GC-MS equipment as used in this investigation) and by retention time matches to available known standards. Compounds were quantified by their total ion abundance relative to that of the internal standard (5 ng/μl of Porapak samples). When comparing the sampling techniques, the detection limit for each compound was set to 0.8 ng/sample. Data were square-root transformed to obtain homogeneity in variance and analyzed by

one-way ANOVA, $\alpha = 0.05$ (Underwood, 1997), followed by a *post hoc* Tukey test (Sigma Stat 2.03, SPSS Inc.).

Electrophysiology. GC-EAD analyses employed a Shimadzu 14A gas chromatograph equipped with the same type of column as in GC-MS analysis (Cp-Wax 58 CB). The SPME samples were injected as above, while the Porapak Q samples were injected manually with the injector temperature set at 200°C. Nitrogen was used as carrier gas, with a flow-rate of 12 cm/sec (at 175°C), and the detector temperature at 230°C. The GC oven temperature program was 30°C for 2 min, then 8°/min to 200°C, 30°/min to 230°C, and isothermal for 3.8 min. Further details on this GC-EAD equipment are given in Pettersson et al. (2001b). For details of the EAD setup with parasitoid antennae, see Pettersson et al. (2000). The *R. tutela* parasitoids used for GC-EAD were 1–16 days old, and each individual was used for only one recording. Ten females were used for the Porapak-samples, and 11 females were exposed to the SPME samples. Before and after the GC-EAD recordings, the responsiveness of each antenna was evaluated by a control electroantennogram (EAG), as described in Pettersson et al. (2000, 2001b). A Kolmogorov-Smirnov normality test (Sigma Stat 2.03, SPSS Inc.) showed that the data met the requirements for parametrical statistics. EAD responses to compounds sampled by different sampling techniques were compared by a one-way ANOVA, $\alpha = 0.05$ (Underwood, 1997), followed by a *post hoc* Tukey test (Sigma Stat 2.03, SPSS Inc.).

RESULTS

Oxygenated monoterpenes of damaged conifers constituted the major group of EAD-active compounds, in both Porapak and SPME samples (Figure 2). Most compounds elicited antennal responses of the same magnitude, irrespective of the sampling method (Figure 3). There was no apparent qualitative difference between the sampling methods. However, the methods differed quantitatively since Porapak samples contained more compounds in greater amounts (>0.8 ng/single sample) than the SPME samples (Table 1, Figure 4). Inexplicably, some compounds elicited different EAD activities in response to Porapak and SPME samples, respectively (Figure 3), although no quantitative difference was detected between the sampling methods (Table 1). Samples taken from entrance holes were weaker than samples from pupal chambers (Figure 4) but did not differ qualitatively. Samples from single bark beetle larvae contained no detectable compounds. Samples taken from 25 pooled larvae contained only hexanal and acetic acid in amounts >0.8 ng/sample; these and all other detected compounds were also found in samples from pupal chambers, with or without larvae. In samples from pupal chambers, only α-pinene, β-pinene, and 3-carene were captured in significantly lower amounts by SPME than by Porapak Q (Table 1).

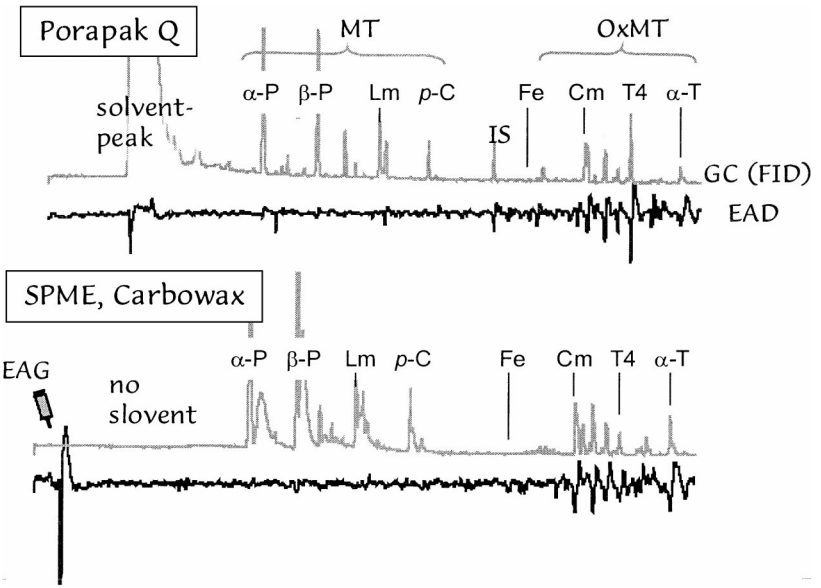


FIG. 2. Representative GC-EAD recordings on female *Rhopalicus tutela* exposed to odor samples from a pupal chamber with bark beetle larva. The Porapak Q-sample was spiked with 5 ng heptyl acetate (IS) for quantification. The double peaks (α-P and β-P) observed in the early MT region of the SPME sample are due to the injector used. Sample compositions are given in Table 1; EAD-active compounds are given in Figure 3. EAG = antennal stimulation by standardized EAG tests indicated by a syringe icon; MT = monoterpenes; OxMT = oxygenated monoterpenes; α-P = α-pinene; β-P = β-pinene; Lm = limonene; *p*-C = *p*-cymene; Fe = fenchone; Cm = camphor; T4 = terpinen-4-ol; α-T = α-terpineol.

DISCUSSION

Most antennal active compounds were oxygenated monoterpenes (Figure 2), indicating damaged spruce trees (Leufvén and Birgersson, 1987), in agreement with previous analyses of volatiles from whole bark beetle infested logs (Pettersson et al., 2000, 2001a). These compounds are also behaviorally active in Y-tube (Sullivan et al., 2000; Pettersson, 2001) and wind-tunnel bioassays (Pettersson et al. 2001a) with bark beetle parasitoids. Although some other parasitoids employ different chemical host location cues at long- and short-range host foraging (Weseloh, 1981), bark beetle parasitoids seems to rely on the same volatile compounds. However, olfaction is probably not the only sensory modality employed in short-range host location. Although metabolic heat or vibrations produced by the host have been ruled out as host location cues (Mills et al., 1991; Pettersson et al., 2001b) for *C. bostrichorum* and *R. tutela* ovipositing through the bark on bark beetle

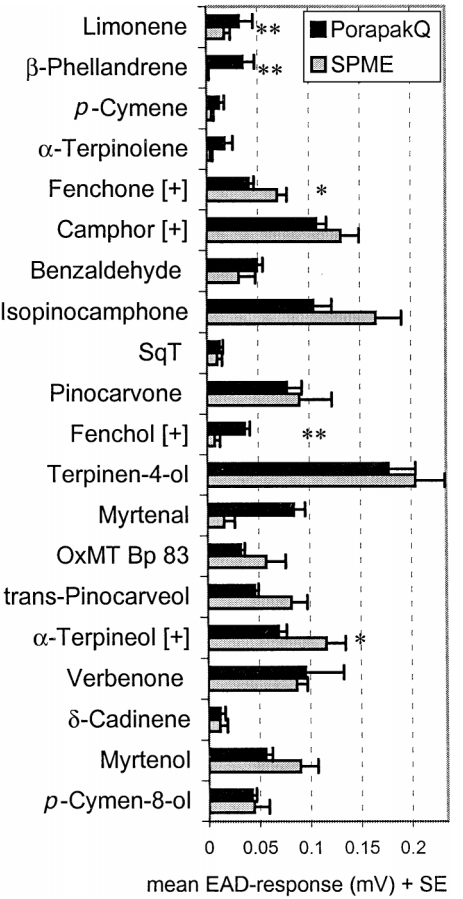


FIG. 3. Mean EAD responses by female *Rhopalicus tutela* to odor samples collected from pupal chambers with bark beetle larvae using either Porapak Q ($N = 10$), or SPME ($N = 11$). Compounds are arranged in order of retention time. [+] = Minor quantities of another compound coeluted on the GC-column: fenchone + nonanal; camphor + pinocamphone; fenchol + bornyl acetate; α-terpineol + borneol. SqT = sesquiterpene; OxMT = oxygenated monoterpene; Bp = base peak, the most abundant ion fragment of the mass spectrum. $^{*}P < 0.05$; $^{**}P < 0.01$; the absence of asterisks indicates that no difference in EAD response was detected ($P > 0.05$).

larvae, vibrations produced by the parasitoid itself may be used in host foraging. The pupal parasitoid *Pimpla turionellae* L. (Hym.: Ichneumonidae), which attacks various lepidopterans, such as *Galleria mellonella*, *Pieris brassicae*, and *Lymantria dispar*, may locate its concealed host by self-produced vibrations (Wäckers et al., 1998), although wind and other factors complicate the interpretation of vibratory

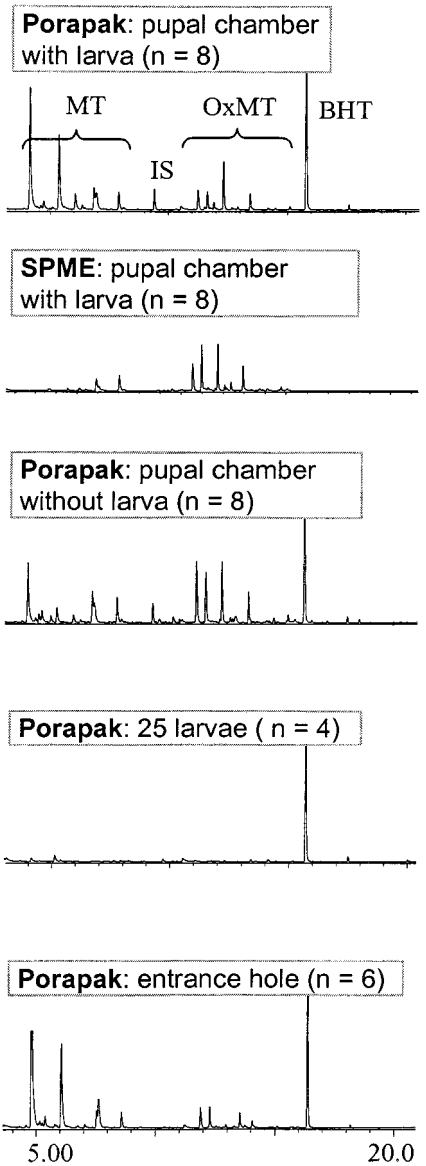


FIG. 4. Volatile samples, collected by Porapak Q or SPME from pupal chambers with or without bark beetle larvae, isolated larvae, and entrance holes into the bark beetle galleries. MT = monoterpenes; IS = 5 ng internal standard, heptyl acetate; OxMT = oxygenated monoterpenes; BHT butylated hydroxytoulene (ether stabilizer).

information (Casas et al., 1998). Other possible close-range host location cues may be chemicals of low volatility requiring contact. While host foraging on the bark surface of trees containing bark beetle larvae, a parasitoid would possibly detect low-volatility compounds by chemoreceptors on the antennae, mouth

TABLE 1. ODOR SAMPLES USED IN GC-EAD ANALYSIS COLLECTED BY PORAPAK AND SPME-SAMPLING METHODS FROM PUPAL CHAMBERS WITH BARK BEETLE LARVAE (C + L) OR WITHOUT BARKBEETLE LARVAE (C)[a]

Compound[b]	Mean abundance[c] (ng) ± SE		
	Porapak C + L	Porapak C	SPME C + L
α-Pinene	34 ± 8.7	24 ± 12	* 2.7 ± 1.5
α-Fenchene	1.8 ± 1.5	0.7 ± 1.4	<
Camphene	4.0 ± 1.2	2.9 ± 1.6	<
β-Pinene	15 ± 7.4	7.9 ± 4.5	* 1.4 ± 1.5
Sabinene	0.6 ± 1.0	<	<
3-Carene	4.9 ± 2.6	1.8 ± 1.2	* 0.8 ± 1.2
β-Myrcene	1.8 ± 0.3	<	1.5 ± 1.3
Limonene	12 ± 5.0	7.5 ± 2.8	10 ± 6.6
β-Phellandrene	11 ± 7.4	8.7 ± 1.1	5.9 ± 1.4
p-Cymene	9.6 ± 4.1	6.2 ± 2.5	8.2 ± 4.0
α-Terpinolene	2.2 ± 1.9	0.6 ± 1.2	2.7 ± 2.2
Fenchone (+Nonanal)	0.5 ± 1.4	0.7 ± 1.4	0.6 ± 0.2
Linalool oxide	1.3 ± 2.2	1.4 ± 0.9	<
Acetic acid	0.6 ± 1.1	0.8 ± 0.9	<
1-methyl-4-(1-methylethenyl)-benzene			1.8 ± 1.1
Sqt [119; 105; 133]	1.4 ± 1.2	0.4 ± 0.7	1.3 ± 1.4
Sqt [105; 119; 204]	0.6 ± 1.1	<	<
Camphor (+Pinocamphone)	21 ± 13.5	21 ± 5.0	19 ± 6.4
Benzaldehyde	<	<	<
Isopinocamphone	19 ± 13.7	20 ± 7.3	22 ± 8.5
Sqt [161; 105; 133]	2.8 ± 2.4	0.8 ± 1.5	4.0 ± 1.7
Pinocarvone	5.3 ± 3.2	1.2 ± 1.3	2.8 ± 2.9
Fenchol + Bornyl acetate	1.9 ± 1.6	0.4 ± 0.7	<
u.i. [107; 149; 91]	0.8 ± 1.3	0.4 ± 0.8	
Terpinen-4-ol	40 ± 6.7	30 ± 11	33 ± 9.8
u.i. [93; 148; 133]	1.6 ± 1.4	0.5 ± 1.0	
Myrtenal	2.8 ± 2.4	1.6 ± 1.1	1.8 ± 1.7
OxMT ketone [83; 95; 109]	2.2 ± 2.0	1.0 ± 1.2	1.2 ± 1.3
trans-Pinocarveol	5.7 ± 5.1	3.4 ± 2.1	5.4 ± 5.2
4-Allylanisole	<	<	0.2 ± 0.6
α-Terpineol (+Borneol)	21 ± 18.1	15 ± 4.7	22 ± 8.6
Verbenone	1.6 ± 1.4	<	<
Stq + Sqt	1.0 ± 1.6	0.6 ± 1.1	1.7 ± 1.1
δ-Cadinene	4.3 ± 3.8	1.6 ± 2.0	4.0 ± 1.9
u.i. [79; 91; 108]	1.6 ± 1.4	<	<
Myrtenol	2.8 ± 2.7	1.4 ± 1.0	3.2 ± 1.6

TABLE 1. CONTINUED

Compound[b]	Mean abundance[c] (ng) ± SE		
	Porapak C + L	Porapak C	SPME C + L
Naphthalene	1.6 ± 1.6	0.4 ± 0.8	<
p-Cymen-8-ol	5.1 ± 4.6	3.2 ± 0.9	2.1 ± 1.4
u.i. [123; 67; 69]	0.8 ± 1.3	0.3 ± 0.6	<
u.i. [93; 121; 79]	3.3 ± 3.0	2.5 ± 0.6	<

[a] Differences in compound concentration in different samples were analyzed by one-way ANOVA. $^{*}P < 0.05$. The only differences detected were between Porapak and SPME sampling methods. Compounds are arranged in order of retention time on the semipolar column used.

[b] u.i. unidentified compound with predominant ion fragments in brackets; Sqt Sesquiterpene; OxMT oxygenated monoterpene.

[c] Mean abundances calculated from eight samples per category, considering compounds more abundant than 0.8 ng/sample; "<", compounds less abundant than 0.8 ng in the samples were not considered in the comparison of sampling methods.

parts, or tarsi (Slifer, 1970). Parasitoids probably detect differences in the concentration of compounds. The bark layer above a pupal chamber is thinner than that above the rest of the bark beetle galleries. The thin bark layer could allow compounds produced in the phloem to pass through readily, allowing for an increase in release rate. Alternatively, it could result in lower release rates since a thin phloem tissue would contain fewer microorganisms, which are the most probable producers of compounds that attract parasitoids. An unidentified compound has been isolated around the pupal chambers of the southern pine beetle (*Dendroctonus frontalis*) in loblolly pine (*Pinus taeda*) (personal communication: G. Birgersson, Göteborg University; M. Dalusky, University of Georgia). The release rate of this compound, as measured on the undamaged bark surface, is high in the area surrounding an oviposition site (a pupal chamber) but decreases at the oviposition site, suggesting that the phloem surrounding pupal chambers emits greater amounts of compounds than the centers of the chambers.

Pupal chambers are the most likely source of host-finding volatiles for parasitoids since pupal chambers emitted larger quantities of volatiles but did not differ qualitatively from samples from entrance holes (Figure 4). However, Porapak samples (using funnels, 2 cm ID) from undamaged bark of spruce trees containing mature bark beetle larvae did not reveal any detectable difference in volatile composition among oviposition sites and other sites (E. M. Pettersson et al., unpublished data). The similarity in chemical composition and EAD activity of samples from pupal chambers, compared to other analyses of bark beetle associated volatiles (Pettersson et al., 2000, 2001a, b), indicates that the volatile composition of the pupal chambers is not altered by the sampling technique.

Odor samples collected from bark beetle larvae did not reveal any compound that was not also present in empty pupal chambers (Figure 4). Thus, these compounds are most likely contaminants from the pupal chamber. Y-tube walking bioassays with the pteromalid bark beetle parasitoid *Ropalicus xylophagorum* showed that bark beetle larvae were not attractive unless they were in the host tree bark, while infested bark deprived of bark beetle larvae was still attractive (Sullivan et al., 2000). Bark subjected to mechanical damage was not attractive and differed in chemical composition from bark beetle infested bark (Sullivan et al., 2000).

Infestation by *Ips typographus* results in microorganisms being inoculated into host tree phloem (Krokene and Solheim, 1996). As the production of oxygenated monoterpenes increases drastically after an attack by bark beetles and their associated microorganisms (Leufvén and Birgersson, 1987), the compounds attracting bark beetle parasitoids seem to arise from the interaction among bark beetles, microorganisms, and the host tree. Interestingly, physiological changes in the tree phloem polyphenolic parenchyma cells, three weeks after bark beetle attack, (Franceschi et al., 2000) coincide with the presence of mature bark beetle larvae susceptible to parasitation. At this time, a marked increase in the concentration of camphor and certain other oxygenated compounds has been found (Pettersson, unpublished data) that elicits EAD activity in *Rhopalicus tutela* and other bark beetle parasitoids (Pettersson et al., 2000, 2001a). It is possible that the phloem polyphenolic parenchyma cells contribute to the increased production of oxygenated monoterpenes.

Few EAD-active compounds elicited antennal responses of different magnitude as a result of different sampling methods (Figure 3). Thus, both SPME and Porapak sampling are useful in GC-EAD analysis of volatiles from bark beetle infested spruce. The long duration of Porapak sampling did not affect the samples significantly, as there was no apparent qualitative difference between the two sampling methods. The major advantage of the more elaborate Porapak sampling is the large quantity of sample obtained, making it possible to identify compounds present at low levels. Furthermore, the same sample can be used in both GC-MS and GC-EAD, facilitating identification of EAD-active compounds. Several insects can be exposed to exactly the same sample, making it possible to detect compounds that elicit minor antennal activity. One drawback of SPME sampling is that a sample can be used only once.

The results show that the volatiles used in host location by parasitoids attacking bark beetle larvae arise from the host tree phloem, rather than from the host itself. The host trees used were naturally inoculated with microorganisms, which may play a role in the production of host location volatiles. The results do not show how parasitoids could use volatile stimuli to pick out the oviposition sites directly above host larvae in pupal chambers. High-resolution sampling around oviposition sites followed by quantitative analyses of the GC-EAD active compounds may resolve this anomaly by disclosing differences in amounts

of behaviorally active compounds that might be used in the precise location of host larvae.

Acknowledgments—I thank my family for providing spruce logs containing parasitoids and Claes Johansson for technical assistance. For helpful comments on previous versions of this manuscript, I thank Hans Alborn at USDA, Florida (USA), Göran Birgersson and Susanna Andersson at Göteborg University , Peter Anderson at SLU (Sweden), and two anonymous reviewers. This work was supported in part by grants from the C. F. Lundström Foundation, A. Ahrenberg Foundation, and W. & M. Lundgren Research Foundation.

REFERENCES

BERISFORD, C. W. 1980. Natural enemies and associated organisms, pp. 31–54, *in* R. C. Thatcher, J. L. Searcy, J. E. Coster, and G. D. Hertel (eds.). The Southern Pine Beetle. USDA Forest Service Science Education Administration Technical Bulletin 1631.

CASAS, J., BACHER, S., TAUTZ J., MEYHOEFER R., and PIERRE, D. 1998. Leaf vibrations and air movements in a leafminer-parasitoid system. *Biol. Control* 11:147–153.

FRANCESCHI, V. R., KROKENE, P., KREKLING, T., and CHRISTIANSEN, E. 2000. Phloem parenchyma cells are involved in local and distant defence responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). *Am. J. Bot*. 87:314–326.

GODFRAY, H. C. J. 1994. Parasitoids: Behavior and Evolutionary Ecology. Princeton University Press, Princeton, New Jersey.

KROKENE, P. and SOLHEIM, H. 1996. Fungal associates of five bark beetle species colonizing Norway spruce. *Can. J. For. Res.* 26:2115–2122.

KROL, A. and BAKKE, A. 1986. Comparison of trap trees and pheromone loaded pipe and traps in attracting *Ips typographus* L. (Coleoptera, Scolytidae). *Pol. Pismo Entomol.* 56:437–446.

LEUFVÉN, A. and BIRGERSSON, G. 1987. Quantitative variation of different monoterpenes around galleries of *Ips typographus* attacking Norway spruce. *Can. J. Bot.* 65:1038–1044.

LEUFVÉN, A. and NEHLS, L. 1986. Quantification of different yeasts associated with the bark beetle, *Ips typographus*, during its attack on a spruce tree. *Microb. Ecol.* 12:237–243.

MADDEN, J. L. 1968. Behavioural responses of parasites to the symbiotic fungus associated with *Sirex noctilio* F. *Nature* 218:189–190.

MILLS, N. J., KRÜGER, K., and SCHLUP, J. 1991. Short-range host location mechanisms of bark beetle parasitoids. *J. Appl. Entomol.* 111:33–43.

PAWLISZYN, J. 1997. Solid Phase Microextraction Theory and Practice. Wiley-VCH, New York.

PETTERSSON, E. M. 2001. Volatile attractants for three pteromalid parasitic wasps attacking concealed spruce bark beetles. *Chemoecology* 11:89–95.

PETTERSSON, E. M., SULLIVAN, B. T., ANDERSON, P., BERISFORD, C. W., and BIRGERSSON, G. 2000. Odor perception in the bark beetle parasitoid *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) exposed to host associated volatiles. *J. Chem. Ecol.* 26:2507–2525.

PETTERSSON, E. M., BIRGERSSON, G., and WITZGALL, P. 2001a. Synthetic attractants for the bark beetle parasitoid *Coeloides bostrichorum* Giraud (Hymenoptera: Braconidae). *Naturwissenschaften* 88:88–91.

PETTERSSON, E. M., HALLBERG, E., and BIRGERSSON, G. 2001b. Evidence for the importance of odor perception in the parasitoid *Rhopalicus tutela* (Walker) (Hymenoptera: Pteromalidae). *J. Appl. Entomol.* 125:293–301.

QUICKE, D. L. J. 1997. Parasitic Wasps. Chapman & Hall, London.

RICHERSON, J. V. and BORDEN, J. H. 1972. Host finding behavior of *Coeloides brunneri* (Hymenoptera: Braconidae). *Can. Entomol.* 104:1235–1250.

RUTLEDGE, C. E. 1996. A survey of identified kairomones an synomones used by insect parasitoids to locate and accept their hosts. *Chemoecology* 7:121–131.

SLIFER, E. H. 1970. The structure of arthropod chemoreceptors. *Annu. Rev. Entomol*. 15:121–142.

SULLIVAN, B. T., PETTERSSON, E. M., SELTMANN, K. C., and BERISFORD, C. W. 2000. Attraction of the bark beetle parasitoid *Roptrocerus xylophagorum* (Hymenoptera: Pteromalidae) to host-associated olfactory cues. *Environ. Entomol.* In press.

UNDERWOOD, A. J. 1997. Experiments in Ecology, Their Logical Design and Interpretation Using Analysis of Variance. Cambridge University Press, Cambridge, United Kingdom.

VINSON, S. B. 1986. The behavior of parasitoids, pp. 417–469, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol 9. Pergamon Press, Elmsford, New York.

VINSON, S. B., BIN, F., and VET, L. E. M. 1998. Critical issues in host selection by insect parasitoids. *Biol. Control* 11:77–78.

WÄCKERS, F. L., MITTER, E., and DORN, S. 1998. Vibrational sounding by the pupal parasitoid *Pimpla* (*Coccygomimus*) *turionellae*: An additional solution to the reliability–detectability problem. *Biol. Control* 11:141–146.

WESELOH, R. M. 1981. Host location by parasitoids, pp. 79–95, *in* D. A. Nordlund, R. L. Jones, and W. J. Lewis (eds.). Semiochemicals, Their Role in Pest Control. Wiley & sons, New York.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

SYSTEMICALLY INDUCED PLANT VOLATILES EMITTED AT THE TIME OF "DANGER"

LETIZIA MATTIACCI,* BETTINA AMBÜHL ROCCA, NADIA SCASCIGHINI, MARCO D'ALESSANDRO, ALAN HERN, and SILVIA DORN

Institute of Plant Sciences/Applied Entomology
Swiss Federal Institute of Technology (ETH)
Clausiusstrasse 25/NW, CH 8092 Zurich, Switzerland

(Received January 26, 2001; accepted July 16, 2001)

Abstract—Feeding by *Pieris brassicae* caterpillars on the lower leaves of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) plants triggers the release of volatiles from upper leaves. The volatiles are attractive for a natural antagonist of the herbivore, the parasitoid *Cotesia glomerata*. Parasitoids are attracted only if additional damage is inflicted on the systemically induced upper leaves and only after at least three days of herbivore feeding on the lower leaves. Upon termination of caterpillar feeding, the systemic signal is emitted for a maximum of one more day. Systemic induction did not occur at low levels of herbivore infestation. Systemically induced leaves emitted green leaf volatiles, cyclic monoterpenoids, and sesquiterpenes. GC-MS profiles of systemically induced and herbivore-infested leaves did not differ for most compounds, although herbivore infested plants did emit higher amounts of green leaf volatiles. Emission of systemically induced volatiles in Brussels sprouts might function as an induced defense that is activated only when needed, i.e., at the time of caterpillar attack. This way, plants may adopt a flexible management of inducible defensive resources to minimize costs of defense and to maximize fitness in response to unpredictable herbivore attack.

Key Words—Tritrophic interactions, plant induced defense, *Cotesia glomerata*, *Pieris brassicae*, *Brassica oleracea*, semiochemicals, pathways, volatiles.

*To whom correspondence should be addressed. E-mail: letizia.mattiacci@ipw.agrl.ethz.ch

0098-0331/01/1100-2233$19.50/0

INTRODUCTION

Plants have evolved a range of defensive mechanisms to guard themselves from the wide variety of pathogens and herbivores with which they are confronted. Physiological changes in plants following damage or stress are generally referred to as induced responses. These are named induced defenses when they reduce the negative consequences in terms of plant fitness of the herbivore attack on plants (Karban and Baldwin, 1997). Recent studies indicate that induced plant responses effectively increase plant fitness in the field both by directly reducing herbivory (Agrawal, 1998; Baldwin, 1998) or indirectly through enhanced parasitization, resulting in increased herbivore mortality (Thaler, 1999). Chemical defenses of plants against herbivorous insects are defined as direct defenses when they are related to a systemic production of toxic metabolites and as indirect defenses when they promote the effectiveness of natural enemies of herbivores (Dicke, 1999).

A well-documented inducible response related to direct defense in insects is the so-called "wound response pathway," in which wounding and/or insect feeding trigger the induction of proteinase inhibitors in solanaceous plants (reviewed by, e.g., Karban and Baldwin, 1997; Maleck and Dietrich, 1999). Inducible responses related to indirect defenses have been known for about a decade. Feeding (e.g., Dicke and Sabelis, 1988; Turlings et al., 1990) and even oviposition (Meiners and Hilker, 1997, 2000) by herbivores induce the emission of blends of plant volatiles that are used by predators or parasitoids (parasitic wasps) as a cue to find their prey or hosts. The importance of induced phytochemicals, such as plant volatiles, for plant defense as well as the mechanisms that regulate their synthesis and release are still poorly understood, however (Paré and Tumlinson, 1999).

Induced responses to injury may be systemic, i.e., a signal produced at the damaged site is transported throughout the plant, causing effects at sites distant from the injury (Karban and Baldwin, 1997). In corn seedlings, the emissions of several volatiles that are highly attractive to parasitic wasps occur a few hours after caterpillars start damaging the plants. The response is systemic, as undamaged leaves of injured plants also emit the volatiles (Turlings and Tumlinson, 1992). Various studies indicate that the systemic responses induced by herbivore feeding occur in several plant species and affect the behavior of predators and parasitoids (Dicke et al., 1990; Potting et al., 1995; Röse et al., 1996, 1998; Souissi et al., 1998; Guerrieri et al., 1999).

In this work, we report on the systemic response of Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*; Brassicaceae) to feeding of *Pieris brassicae* (Lepidoptera: Pieridae) caterpillars. In laboratory behavioral assays, the parasitic wasp *Cotesia glomerata* (Hymenoptera: Braconidae) was used as a biological sensor to determine the dynamics of the plant systemic response. In addition we carried out GC-MS identification and quantification of the volatiles emitted by induced plants.

METHODS AND MATERIALS

Rearing Procedures

Brussels sprouts seedlings were individually transplanted into 400-ml pots two weeks after they had been sowed on a commercially available mixture of peat and clay (Optima-Werke, H. Gilgen, Germany). The substrate contained 400 mg/liter total nitrogen, 200 mg/liter phosphorus, 370 mg/liter potassium, 2200 mg/liter lime, and 0.32 mg/liter magnesium. Plants were reared in a walk-in climate room under high pressure sodium lamps (4×400 W, for a total of $10{,}000 \pm 1000$ lux) at 16°C (night), 24°C (day), 50–70% relative humidity, 16L : 8D photoperiod. Leaves from 8- to 10-week-old Brussels sprouts plants with five to eight fully expanded leaves were used for experiments. Plants used were similar with respect to appearance, size, and number of leaves. After treatment, they were kept under the environmental conditions described above and transferred to the bioassay room 30 min prior to the experiment.

Herbivores (*P. brassicae*) and parasitoids (*C. glomerata*) were reared as previously described (Gu and Dorn, 2000). *P. brassicae* larvae were maintained on Brussels sprouts plants, at 21 ± 1°C, 50–70% relative humidity, and 16L : 8D photoperiod. *C. glomerata* were reared in a climate room at 15°C, 90% relative humidity, and a 16L : 8D photoperiod. For parasitization, first- or second-instars of *P. brassicae* were exposed to 4- to 10-day-old parasitoids for 15 min. This method ensures the highest acceptance/rejection ratio (Brodeur and Geervliet, 1992) and decreases risk of multiparasitism. Parasitised hosts were subsequently fed on an ample amount of Brussels sprouts plants and, after they reached the fourth instar, on Savoy cabbage leaves (*Brassica oleracea* var. *sabauda*). Upon egression, parasitoid cocoons were separated from the remains of the host and transferred into a Petri dish. Newly emerged adults were transferred into a nylon gauze cage ($30 \times 30 \times 30$ cm) in groups of uniform age. For all experiments, parasitoids had access to water and honey, and they were able to perform normal flight activities and to mate at random. Females were never exposed to host larvae or plants before bioassay, and, therefore, they were considered "naive."

Behavioral Bioassay

Behavioral experiments were conducted in a Plexiglas wind tunnel that measured $150 \times 35 \times 35$ cm. Air was pulled through the wind tunnel via an activated charcoal filter (0.5 cm thick) at a wind speed of 26–30 cm/sec. The exhaust air was vented outside the bioassay room. A smoke test was carried out to obtain laminar airflow and to ensure that the release point of wasps was within the odor plume. A light intensity of 750–950 lux at the release point and 750–1000 lux at the landing

point was obtained with high-frequency fluorescence tubes. The conditions inside the tunnel were 22 ± 2°C and 50 ± 5% relative humidity.

Brussels sprouts leaves were offered to parasitoids in a dual-choice situation as odor sources. Two sets of two leaves each were excised just before the experiment, their petioles were wrapped in cotton wool, and they were placed in separate 100-ml Erlenmeyer flasks filled with water. Flasks were transferred to the floor of the tunnel, 20 cm apart, so that they formed an isosceles triangle with 30-cm sides with the release point of parasitoids. In order to minimize visual stimuli, white cotton gauze was suspended in front of the leaves. For the bioassay, 4- to 5-day-old female parasitoids were used. Steinberg et al. (1992) showed that wasps of this age offer the strongest and most consistent response to the volatiles. The evening before the experiment, 25 wasps were selected at random in the cage and transferred individually into cotton wool-stoppered glass vials (6 ml) provided with a droplet of honey. The vials were held overnight in a dark box in a climate room and transferred to the bioassay room 30 min prior to the experiment.

For the bioassay, individual females were gently introduced into the wind tunnel and released in the middle of a platform placed 15 cm from the bottom of the tunnel floor. Each wasp was allowed two flight attempts. Based on preliminary observations, most females would not fly toward the odor sources at the first flight attempt. Wasps that did not fly within 5 min from being released in the wind tunnel were discarded. A choice was recorded when wasps completed a flight, landing on the nylon gauze in front of one of the two odor sources. Landing elsewhere in the tunnel was recorded as no choice. The two odor sources were exchanged after testing three wasps to compensate for possible asymmetry in the setup. In order to avoid day-to-day variation in the response of the wasps, we conducted every bioassay on at least two days. A total of 50 wasps were tested for every experiment (one combination of odor sources).

When less than 40% of the females tested responded in a bioassay, the same parasitoids were immediately retested using a leaf infested with approximately 100 first or second instar *P. brassicae* as a test odor source and an undamaged leaf as a control. This standard bioassay was used to assess if the low responsiveness was due to either insufficient concentration of volatiles or to low motivation of the wasps, possibly due to a fluctuation in barometric pressure (Steinberg et al., 1992). We expected a significant increase in responsiveness only in case of problems of odor concentration in the previous bioassay.

Plant Treatments

Timing and Pattern of Systemic Induction. At different time intervals before the bioassay (see below), two basal leaves of a plant were infested each with 100 first- or second-instar *P. brassicae* caterpillars. Herbivores were prevented from feeding on other parts of the plant by sticky strips wrapped around the leaf

petiole. For the bioassays, two uninfested leaves of caterpillar-infested plants were excised and used as odor sources as explained above. These leaves, which we named "induced leaves," were always those immediately above the infested ones. As a control odor source, we used two leaves of an uninfested plant that were approximately the same size and position as those on the infested plant.

In order to investigate the conditions under which Brussels sprouts plants emit systemically induced volatiles, we tested several combinations of herbivore damage on the infested leaves and mechanical damage on the systemically induced leaves. Mechanical damage was inflicted, with the same method and timing, also on leaves excised from control plants. The perimeter of the wound opened by caterpillars on the leaf ranged from 10 (one day feeding) to 40 cm (three days feeding). The perimeter of the mechanically damaged leaves ranged from 15 to 25 cm. This was obtained by punching several 0.5-cm-diam. holes on the leaf surface with a hole-punch. Plants were kept in a common climatic chamber to ensure that possible adsorption of herbivore induced volatiles would be equal for control and test plants (Turlings and Tumlinson, 1992).

Figure 1 depicts an overview of the plant treatments. Each combination of herbivore-induced and mechanical damage was tested in separate bioassays. A first set of bioassays was carried out in order to assess the level of caterpillar infestation and the timing of mechanical damage after initial infestation (one day of feeding, Figure 1A). One hundred caterpillars were allowed to feed for approximately one day (20 hr). Mechanical damage, consisting of 10 holes/leaf, was inflicted (1) 20 hr before the bioassay; or (2) during the bioassay, that is 1 hole/15 min, starting 1 hr before the bioassay; or (3) immediately before the bioassay. In the last case, the experiment was repeated with a higher level of infestation. This was obtained by placing caterpillars (approx. 500) all over the plant except on the two leaves used for the bioassay. The two induced leaves were protected from caterpillars by sticky strips wrapped around the petiole.

A second set of bioassays was carried out to assess the effect of different duration of caterpillar feeding (Figure 1B). One hundred caterpillars were allowed to feed for approximately two (40 hr) or three days (60 hr) before the bioassay. On the induced leaves of plants infested for two days, mechanical damage was inflicted immediately before the bioassay (10 holes/leaf). On the systemically induced leaves of plants infested for three days, mechanical damage was inflicted as follows: (1) no damage, (2) damage one day before the bioassay, (3) damage immediately before the bioassay, or (4) damage of 16 holes immediately before the bioassay. Sixteen holes represent the maximum amount of mechanical damage that can be inflicted on a single leaf without complete alteration of its shape.

A third set of bioassays was carried out to assess the permanence of the induced signal after termination of caterpillar feeding (Figure 1C). One hundred caterpillars were allowed to feed for one or three days before the bioassay and were then removed by means of a brush. Bioassays were then carried out one,

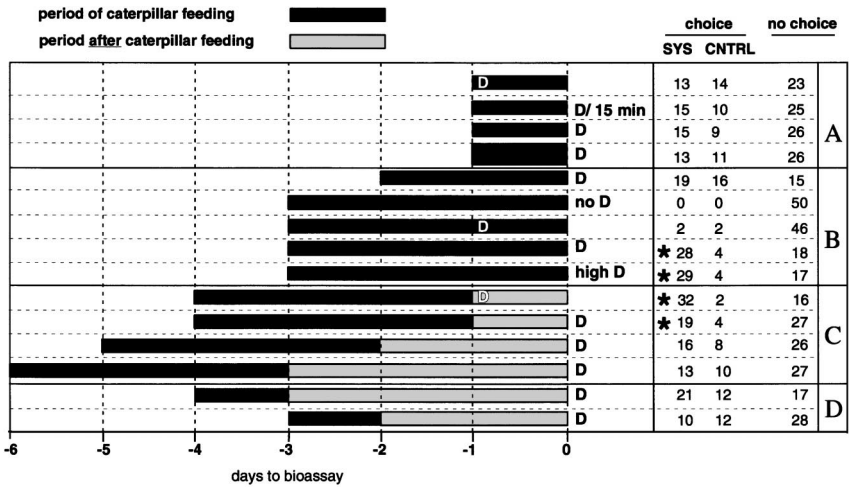


FIG. 1. Response of female *C. glomerata* to systemically induced Brussels sprouts leaves in the wind tunnel. Leaves were excised from plants where caterpillars were released on two different lower leaves one to six days before bioassay, but fed for a maximum of three days. Bioassays were carried out at day 0. Dark gray bars indicate the caterpillar feeding period. The wider bar (4th from top, section A) symbolizes a higher level of caterpillar infestation, i.e., the whole plant was infested except the leaves used in the bioassay. Light grey bars indicate that the bioassay was carried out one to three days after termination of caterpillar feeding. The letter "D" within a bar indicates mechanical damage inflicted one day before the bioassay (10 holes/leaf), "D 15/min" indicates damage inflicted throughout the bioassay (1 hole/15 min), "D" at the right end of a bar indicates damage inflicted just before the bioassay (10 holes/leaf), "no D" indicates no mechanical damage, "high D" indicates maximum possible damage on a leaf (16 holes). The actual number of females choosing either systemically induced leaves (SYS) or control (CNTRL) or making no choice is indicated on the right inside of the figure (sample size $= 50$ per treatment). Asterisks indicate significant differences within the choice test at $P < 0.001$. [Chi-square test for goodness-of-fit; Sokal and Rohlf (1981)].

two, or three days after removal of the caterpillars. Mechanical damage (10 holes) was inflicted just before the bioassay. In one bioassay, mechanical damage was inflicted immediately after caterpillar removal, that is, approximately 20 hr before the bioassay.

A fourth set of bioassays was carried out to assess whether the occurrence of systemic induction depended on the time lag after initial infestation rather than on the duration of feeding (Figure 1D). One hundred caterpillars were allowed to feed for approximately one day (20 hr) and then removed by means of a brush. Bioassays were then carried out two or three days after removal of

the caterpillars. Mechanical damage (10 holes/leaf) was inflicted just before the bioassay.

Plant Age and Systemic Induction. One additional experiment was carried out to study the effect of plant age on systemic emission of volatiles. One hundred caterpillars were allowed to feed for three days on the lower leaves of either a 2- or a 6-month-old plant. Two sets of two leaves were excised from the two plants and offered to parasitoids as explained above. Mechanical damage (10 holes/leaf) was inflicted just before the bioassay.

Level of Infestation for Systemic Induction. To assess the level of infestation necessary to obtain systemic induction of volatiles, 8-week-old Brussels sprouts plants were infested on one single lower leaf for three days with 5, 25, 50, or 100 caterpillars. In preliminary experiments, we observed that five caterpillars feeding for three days will inflict a damage of approximately 2 cm^2 and that a wound of this dimension is sufficient to elicit local emission of attractive volatiles. Two noninfested upper leaves were excised and mechanically damaged (10 holes) immediately before the wind-tunnel bioassay as explained above. As a control odor source, we used two mechanically damaged leaves from an uninfested plant that were approximately the same size and position as those on the infested plant.

Data Analysis. Parasitoid choice response was analyzed with a chi-square test (Sokal and Rohlf, 1981) to determine if the response differed from a 50 : 50 distribution of wasps over the two odor sources. Wasps that did not make a choice within 5 min were excluded from statistical analysis.

Volatile Sampling

In order to collect headspace volatiles from single leaves of living cabbage plants, we used a glass–Teflon chamber similar to others previously described (Heath and Manukian, 1994; Agelopoulos et al., 1999). The chamber consisted of a 10-cm-diam. × 11-cm-high bell-shaped jar in which the two leaves from the treated plant were introduced. Air entered the top of the jar through an activated charcoal filter. At the bottom of the jar, a split Teflon plate with a hole in the center closed loosely around the petioles of the leaves like a guillotine, which prevented air from flowing back into the jar from outside.

The base of the jar allowed the connection of Tenax traps that were used to adsorb the volatiles. Each trap contained 300 mg of Tenax-GR (mesh size 60/80, Alltech Associates) and was thermally conditioned prior to each collection for 4 hr at 300°C with a flow of dried filtered helium (initial purity 99.96%, 50–60 ml/min). Traps analyzed with the Unity thermaldesorber (see below) were conditioned for 15 hr. During collection, air was pulled trough the Tenax-GR trap at 240–340 ml/min. Three plant treatments were sampled for volatiles over 24 hr: (1) A plant-herbivore-complex (PHC) consisted of two infested leaves where first instars (100 leaf) were allowed to feed for 16 hr before volatile collection

and throughout the 24 hr of headspace sampling. The duration of feeding was chosen to ensure that the amount of damage on PHC plants would be comparable to the mechanical damage inflicted on systemically induced ones. (2) Systemically induced leaves (SYS+D) consisted of two noninfested, artificially damaged leaves from an infested plant. The plant was infested with 100 caterpillars/leaf on two basal leaves for 60 hr. Volatiles were sampled from the two leaves located immediately above the infested ones. Artificial damage was inflicted with a hole-punch (10 holes/leaf) just before sampling as described above. (3) Mechanically damaged leaves (MD) consisted of two noninfested, artificially damaged leaves from an uninfested plant. Artificial damage was inflicted with a hole-punch (10 holes/leaf) just before sampling. Headspace sampling was replicated five times for each treatment. This set of samples was analyzed with manual thermaldesorption (see below)

In subsequent expriments, volatiles were collected as described above but with a 340 ml/min airflow through the traps. Three plant treatments were sampled for volatiles over 24 hr: (1) Systemically induced and undamaged leaves (SYS_UND) consisted of two noninfested, leaves from an infested plant. The plant was infested with 100 caterpillars/leaf on two basal leaves for 60 hr. Volatiles were sampled from the two leaves located immediately above the infested ones. (2) Systemically induced and damaged leaves (SYS+D) were as described above. (3) Undamaged leaves (UND) consisted of two leaves from an uninfested, undamaged plant. All sampling procedures were repeated five times. This set of samples was analyzed with automatic thermaldesoprtion (see below).

All collections were carried out in a climate room at 20°C, 60% relative humidity, and a 16L : 8D photoperiod. Light was provided by "Very High Output" Sylvania fluorescent lamps (cold light, 10,000 ± 1000 lux). Headspace collections were started 2–3 hr after beginning of the photophase in the chamber to minimize variations due to changes in plant volatile emissions throughout the day (see e.g., Loughrin et al., 1997).

Volatile Analysis

Manual Thermodesorption. Volatiles were eluted from the Tenax traps with a thermal desorption procedure after headspace sampling. Traps were connected to the inlet of a gas chromatograph (Hewlett Packard 5890 Series II +) by Swagelock connections. The exhaust of the trap was connected to flushed copper tubing and exited the GC oven through the dismantled detector. The oven was programmed with an initial temperature of 100°C, then to 300°C at 45°C/min. The inlet was programmed in the same manner. The GC was operated with a constant pressure (3 kPa) of helium that resulted in a flow rate through the Tenax trap of ca. 20 ml/min at ambient temperature (ca. 25°C). The exhaust from the trap was bubbled into a 2-ml vial containing 500 μl of hexane (Fluka, purity 99.5%) that was kept in an

acetone–ice bath. Samples were stored at −60°C in glass vials with PTFE silicon septa after being concentrated to 15 μl for 5–10 min using a stream of purified nitrogen.

Collected volatiles were analyzed using GC-MS (Hewlett Packard GC model 6890; MSD 5973). Analyses were carried out using a 0.25-mm-diam. × 30-m long HP1, polydimethyl siloxane column with nominal film thickness of 1 μm. Initial oven temperature was 55°C rising 2°C/min up to 102°C, and then to 250°C at 60°C/min. A postrun time of 15 min at 320°C was run to remove impurities from the column. The inlet was operated in the splitless injection mode at a temperature of 250°C with a constant column flow of 1.0 ml/min helium. The effluent from the column was transferred to the MS detector via a transfer line (280°C) and the GC-MS electron impact source was operated in the scan mode with the MS source temperature at 230°C and the MS Quad at 150°C.

Automatic Thermodesorption. In subsequent experiments, volatiles were analyzed with a commercial system (Unity, Markes Int. Ltd.). Using the Unity system with a double split (split ratio 25 : 1, desorption flow 50 ml/min, split flow 30 ml/min), volatiles were desorbed from the Tenax trap for 5 min, starting at 50°C, then up to 300°C at ∼20°C/min, and transferred to the cold trap (50 : 50 Tenax TA + Carbopack B), which was cooled at −10°C. The cold trap was subsequently heated for 3 min to 300°C at ∼60°C/min. The fused silica transfer line to the GC was kept at 200°C. Analyses were carried out with the Hewlett Packard GC-MS and HP column described above. The GC oven was set at 40°C, rising to 300°C at 8°C/min, initial flow of approx. 2.0 ml/min.

Identification of volatiles was achieved by comparing the sample spectra with those in the NIST98 library for all samples. Subsequently, spectra were compared with those of authentic standards and identification was confirmed by coinjection. Quantification of constituents was based on the peak area of an internal standard (hexylbenzene) that was added to the hexane extract (100 ng/μl sample) or injected directly into the Tenax-GR trap (200 ng in 2 μl ethyl acetate). As no response factors were determined, amounts given are only rough estimates.

Data Analysis. Comparisons of the amounts of individual volatiles resulting from the different treatments were analyzed with one-way ANOVA or Kruskal-Wallis, for data not normally distributed, followed by Student-Newman-Keuls test for pairwise comparison between single compounds emitted by plants that underwent different treatments (Sokal and Rohlf, 1981).

RESULTS

Behavioral Bioassays. Emission of systemically induced volatiles from *B. oleracea* varied depending on the duration of caterpillar feeding (one to three days). In the wind tunnel, *C. glomerata* females were not attracted to upper

uninfested leaves (induced) if *P. brassicae* caterpillars had been feeding on the lower leaves (infested) for only one day (Figure 1A). Regardless of the timing of infliction of mechanical damage or the level of infestation, parasitoid choice was equally distributed over the test and control.

After two days of caterpillar feeding, again no preference for induced versus control leaves was observed. In contrast, parasitoid choice shifted significantly to induced leaves when caterpillar feeding time was prolonged to at least three days (Figure 1B). Parasitoids were attracted ($P < 0.001$) to induced leaves if mechanical damage was inflicted immediately before the bioassay regardless of the level of mechanical damage inflicted (see "high D" bar, Figure 1B). Remarkably, parasitoids were not attracted to leaves without mechanical damage or with damage inflicted one day before the bioassay. However, the same parasitoids tested in the standard bioassay preferred an herbivore-infested leaf to an uninfested leaf (56% vs. 24%, with 20% of wasps making no choice), indicating that the induced leaves in the previous bioassays emitted an extremely low concentration of volatiles.

The permanence of the systemic signal after termination of caterpillar feeding is depicted in Figure 1C. One day after removal of caterpillars from the plant, parasitoids preferred the induced leaves over control leaves, even when mechanical damage was inflicted one day before the bioassay. No preference was observed for induced leaves tested two or three days after caterpillar removal, indicating a relevant decrease of the emitted signal.

Parasitoids were not attracted to leaves induced for one day and tested two or three days after termination of caterpillar feeding (Figure 1D). This indicates that, for a detectable systemic response, the duration of feeding is more important than the time lag between infestation and mechanical damage. Furthermore, we did not observe any effect of plant age on systemic emission of volatiles (results not shown). Parasitoids did not prefer induced leaves excised from two-month-old Brussels sprouts over induced leaves excised from 6-month old plants (18 vs. 14, with 18 females making no choice).

The effect of the level of infestation on the emission of systemically induced volatiles is shown in Figure 2. Systemic induction did not occur at low levels of infestation even if caterpillars were feeding on the same leaf for a prolonged time (five caterpillars, three days).

Volatile Analysis. The composition and quantification of the volatile blend emitted by the plant–herbivore complex (PHC), systemically induced and damaged (SYS+D) leaves, or mechanically damaged (MD) leaves of Brussels sprouts plants are depicted in Figure 3. The profiles of systemically induced and caterpillar infested leaves were almost identical, although systemically induced leaves emitted smaller amounts of (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate. Mechanically damaged leaves emitted less (*Z*)-3-hexenyl acetate than infested leaves and did not emit (*Z*)-3-hexen-1-ol or (*E*)-β-caryophyllene.

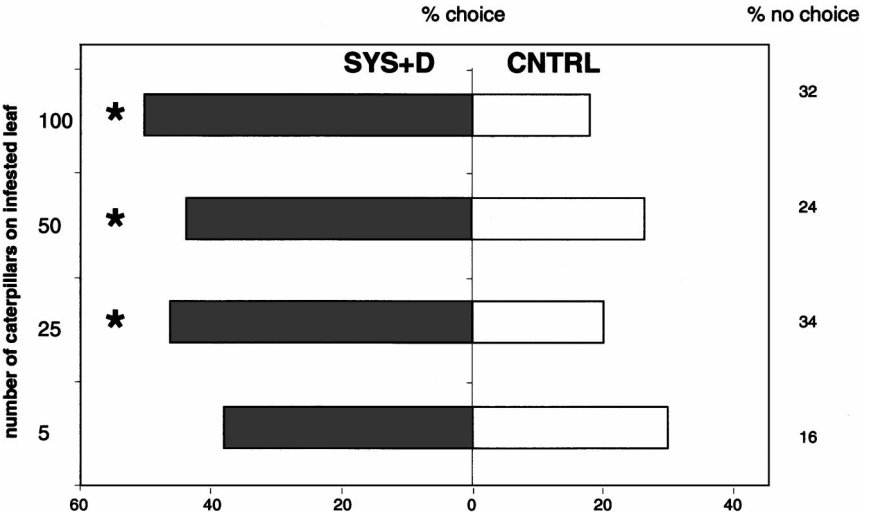


FIG. 2. Level of infestation necessary to obtain systemic induction of volatiles in 8-week-old Brussles sprouts plants. Bars indicate the choice response of female *C. glomerata* to upper leaves of plants that were infested on the lowest leaf by different numbers of *P. brassicae* caterpillars (5–100) for three days. Asterisks indicate significant differences within the choice test at $P < 0.05$ [chi-square test for goodness-of-fit; (Sokal and Rohlf, 1981)]. SYS+D = systemically induced and damaged; CNTRL = noninduced and mechnically damaged. (sample size = 50 per treatment). See Methods and Materials for further explanations.

The composition and quantification of the volatile blend emitted by systemically induced and damaged leaves (SYS+D), systemically induced and undamaged leaves (SYS), or noninduced and undamaged leaves (UND) of Brussels sprouts plants are given in Table 1. Systemically induced but undamaged leaves emit a volatile profile that is similar to that of noninduced undamaged leaves. Compared to undamaged leaves, systemically induced and damaged leaves are characterized by increased amounts of green leaf alcohols (1-hexanol, (*Z*)-3-hexen-1-ol), green leaf esters [(*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl butyrate, (*Z*)-3-hexenyl isovalerate] and sabinene. Interestingly, in both sets of analyses, α-humulene and (*E*)-β-caryophyllene appear to be the only terpenoids specifically associated with induced responses.

DISCUSSION

Brussels sprouts plants, attacked by *Pieris brassicae* caterpillars, emit volatiles that attract a natural antagonist of the herbivore, the parasitoid *Cotesia*

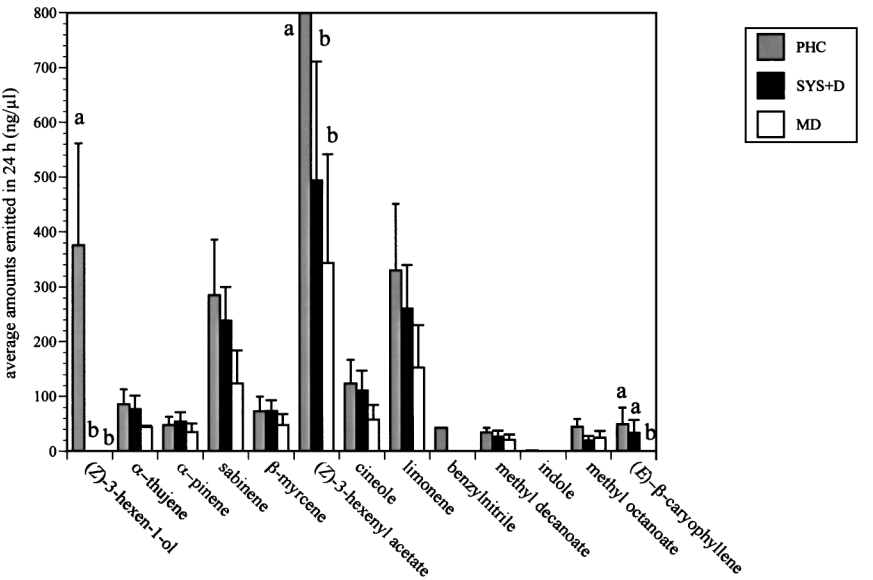


FIG. 3. Quantification of major volatile blends emitted during 24 hr by two leaves of Brussels sprouts plants that were either infested by caterpillars (PHC = plant–herbivore complex), systemically induced and damaged (SYS+D), or mechanically damaged (MD). Samples were analyzed by manual thermaldesorption. Compounds are listed based on their retention time. Different letters close to bars indicate significant differences at $P < 0.05$ (Student-Neuman-Keuls test). The concentration of (*Z*)-3-hexenyl acetate [(avg. 3608 ± 2076 (SE) ng/μl/24 hr)] emitted by PHC has been overscaled to show minor compounds. Statistics and error bars are presented only for compounds that appeared in more than one treatment.

glomerata. Chemical and behavioral experiments show that the induced emission of volatiles is not limited to the site of damage, but occurs throughout the plant. In Brussels sprouts, the systemic emission of volatiles appears only when the surface of uninfested leaves is damaged (Figure 1B). This contrasts with induced responses observed in beans, cassava, and corn, where undamaged leaves of herbivore-infested plants release detectable amounts of volatiles that are attractive to the natural enemies of the herbivores (Table 2). In previous experiments, we observed that detached cabbage leaves that were incubated in a solution of caterpillar gut regurgitate released a volatile blend similar to that of herbivore-damaged plants. The release was detectable only if the leaf surface were wounded prior to bioassay (Mattiacci et al., 1994) . Similarly, leaves that were incubated in a solution of β-glucosidase, an elicitor of volatile emission present in caterpillar salivary secretions, released detectable amounts of volatiles only after mechanical damage

TABLE 1. VOLATILE BLEND EMITTED BY SYSTEMICALLY INDUCED AND DAMAGED (SYS+D), SYSTEMICALLY INDUCED AND UNDAMAGED (SYS_UND) OR NONINDUCED AND UNDAMAGED (UND) LEAVES OF BRUSSELS SPROUTS[a]

Compounds	Odor sources		
	SYS+D	SYS_UND	UND
Aldehydes			
Nonanal	tr	tr	tr
Decanal	37.0 ± 16.2	102.3	73.6 ± 23.2
Benzaldehyde	13.3 ± 4.0	20.8 ± 1.5	12.5 ± 2.3
Alcohols			
1-hexanol	tr		
(*Z*)-3-Hexen-1-ol	90.7 ± 11.2		
Esters			
(*Z*)-3-Hexenyl acetate*	645.8 ± 56.5	153.1 ± 37.7	57.5 ± 11.3
(*Z*)-3-Hexenyl butyrate	23.3 ± 4.1		
(*Z*)-3-Hexenyl isovalerate	27.7 ± 7.6		
Terpenoids			
α-Thujene	169.2 ± 15.1	149.6 ± 21.5	90.7 ± 11.6
α-Pinene	101.5 ± 6.2	99.0 ± 10.1	52.5 ± 5.2
Sabinene*	878.4 ± 75.3	495.9 ± 54.0	377.5 ± 47.4
β-Myrcene	227.8 ± 21.1	169.0 ± 20.0	102.1 ± 11.1
Cineole	331.0 ± 45.3	229.6 ± 37.4	151.7 ± 15.5
Limonene	1030.8	1095.1	tr
(*E*)-β-Caryophyllene	433.6 ± 140.8	441.3	
α-Humulene	25.2 ± 6.4	41.6	
3-Carene	88.5 ± 11.4		83.0 ± 10.0
α-Terpinene	73.7 ± 7.2	90.3 ± 22.5	45.1 ± 7.5

[a] Average amounts (ng ± SE) were calculated based on the peak area of an internal standard (hexylbenzene). Absolute amount is given for compounds that were detected only in one sample. $N = 5$ for each treatment. *Significant effect of plant treatment (Kruskal-Wallis one-way ANOVA on ranks, $P < 0.05$). Samples were analyzed by automatic thermaldesorption. Statistics are presented only for compounds that appeared in more than one treatment. tr = trace compounds.

(Mattiacci et al., 1995). This indicates that in cabbage a mechanical damage is needed either to release volatiles stored in the induced leaf and/or to activate their precursors.

Induced direct defenses to herbivores have been found in response to both mechanical damage and actual herbivory on plants (Edwards and Wratten, 1987; Alborn et al., 1996; McCloud and Baldwin, 1997). Comparatively indirect defenses, such as the emission of plant volatiles attracting predatory and parasitic arthropods, have been associated with mechanical damage and with herbivory, albeit with different levels of specificity. The majority of plant species studied so far, emit, at the site of herbivory, volatiles that are different from those of

TABLE 2. COMPARISON OF KNOWN MECHANISMS OF EMISSION OF SYSTEMICALLY INDUCED VOLATILES IN DIFFERENT PLANT SPECIES[a]

Plant	Time of emission after herbivore injury	Mechanical damage necessary?	Volatiles emitted at the site of damage	Systemically emitted volatiles	References
Brussels sprouts	3 days	Yes	GLVs Cyclic monoterpenes Sesquiterpenes Nitriles, isothiocyanates	(*Z*)-3-Hexenyl acetate, butyrate, Isovalerate Cyclic monoterpenes Sesquiterpenes	Mattiacci et al. (1994)
Broad beans	2–3 days	No	GLVs Acyclic monoterpenes Sesquiterpenes	n.a.	Du et al. (1998), Guerrieri et al. (1999)
Cassava	3 days	No	n.a.	n.a.	Souissi et al. (1998)
Cotton	3–4 days	No	GLVs Cyclic + acyclic monoterpenes Sesquiterpenes Homoterpenes Indole	(*Z*)-3-Hexenyl acetate Acyclic monoterpenes Sesquiterpenes Homoterpenes	Röse et al. (1996, 1998)
Lima beans	7 days	No	GLVs Acyclic monoterpenes Homoterpenes Sesquiterpenes Methyl salicylate Indole	GLVs Acyclic monoterpenes Homoterpenes Sesquiterpenes Methyl salicylate Indole	(Dicke et al. 1990, 1998[b])
Corn	6–8 hr	No	GLVs Cyclic + acyclic monoterpenes Sesquiterpenes Homoterpenes Indole	(*Z*)-3-hexenyl acetate Acyclic monoterpenes Sesquiterpenes Homoterpenes Indole	Turlings and Tumlinson (1992)

[a]GLVs = green leaf volatiles; n.a. = identification of volatiles not available.
[b]Systemic induction elicited through application of jasmonic acid and not by herbivore feeding.

mechanically damaged plant parts. The composition of herbivore-induced volatile blends might vary even with different herbivore species or with different larval instars attacking the same plant (reviewed by Dicke and Vet, 1999; Paré and Tumlinson, 1999; Hern and Dorn, 2001). In contrast, some species emit qualitatively similar chemical blends, irrespective of the damaging agent (Dicke and Vet, 1999). In Brussels sprouts, there is no striking qualitative difference in GC profiles produced in response to herbivory or mechanical damage (Agelopoulos and Keller, 1994; Mattiacci et al., 1994), and it is, therefore, not surprising that volatiles associated with mechanical damage are an important signal for *C. glomerata* (see also Steinberg et al., 1993). In general, the ecological function of volatiles associated with mechanical damage should not be overlooked, as generalist carnivores might use volatiles associated with mechanical damage even when the volatile profiles of herbivore-infested and mechanically damaged plants differ. For example, a generalist parasitoid, *Cotesia marginiventris*, is attracted to volatiles typically emitted by artificially damaged cotton plants, while a specialist, *Microplitis croceipes*, is attracted to volatiles typically induced by herbivores (Röse et al., 1998).

While in cabbage the systemic response is detectable only upon mechanical damage, and in corn the response is detectable without damage, cotton seems to have an intermediate mechanism of response. In cotton, inducible terpenoids and (*Z*)-3-hexenyl acetate are released from undamaged, systemically induced leaves, while several hexenyl butyrates, known to be associated with caterpillar herbivory, are emitted only upon mechanical damage. In addition, the amounts of constitutive compounds emitted by systemically induced and mechanically damaged leaves are larger than those emitted by systemically induced undamaged leaves (Röse et al., 1996).

Time-course experiments on the emission of carnivore-attracting volatiles are available only for corn, cotton, and broad bean (Table 2). Different plant species express a systemic response after a variable time lag from initiation of herbivore feeding, ranging from a few hours (corn) to several days (lima bean). The observed delay in the systemic release could be due to the capability of plants to minimize the costs of defense by activating a systemic response only upon higher levels of herbivore damage or prolonged periods of feeding. Theory predicts that inducible defenses should allow plants to allocate limited resources so as to maximize fitness in response to current unpredictable conditions (see for review Karban and Baldwin, 1997). Broad bean plants emit systemically induced volatiles only when attacked above a certain density of *Acyrthosiphon pisum* aphids and only after 48–72 hr of infestation (Guerrieri et al., 1999). Brussels sprouts seem to minimize the cost of defense even more effectively. Not only are the systemically induced volatiles detectable after at least three days of continuous feeding, but when no further attack occurs (e.g., when caterpillars are removed), the volatile emission stops after one day (see Figure 1). In addition, a systemically induced response does not occur if few caterpillars (5) are feeding on a leaf (Figure 2). Thus, the plant

does not invest in an induced defense when the attack is of short duration or of low intensity. In addition, as no attractive induced volatiles are emitted without damage on the systemically induced parts of the plant, it is likely that the metabolites stored in the induced leaves are reutilized for other biosynthetic needs.

The chemical structures of the volatile compounds emitted by arthropod-damaged leaves have notable similarities in all plants reported so far (Table 2). This structural uniformity has been suggested as evidence for the activation of a common set of biosynthetic pathways shared by a wide range of plant families and used by a broad spectrum of insect parasitoids and predators (Paré and Tumlinson, 1999).

The volatiles emitted upon herbivory have been categorized into three basic groups (Paré and Tumlinson, 1996): lipoxygenase-derived green leaf volatiles, isoprenoid-derived terpenoids, and shikimic acid-derived aromatics such as indole and methyl salicylate. Green leaf volatiles such as (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexenyl acetate are emitted at the site of herbivory by several plants, including Brussels sprouts, corn, cotton, and beans (Table 2). Remarkably, in corn, cotton, and our samples, (*Z*)-3-hexenyl acetate is the only lipoxygenase product that is systemically released by uninfested but undamaged leaves of infested plants. Based on these results, it has been hypothesized that lipoxygenase products need to be converted to the acetate form to be emitted in induced leaves (Paré and Tumlinson, 1999). In our samples, other green leaf esters (Figure 3 and Table 1) are probably associated with fresh mechanical damage, since these compounds are not emitted by systemically induced and undamaged leaves. In addition, systemically induced leaves emit esters in amounts not substantially different from mechanically damaged leaves.

The terpenoids detected in herbivore-damaged plants are products of more than one biosynthetic route or different steps within one pathway (Paré and Tumlinson, 1996). A flexible management of these defensive compounds has been recently elucidated in lima bean. The production of induced terpenoids is regulated by two pathways, of which one is mevalonate-dependent and the other is mevalonate-independent (deoxyxylulose pathway). The mevalonate-independent pathway appears to be important in the release of inducible monoterpenes after elicitation with jasmonic acid (Piel et al., 1998), which has a similar effect as herbivore infestation (Dicke et al., 1998). In contrast, constitutive compounds are synthesized through the mevalonate-dependent pathway and need no elicitation for emission by leaves or flowers (Piel et al., 1998).

In cotton, the acyclic monoterpene β-ocimene, the sesquiterpenes α- and β-farnesene, and the homoterpene (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene are systemically released by herbivore-induced plants. Cyclic and acyclic monoterpenes, such as α- and β-pinene, limonene, and myrcene, and the sesquiterpene (*E*)-β-caryophyllene are released at the site of damage, but not systemically (reviewed by Paré and Tumlinson, 1999). In contrast to cotton, Brussels sprouts emit

α- and β-pinene, limonene, myrcene, sabinene, and (*E*)-β-caryophyllene both systemically and at the site of damage (Figure 3).

The acyclic homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene have been repeatedly identified in the headspace volatiles of herbivore-infested plants, including lima bean, apple, cowpea, cucumber, corn, and cotton (see, e.g., Turlings et al., 1991; Dicke, 1994; Loughrin et al., 1994; Bouwmeester et al., 1999). Therefore, these compounds have been suggested as a general signal of insect infestation (Dicke, 1994). However, virtually no homoterpenes and only trace amounts of indole, a derivative of the shikimic acid pathway, were detected in our study or in previous analyses of cabbage headspace volatiles (Blaakmeer et al., 1994; Mattiacci et al., 1994; Geervliet et al., 1997).

These differences among the systemically induced responses of different species led to the hypothesis that plants might utilize a defensive strategy that is more complex than a common set of biosynthetic pathways shared by a range of plant families. In fact, specific pathways might be activated based on the available "defensive arsenal" of inducible chemicals of a certain plant species (Figure 4). Accordingly, some plant species might utilize different pathways to produce different sets of compounds, of which some are used for local and some for systemic defense.

This could be the case in cotton, where cyclic monoterpenes are emitted locally and acyclic monoterpenes are emitted systemically. Other plants might utilize

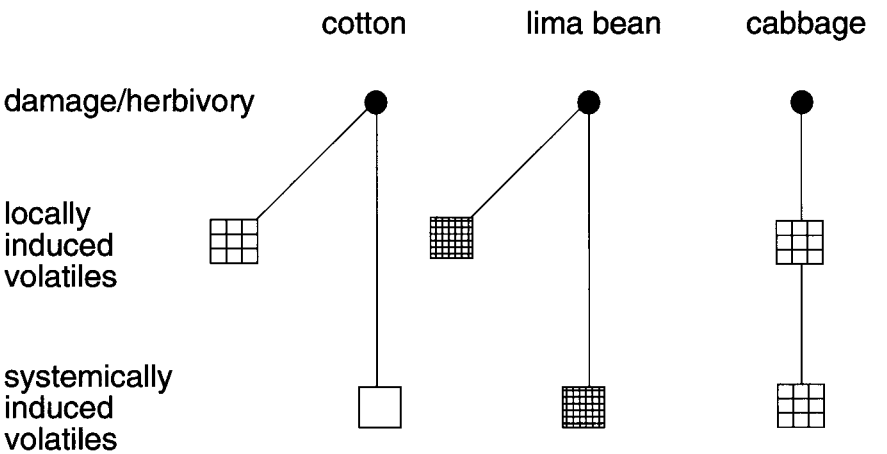


FIG. 4. A hypothesis of flexible use of inducible pathways for the biosynthesis of locally and systemically emitted plant volatiles. Black dots indicate damage or herbivory as the phenomenon triggering volatile emission. Lines indicate biosynthetic pathways. Different squares indicate different chemical compounds emitted by plants.

different pathways to produce the same compounds, for example, the acyclic homoterpenes in lima beans. Other species, with limited biosynthetic capabilities for inducible defensive compounds could be expected to use the same compounds—and possibly the same pathway—at all levels of defense, local and systemic. It would be interesting to investigate whether this is the case for locally and systemically induced cyclic terpenoids in Brussels sprouts.

Understanding the complexity of the changes occurring in plants after herbivore attack is in its infancy. More investigation on the systemically induced volatile emission of different species will prove useful in shedding light on the ecological significance and regulation mechanisms of these defensive responses in plants.

Acknowledgments—We are grateful to R. Camenzind, T. Christoffel, A. Dutton, C. Fornallaz, M. Gandolfi, H. Gu, M. Hollenstein, R. de Jong, and S. Töpfer for help with insect rearing, data analysis, and useful comments on a previous version of the manuscript. We thank J. Tumlinson and H. Alborn for help with identification of homoterpenes.

REFERENCES

AGELOPOULOS, N. G. and KELLER, M. A. 1994. Plant-natural enemy association in tritrophic system, *Cotesia rubecula–Pieris rapae* (Brassicaceae Cruciferae). III: Collection and identification of plant and frass volatiles. *J. Chem. Ecol*. 20:1955–1967.

AGELOPOULOS, N. G., HOOPER, A. M., MANIAR, S. P., PICKETT, J. A., and WADHAMS, L. J. 1999. A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. *J. Chem. Ecol*. 25:1411–1425.

AGRAWAL, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 279:1201–1202.

ALBORN, H. T., RÖSE, U. S. R., and MCAUSLANE, H. J. 1996. Systemic induction of feeding deterrents in cotton plants by feeding of *Spodoptera* spp. larvae. *J. Chem. Ecol*. 22:919–932.

BALDWIN, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc. Natl. Acad. Sci. USA* 95:8113–8118.

BLAAKMEER, A., GEERVLIET, J. B. F., VAN LOON, J. J. A., POSTHUMUS, M. A., VAN BEEK, T. A., and DE GROOT, A. E. 1994. Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: Consequences for in-flight host location by *Cotesia* parasitoids. *Entomol. Exp. Appl*. 73:175–182.

BOUWMEESTER, H. J., VERSTAPPEN, F. W. A., POSTHUMUS, M. A., and DICKE, M. 1999. Spider mite-induced (3*S*)-(*E*)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. *Plant Physiol*. 121:173–180.

BRODEUR, J. and GEERVLIET, J. B. F. 1992. Host species affecting the performance of the larval parasitoid *Cotesia glomerata* and *Cotesia rubecula* (Hymenoptera: Braconidae). I. Preference for host developmental stage of *Pieris* (Lepidoptera: Pieridae). *Med. Fac. Landb. Univ. Gent*. 57/2B:543–545.

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant–carnivore mutualism. *J. Plant Physiol*. 43:465–472.

DICKE, M. 1999. Evolution of induced indirect defense of plants, pp. 62–88, *in* R. Tollrian and, C. D. Harvell (eds.). The Ecology and Evolution of Inducible Defenses. Princeton University Press, Princeton, New Jersey.

DICKE, M. and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool*. 38:148–165.

DICKE, M. and VET, L. E. M. 1999. Plant-carnivore interactions: Evolutionary and ecological consequences for plant, herbivore and carnivore, pp. 483–520, *in* H. Olff, V. K. Brown, and R. H. Drent (eds.). Herbivores Between Plants and Predators. Blackwell Science.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990. Plant strategies of manipulating predator-prey interactions through allelochemicals: Prospects for application in pest control. *J. Chem. Ecol.* 16:3091–3118.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1998. Jasmonate and herbivory differentially induce carnivore-attracting plant volatiles. *J. Chem. Ecol*. 25:1907–1922.

DU, Y., POPPY, G. M., POWELL, W., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1998. Identification of semiochemicals released during aphid feeding that attract parasitoid. *Aphidius ervi*. *J. Chem. Ecol.* 24:1355–1367.

EDWARDS, P. J. and WRATTEN, S. D. 1987. Ecological significance of wound-induced changes in plant chemistry, pp. 213–219, *in* V. Labeyrie, G. Fabres, and D. Lachaise (eds.). Insects–Plants. Dr. W. Junk Publishers, Dordrecht, The Netherlands.

GEERVLIET, J. B. F., POSTHUMUS, M. A., VET, L. E. M., and DICKE, M. 1997. Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *J. Chem. Ecol*. 23:2935–2954.

GU, H. and DORN, S. 2000. Genetic variation in behavioural response to herbivore infested plants in the parasitic wasp *Cotesia glomerata*. *J. Insect Behav.* 13:141–157.

GUERRIERI, E., POPPY, G. M., POWELL, W., TREMBLAY, E., and PENNACCHIO, F. 1999. Induction and systemic release of herbivore-induced plant volatiles mediating in-flight orientation of *Aphidius ervi*. *J. Chem. Ecol*. 25:1247–1261.

HEATH, R. R. and MANUKIAN, A. 1994. An automated system for use in collecting volatile chemicals released from plants. *J. Chem. Ecol*. 20:593–608.

HERN, A. and DORN, S. 2001. Induced emissions of apple fruit volatiles by the codling moth: Changing patterns with different time periods after infestation and different larval instars. *Phytochemistry* 57:409–416.

KARBAN, R. and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, Illinois.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., and TURLINGS, T. C. J. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. *Proc. Natl. Acad. Sci. USA* 91:11836–11840.

LOUGHRIN, J. H., POTTER, D. A., HAMILTON KEMP, T. R., and BYERS, M. E. 1997. Diurnal emission of volatile compounds by Japanese beetle-damaged grape leaves. *Phytochemistry* 45:919–923.

MALECK, K. and DIETRICH, R. A. 1999. Defense on multiple fronts: How do plants cope with diverse enemies? *Trends Plant Sci*. 4:215–219.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1994. Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of *Pieris brassicae* larvae: Role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20:2229–2247.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1995. β-glucosidase: An elicitor of herbivore induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92:2036–2040.

MCCLOUD, E. S. and BALDWIN, I. T. 1997. Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris. Planta* 203:430–435.

MEINERS, T. and HILKER, M. 1997. Host location in *Oomyzus gallerucae* (Hymenoptera: Eulophidae), an egg parasitoid of the elm leaf beetle *Xanthoglaeruca luteola* (Coleoptera: Chrysomelidae). *Oecologia* 112:87–93.

MEINERS, T. and HILKER, M. 2000. Induction of plant synomones by oviposition of a phytophagous insect. *J. Chem. Ecol.* 26:221–232.

PARÉ, P. W. and TUMLINSON, J. H. 1996. Plant volatile signals in response to herbivore feeding. *Fla. Entomol.* 79:93–103.

PARÉ, P. W. and TUMLINSON, J. H. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol*. 121:325–331.

PIEL, J., DONATH, J., BANDEMER, K., and BOLAND, W. 1998. Mevalonate-independent biosynthesis of terpenoid volatiles in plants: Induced and constitutive emission of volatiles. *Angew. Chem. Int. Ed.* 37:2478–2481.

POTTING, R. P. J., VET, L. E. M., and DICKE, M. 1995. Host microhabitat location by stem-borer parasitoid *Cotesia flavipes*: The role of herbivore volatiles and locally and systemically induced plant volatiles. *J. Chem. Ecol*. 21:525–539.

RÖSE, U. S. R., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1996. Volatile semiochemicals released from undamaged cotton leaves: A systematic response of living plants to caterpillar damage. *Plant Physiol*. 111:487–495.

RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1998. Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. *J. Chem. Ecol.* 24:303–319.

SOKAL, R. R. and ROHLF, F. J. 1981. Biometry. W. H. Freeman and Co, New York.

SOUISSI, R., NENON, J. P., and LE RU, B. 1998. Olfactory responses of parasitoid *Apoanagyrus lopezi* to odor of plants, mealybugs, and plant-mealybug complexes. *J. Chem. Ecol.* 24:37–48.

STEINBERG, S., DICKE, M., VET, L. E. M., and WANNINGEN, R. 1992. Response of the braconid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: Effects of bioassay set-up, parasitoid age and experience and barometric flux. *Entomol. Exp. Appl*. 63:163–175.

STEINBERG, S., DICKE, M., and VET, L. E. M. 1993. Relative importance of infochemicals from first and second trophic level in long-range host location by the larval parasitoid *Cotesia glomerata*. *J. Chem. Ecol.* 19:47–60.

THALER, J. S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399:686–688.

TURLINGS, T. C. J. and TUMLINSON, J. H. 1992. Systemic releases of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. USA* 89:8399–8402.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., TUMLINSON, J. H., HEATH, R. R., PROVEAUX, A. T., and DOOLITTLE, R. E. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

PINE WEEVIL (*Hylobius abietis*) ANTIFEEDANTS FROM LODGEPOLE PINE (*Pinus contorta*)

KATHARINA BRATT,[1] KERSTIN SUNNERHEIM,[1,*] HENRIK NORDENHEM,[2] GÖRAN NORDLANDER,[2] and BO LÅNGSTRÖM[2]

[1]*Institute of Chemistry*
Uppsala University
Box 531, SE-751 21 Uppsala, Sweden

[2]*Department of Entomology*
Swedish University of Agricultural Sciences
Box 7044, SE-750 07 Uppsala, Sweden

(Received September 7, 2000; accepted July 1, 2001)

Abstract—Pine weevils (*Hylobius abietis*) fed less on bark of lodgepole pine (*Pinus contorta*) than on bark of Scots pine (*P. sylvestris*). Two pine weevil antifeedants, ethyl *trans*-cinnamate and ethyl 2,3-dibromo-3-phenyl-propanoate, were isolated from bark of lodgepole pine. These two compounds significantly reduced pine weevil feeding in a laboratory bioassay. In field assays, the second compound significantly decreased pine weevil damage on planted seedlings. Ethyl 2,3-dibromo-3-phenylpropanoate has not previously been reported as a natural product.

Key Words—Antifeedant, ethyl 2,3-dibromo-3-phenylpropanoate, ethyl *trans*-cinnamate, *Hylobius abietis*, lodgepole pine, pine weevil, *Pinus contorta*, *Pinus sylvestris*, Scots pine.

INTRODUCTION

Seedling mortality resulting from stem-feeding by adult *Hylobius* weevils (*Col., Curculionidae*) is a major silvicultural problem wherever clear-felling followed by planting of conifer seedlings is practiced in temperate regions of the Northern Hemisphere. All over Europe, the pine weevil *Hylobius abietis* (L.) is the principal noxious species, and seedling mortality due to this insect leads to considerable economic loss. Permethrin has been used to control pine weevil damage in

*To whom correspondence should be addressed. E-mail: ks@kemi.uu.se

0098-0331/01/1100-2253$19.50/0

Sweden, but the use of insecticides for this purpose will soon be prohibited. Alternative measures to manage the pine weevil problem are, therefore, urgently needed.

There is strong evidence that plants have evolved defense systems against predators and that these systems, to a large extent, are based on the production of secondary metabolites (Rosenthal, 1986; Harborne, 1988, and references therein; Sunnerheim-Sjöberg, 1991; Daurade-Le Vagueresse and Bounias, 1992; Suga et al., 1993). Even high priority food plants produce defense compounds against herbivores—presumably to prevent overbrowsing. Although the polyphagous pine weevil prefers pines to a multitude of other plant species (Eidmann, 1974), pines can be expected to contain some antifeedants against pine weevils. However, different species of pines and different parts of a pine tree may contain varying amounts of defense compounds.

Preliminary feeding tests indicated that pine weevils fed less on bark from lodgepole pine, *Pinus contorta*, than on bark from Scots pine, *P. sylvestris*. This result prompted us to look for compounds possessing antifeedant activity derived from *P. contorta*. Scots pine is the most abundant native pine species in Sweden. Lodgepole pine has been introduced from North America and planted on large areas in northern Sweden.

The aims of this study were to determine if compounds (either endogenous or spontaneously modified) possessing antifeedant activity could be isolated from coniferous hosts, and if these compounds could be exploited to reduce weevil damage. We:

- compared the feeding preference of *H. abietis* for bark from Scots and Lodgepole pine;
- isolated, identified, and bioassayed two antifeedants, ethyl *trans*-cinnamate (**1**) and ethyl 2,3-dibromo-3-phenylpropanoate (**2**), from Lodgepole pine;
- performed syntheses and field trials of compound (**2**).

METHODS AND MATERIALS

Plant Material. Stem samples of lodgepole and Scots pine were collected from plantations in northern Uppland (seed sources unknown). All fractions and extracts were subjected to feeding trials, and the results were used for selection of fractions for further fractionation. Fresh inner bark (500 g) was cut into small pieces and extracted in methanol for 24 hr. The extract was filtered, and the plant residue was discharged. The methanol was evaporated, keeping the temperature below 40°C. Water (200 ml) was added, and the suspension extracted with pentane (2×150 ml) followed by ethyl acetate (2×150 ml). The pentane and ethyl acetate fractions were dried over $MgSO_4$, and solvent was evaporated. Extracts of *P. sylvestris* were obtained in the same manner as described for *P. contorta*.

The ethyl acetate extract (8.7 g) was dissolved in ethanol (99.7%, 15 ml) and subsequently fractionated on Sephadex LH-20 using water (250 ml), water–ethanol (7 : 3, 400 ml), water–ethanol (4 : 6, 400 ml), and finally ethanol (99.7%, 400 ml) as eluent. Seven fractions were collected, and the solvent was evaporated (12.1 g, 1.80 g, 1.93 g, 0.33 g, 0.08 g, 0.07 g, and 0.03 g, respectively).

Fraction 2 was dissolved in ethyl acetate (200 ml) and extracted with acid (0.1 M HCl, 2 × 100 ml) followed by base [0.1 M Na_2CO_3 (aq), 2 × 100 ml]. The neutral fraction (0.154 g) was further fractionated on silica using CH_2Cl_2 : $CHCl_3$: MeOH : H_2O (80 : 11 : 8 : 1) as eluent. Four fractions were collected.

Spectroscopy. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.5 MHz) spectra were recorded on a Varian Unity 400 using residual solvent peaks as internal standards.

Chromatography. GC-MS was performed on a Finnigan MAT GCQ Plus system (EI; 70 ev). Thin-Layer chromatography was performed on Merck HF-254 silica gel plates. Merck Kieselgel 60 (230–400 mesh) or Sephadex LH-20 (Pharmacia Fine Chemicals) was used for column chromatography. HPLC was performed on a Waters chromatograph equipped with an autosampler and a diode array detector. Column: LichroCart 125-4 Lichrospher 100 RP-18 (5 μm) (Merck). The mobile phase consisted of solvent A: ammonium formiate buffer pH 3.4, and solvent B: acetonitrile. The gradient program was 0–30 min 100% A to 40% followed by 5 min at 40%, and 35–40 min 40% A to 10%. The flow rate was 1 ml/min.

Isolated Compounds. Ethyl *trans*-cinnamate, [(*E*)-3-phenyl-2-propenoic acid ethyl ester, **1**, 2 mg)] was the main compound in the first fraction, identified by GC-MS and NMR (spectral data were in accordance with Cristau and Taillefer, 1998). Ethyl 2,3-dibromo-3-phenylpropanoate (**2**, 5 mg) was the main compound in the second fraction, identified by GC-MS and NMR (spectral data were in accordance with Kabalka et al., 1998).

Synthesis of Ethyl 2,3-Dibromo-3-Phenylpropanoate (***2***). A racemic mixture of (2*R*,3*S*)- and (2*S*,3*R*)-**2** was prepared by bromine addition to **1** according to standard procedures (Vogel, 1989). Spectral and chromatographic data for the synthesized compound were identical to those obtained from the isolated ones.

Ethyl *trans*-cinnamate was purchased from Aldrich (99%). Bromine (Aldrich). Ethanol (ETAX 99.7 vol-%). Acetonitrile HPLC-grade (Aldrich). Pentane, ethyl acetate, and dichloromethane were dried and distilled according to standard procedures.

Extracts were tested in concentrations slightly higher than the naturally occurring amounts. The amounts to be tested were calculated with the equation: $(E \times B_t \times 4)/B_e$ where E is the dry weight of extract, B_t is the dry weight of extracted bark, B_e is the estimated dry weight of test-bark, and 4 is a factor compensating for losses during the fractionation and the dilution by distribution of compounds over several fractions, and hopefully increases the concentration to slightly above natural.

Test of Host Preference. Pine weevil feeding preference was studied in a two-choice laboratory test. One pine weevil was placed in each of 20 boxes, together with one fresh twig of each of the two pine species. After three days, the feeding area on the twigs was measured with the aid of 1-mm-grid graph paper.

Bioassay. Extracts, fractions thereof, and solutions of single compounds were presented to single pine weevils in two-choice laboratory tests with a treatment and a control area. The bioassay was designed to allow use of small test samples applied on a specified area of bark, i.e., the amount of substance per unit area can be accurately calculated (Nordlander et al., 2000). Fresh pieces of Scots pine twigs (50 mm long, ca. 15 mm diam.) were split, and each half (= test twig) was wrapped in aluminum foil. In each test twig, two metal rings (5 mm diameter) were punched through the foil and into the bark at 25 mm distance. After removal of the aluminum foil inside the ring, 100 μl of the extract/compound to be tested were applied on the bark in one of the two rings. In the other ring, the same amount of the solvent was applied (control). After the solvent had evaporated, the metal rings were removed. Each test twig was placed on moist filter paper in a 142-mm-diam Petri dish with one weevil in each dish. Forty replicates were used, 20 with females and 20 with males. Generally, there was no significant difference in response between the sexes, and, therefore, the data presented are pooled. Each weevil was used only once. The weevils were all in the reproductive phase of their life cycle and were starved for 24 hr before the test period. The bioassays were conducted at room temperature (ca. 22°C).

The amount of feeding on the treatment and control area of each test twig was recorded after 6 and 24 hr. The test areas were commonly either not fed upon or completely consumed before the end of the 24 hr test period. Therefore, we chose to present data based only on the presence or absence of feeding scars, which is a more conservative measure of antifeedant effect than the amount of consumed bark. The effect of the various treatments on the initiation of feeding is described by the index: $[(C - T) \times 100]/(C + T)$ where C is the number of control surfaces with feeding scars and T the number of treated surfaces with feeding scars ($N = 40$). Thus, an antifeedant effect gives positive values up to a maximum of 100. Statistical differences between treatment and control were tested with a chi-square test of a 2×2 table (not continuity corrected): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (null hypothesis: no difference).

Field Test. The antifeedant effect of ethyl 2,3-dibromo-3-phenylpropanoate applied on stems of Norway spruce seedlings was assessed in a field test. The active substance was dissolved in methanol and to this solution a liquid wax (AGS 3512 Ultra, Trion Tensid AB, Uppsala, Sweden) was added in the proportion 3 : 1 methanol–AGS), giving 0.056 g active substance per ml of the final mixture. About 2 ml of this mixture (containing 0.11 g active substance) were applied on each seedling in a way that it completely covered the lower half of the stem. Seedlings treated this way were compared with seedlings similarly treated

with the same methanol–AGS mixture without the active substance and with untreated seedlings. A randomized block design was used with each of the 80 blocks containing one seedling of each of the three treatments. The distance between seedlings within a block was about 0.5 m, and the distance between blocks about 2 m. The seedlings were planted on a fresh clear-cutting 13 km NNE Uppsala, Sweden, on June 11, 1999. The amount of pine weevil feeding was recorded for each seedling after 19 and 36 days.

RESULTS

Pine weevils fed about twice as much on *P. sylvestris* twigs as on *P. contorta*. The mean (±SE) bark area consumed per weevil during three days was 43.0 ± 5.1 mm^2 for *P. sylvestris* and 23.0 ± 4.9 mm^2 for *P. contorta* ($P = 0.026$, paired *t* test).

The methanol extracts of the two pine species were divided into ethyl acetate, pentane, and water. The water extracts of both species showed a strong stimulating effect on feeding (Figure 1), whereas the pentane extracts slightly reduced feeding. The ethyl acetate extracts of *P. contorta* had a significant antifeedant effect. In contrast, no significant effect on feeding was found for the EA extract of *P. sylvestris*.

The ethyl acetate extract from *P. contorta* was subjected to Sephadex LH-20 chromatography, and seven fractions were collected. Fraction 2 was the only fraction found to possess significant antifeedant activity and was, therefore, chosen for further fractionation. It was divided into acidic, basic, and neutral phases by

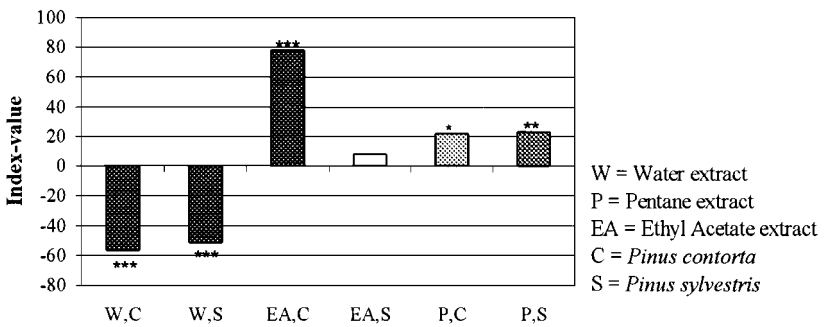


FIG. 1. Results of bioassays of water, ethyl acetate, and pentane extracts of *P. contorta* and *P. sylvestris* after 6 hr of feeding. Note that positive index values indicate antifeedant effect and negative values a stimulating effect on feeding. Shading of the columns highlights significance levels. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

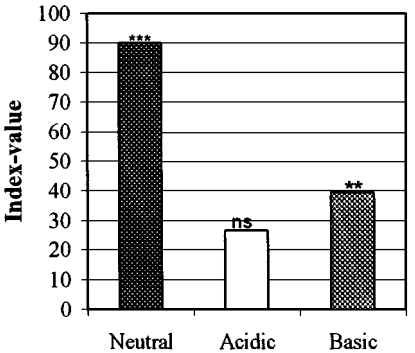


FIG. 2. Results of bioassays of the acidic, basic, and neutral fractions after 6 hr. $*P < 0.01$; $**P < 0.001$.

extraction. All fractions tended to reduce feeding, and a strong antifeedant effect was found for the neutral extract (Figure 2).

The neutral fraction consisted of three major constituents and some minor ones. Two of the major compounds were identified as ethyl *trans*-cinnamate (**1**) and ethyl 2,3-dibromo-3-phenylpropanoate (**2**) (Figure 3). The other compounds in the mixture were not identified.

Both ethyl *trans*-cinnamate and ethyl 2,3-dibromo-3-phenylpropanoate showed high antifeedant activity in the bioassays. After 24 hr the activity was still high for both compounds (Figure 4).

To ensure that 2,3-dibromo-3-phenylpropanoate emanated from the pine bark and was not an artifact, a new methanol extract of *P. contorta* was made. This extract was divided into pentane, ethyl acetate, and water as described above, and the ethyl acetate phase was analyzed using HPLC equipped with a diode array detector; synthetic 2,3-dibromo-3-phenylpropanoate was used as reference compound. A peak with retention time and UV spectrum identical to the reference compound was obtained. A new extract of *P. sylvestris* was treated the same way, and no ethyl 2,3-dibromo-3-phenylpropanoate could be detected.

Field Test. In field tests, ethyl 2,3-dibromo-3-phenylpropanoate significantly reduced the amount of pine weevil feeding on spruce stems (Table 1). After five

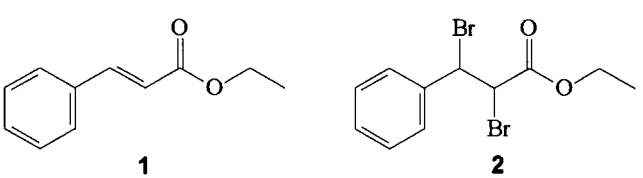


FIG. 3. Structures of ethyl *trans*-cinnate and ethyl 2,3-dibromo-3-phenylpropanoate.

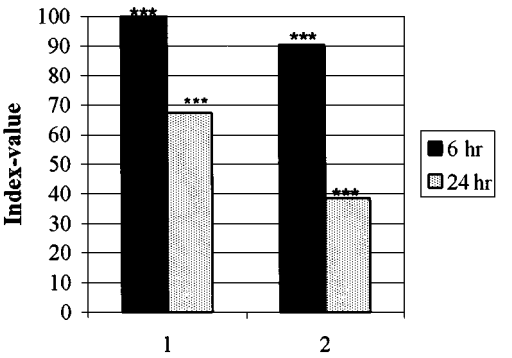


FIG. 4. Results of bioassays of ethyl 2,3-dibromo-3-phenylpropanoate (**1**) ($c = 50$ mM) and ethyl *trans*-cinnamate (**2**) ($c = 50$ mM) after 6 and 24 hr. $^{***}P < 0.001$.

weeks, the damage level was four times higher on control seedlings treated with methanol–AGS without the active substance and three times higher on untreated seedlings. Thus, methanol–AGS alone tended to increase damage, but this effect was counteracted when the active substance was added.

DISCUSSION

Host plants can simultaneously produce both attractant and deterrent compounds. The host monoterpene limonene can, for example, inhibit the attraction of pine weevils to the attractant monoterpene α-pinene and other host volatiles (Nordlander, 1990, 1991). Verbenone is also known to reduce feeding by pine weevils (Lindgren et al., 1996). Other natural plant compounds that have been found to possess antifeedant activity against *Hylobius* are limonin, carvone,

TABLE 1. PINE WEEVIL FEEDING IN FIELD ON SEEDLINGS TREATED WITH ETHYL 2,3-DIBROMO-3-PHENYLPROPANOATE (**2**) AND ON CONTROL SEEDLINGS OF TWO DIFFERENT TYPES[a]

Treatment of seedlings	No. of attacked seedlings ($N = 80$)		Mean (SE) debarked area (cm^2) per seedling ($N = 80$)	
	19 days	36 days	19 days	36 days
2 + MeOH + AGS	27	63	0.62 (0.14) a	1.63 (0.22) a
MeOH + AGS	58	71	4.66 (0.46) b	6.53 (0.41) b
Untreated	47	69	3.28 (0.40) c	5.09 (0.38) b

[a] Column means followed by the same letter are not significantly different ($P < 0.05$) (ANOVA (GLM Proc., SAS) of log-transformed data followed by Tukey's HSD test).

curcubitacin, and coumarin (Salom et al., 1994, 1996; Klepzig and Schlyter, 1999).

We confirm in this study that pine weevils feed less on bark from *P. contorta* than on bark from *P. sylvestris.* (The strong attractivity of the two water extracts was probably due to the content of low-molecular-weight carbohydrates in those fractions (Theander, 1982).) Two compounds possessing antifeedant activities were isolated from *P. contorta*, ethyl *trans*-cinnamate (**1**) and ethyl 2,3-dibromo-3-phenylpropanoate (**2**). Compound **2**, found in *P. contorta* and not in *P. sylvestris*, might also play a role in feeding preference for *P. sylvestris*. Several other factors also may, be responsible for *P. sylvestris* preference over *P. contorta*. *P. sylvestris* may contain more feeding stimulants, and/or additional compounds with antifeedant activity may exist in *P. contorta*.

Ethyl cinnamate has been isolated from several plant species (Pandji et al., 1993; Aubry et al., 1997; Mallavarapu et al., 1999) but not to our knowledge from any pine species. Ethyl cinnamate has not been reported to be an antifeedant but is known to possess other defensive activities (Kiuchi et al., 1988; Pandji et al., 1993). Ethyl 2,3-dibromo-3-phenylpropanoate has been synthesized and shown to possess antibacterial and antifungal activities (patent: US patent 4,397,851). To our knowledge, it has not been detected previously in living tissue.

The existence of a bromine compound in lodgepole pine bark was surprising. However, ponderosa pine (*Pinus ponderosa*) also contains some bromocompounds, since small amounts of bromine were detected in gases from burning biomass (Turn et al., 1997). Bromines emanate from man-made chemicals (Morris et al., 1993) and natural sources (Gribble, 2000; von Sydow et al., 2000; Keppler et al., 2000); however, brominated natural products are not common. Biologically, organobromine compounds are mainly produced by marine organisms and less commonly by plants and other terrestrial organisms. Many of these compounds possess biological activity (e.g., Gribble, 2000).

Host-derived antifeedant compounds of the type identified in this study may be useful for the protection of conifer seedlings against pine weevil damage. However, it is not yet clear whether long-term effects can be achieved with these compounds.

Acknowledgments—This investigation was supported by a grant from SJFR (Swedish council for Forestry and Agricultural Research), which is gratefully acknowledged.

REFERENCES

AUBRY, V., ETIÉVANT, P. X., GINIÈS, C., and HENRY, R. 1997. Quantitative determination of potent flavor compounds in Burgundy Pinot Noir wines using a stable isotope dilution assay. *J. Agric. Food Chem.* 45:2120–2123.

CRISTAU, H. J. and TAILLEFER, M. 1998. Reactivity of substituted and unsubstituted diphenylphosphonium diylides towards carbonic acids derivatives. *Tetrahedron* 54:1507–1522.

DAURADE-LE VAGUERESSE, M. H. and BOUNIAS, M. 1992. Résponse du β-glucosyle de l'alcool conifĕrylique du *Pinus sylvestris* à l'inoculation du *Ceratocystis bruneociliata* au liber. *Can. J. For. Res.* 22:1184–1191.

EIDMANN, H. N. 1974. HYLOBIUS Schönh, pp. 275–293, *in* W. Schwenke (ed.). Die Forstshädlinge Europas. 2 Käfer, Hamburg, Germany.

GRIBBLE, G. J. 2000. The natural production of organobromine compounds. *Environ. Sci. Pollut. Res.* 7:37–49.

HARBORNE, J. B. 1988. Introduction to Ecological Biochemistry, 3rd ed. Academic Press, London.

KABALKA, G. W., YANG, K., REDDY, N. K., and NARAYANA, C. 1998. Bromination of alkenes using a mixture of sodium bromide and sodium perborate. *Synth. Commun.* 28:925–929.

KEPPLER, F., EIDEN, R., NIEDAN, V., PRACHT, J., and SCHOLER, H. F. 2000. Halocarbons produced by natural oxidation processes during degradation of organic matter. *Nature* 403:298–301.

KIUCHI, F., NAKAMURA, N., TSUDA, Y., KONDO, K., and YOSHIMURA, H. 1988. Studies on crude drugs effective on visceral larva migrans. II. Larvacidal principles in Kaempferiae rhiozoma. *Chem. Pharm. Bull.* 36:412–415.

KLEPZIG, K. D. and SCHLYTER, F. 1999. Laboratory evaluation of plant-derived antifeedants against the pine weevil *Hylobius abietis* (Coleoptera: Curculionidae). *J. Econ. Entomol.* 92:644–650.

LINDGREN, B. S., NORDLANDER, G., and BIRGERSSON, G. 1996. Feeding deterrence of verbenone to the pine weevil, *Hylobius abietis* (L.) (Col., Curculionidae). *J. Appl. Entomol.* 120:397–403.

MALLAVARAPU, G. R., KULKARNI, R. N., BASKARAN, K., RAO, L., and RAMESH, S. 1999. Influence of plant growth stage on the essential oil content and comosition in davana (*Artemisia pallens* Wall.). *J. Agric. Food Chem.* 47:254–258.

MORRIS, P. J., QUENSEN, J. F., TIEDJE, J. M., and BOYD, S. A. 1993. An assessment of the reductive debromination of polybrominated biphenyls in the pine river reservoir. *Environ. Sci. Technol.* 27:1580–1586.

NORDLANDER, G. 1990. Limonene inhibits attraction to α-pinene in the pine weevils *Hylobius abieis*, and *H. pinastri. J. Chem. Ecol*. 16:1307–1320.

NORDLANDER, G. 1991. Host finding in the pine weevil *Hylobius abietis*—effects of conifer volatiles and added limonene. *Entomol. Exp. Appl*. 59:229–237.

NORDLANDER, G., NORDENHEM, H., BORG-KARLSON, A.-K., and UNELIUS, R. 2000. Use for conifer sapling protection. *Swedish and PCT Patent Application WO 0056152 A1*.

ROSENTHAL, G. A. 1986. The chemical defenses of higher plant. *Sci. Am.* 254:76–81.

PANDJI, C., GRIMM, C., WRAY, V., WITTE, L., and PROKSCH, P. 1993. Insecticidal constituents from four species of the Zingiberaceae. *Phytochemistry* 34:415–419.

SALOM, S. M., CARLSON, J. A., ANG, B. N., GROSMAN, D. M., and DAY, E. R. 1994. Laboratory evaluation of biologically-based compounds as antifeedants for the pales weevil, *Hylobius pales* (Herbst) (Coleoptera: Curculionidae) *J. Entomol. Sci.* 29:407–419.

SALOM, S. M., GRAY, J. A., ALFORD, A. R., MULESKY, M., FETTIG, C. J., and WOODS, S. A. 1996. Evaluation of natural products as antifeedants for the pales weevil (Coleoptera: Curculionidae) and as fungitoxins for *Leptographium procerum. J. Entomol. Sci.* 31:453–365.

SUGA, T., OHTA, S., MUNESADA, K., IDE, N., KURAKAWA, M., SHIMIZU, M., and OHTA, E. 1993. Endogenous pine wood nematodical substances in pines, *Pinus massoniana*, *P. strobus* and *P. palustris. Phytochemistry* 33:1395–1401.

SUNNERHEIM-SJÖBERG, K. 1991. Chemical Studies of Secondary Metabolites in *Betula* and *Pinus*—with emphasis on defence against mammalian herbivores. Thesis. Department of Chemistry, University of Agricultural Sciences, Uppsala, Sweden.

THEANDER, O. 1982. Hydrophilic extractives from the needles of Scots pine and Norway spruce. *Svensk Papperstidning.* 85:64–68.

TURN, S. Q., JENKINS B. M., CHOW J. C., PRITCHETT, L. C, CAMPBELL, D., CAHILL, T., and WHALEN, S. A. 1997. Elemental characterization of particulate matter emitted from biomass burning: Wind tunnel derived source profiles for herbaceous and wood fuels. *J. Geophys. Res. Atm.* 102:3683–3699.

VOGEL, A. I. 1989. Practical Organic Chemistry. Longman Scientific & Technical, New York, pp. 510–511.

VON SYDOW, L. M., NIELSEN, A. T., GRIMVALL, A. B., and BREN, H. B. 2000. Chloro- and bromoacetates in natural archives of firn from Antarctica. *Environm. Sci. Technol.* 34:239–245.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

EXTRACTS OF *Flourensia cernua* (L): VOLATILE CONSTITUENTS AND ANTIFUNGAL, ANTIALGAL, AND ANTITERMITE BIOACTIVITIES[1]

MARIO TELLEZ,[2,*] RICK ESTELL,[3] ED FREDRICKSON,[3] JANINE POWELL,[4] DAVID WEDGE,[2] KEVIN SCHRADER,[2] and MOZAINA KOBAISY[2]

[2]*USDA/ARS Natural Products Utilization Research Unit University, Mississippi 38677*

[3]*USDA/ARS Jornada Experimental Range Las Cruces, New Mexico 88003*

[4]*USDA-ARS Biological Control & Mass Rearing Research Stoneville, Mississippi 38776*

(Received November 7, 2000; accepted July 15, 2001)

Abstract—The chemical components of tarbush (*Flourensia cernua*) leaves were fractionated by extracting successively with hexanes, diethyl ether, and ethanol. Volatile profiles of each fraction were identified by using GC-MS. The hexanes fraction contained mostly monoterpenoids, while the ethanol fraction volatiles were primarily sesquiterpenoids. Crude fractions were tested for activity against fungi, algae, and termites. Application of as little as 1 μg of the essential oil from the hexanes fraction was sufficient to provide visible antifungal activity in bioautography assays. The diethyl ether fraction showed selective activity against the cyanobacterium responsible for the 2-methylisoborneol-induced off-flavor sometimes associated with catfish farming operations. All three fractions exhibited a high degree of antitermite activity.

Key Words—*Flourensia cernua*, fractionation, activity, fungi, cyanobacteria, algae, termite, *Colletotrichum*, *Oscillatoria*, *Selenastrum*, *Reticulitermes*.

*To whom correspondence should be addressed. E-mail: matellez@olemiss.edu

[1]Mention of a trade name, proprietary product or vendor does not constitute a warranty of the product by the USDA or imply its approval to the exclusion of other products or vendors that may also be suitable.

INTRODUCTION

At the Natural Products Utilization Research Unit (NPURU), Oxford, Mississippi, we are interested in finding novel uses for products from natural sources, especially as they relate to pest management. *Flourensia cernua* DC (tarbush, hojasen) is an aromatic deciduous shrub endemic to the Chihuahuan Desert of the southwestern United States and northern Mexico. Tarbush flowers are reported to be acutely toxic to livestock (Mathews, 1944; Dollahite and Allen, 1975) when consumed in large amounts (approximately 1% of body weight per day). Additionally, leaves and flower heads are sold in drug markets in Mexico (Dillon, 1984) and local farmers' markets in the United States as a remedy for indigestion. Tarbush is increasing in dominance within the Chihuahuan Desert and is currently the target of several studies involving interactions of herbivory and shrub chemistry at the Jornada Experimental Range (JER), Las Cruces, New Mexico. We previously reported the chemical profile of the essential oil of *F. cernua* (Tellez et al., 1997). In collaboration with the JER, we examined a set of crude extracts obtained from tarbush leaves by sequential extractions with solvents of widely differing properties (hexanes, ether, and ethanol). Bioassays conducted at the JER show all three crude fractions deter consumption by sheep when applied to alfalfa pellets (Estell et al., 2001). Bioassays conducted at the NPURU have shown that the crude hexanes and ether extracts are phytotoxic, whereas the ethanol fraction is inactive (Dayan and Tellez, 1999). Little else is known of the bioactivity of tarbush constituents.

Because volatile chemicals are likely the first line of exposure of a plant to herbivores, one objective of this research was to examine volatile chemical profiles of the three fractions that were deterrent to consumption by sheep (Estell et al., 2001). A second objective was to test these fractions for antifungal, antialgal, and antitermitic activity in an effort to identify potential agricultural and pest management uses of material from this invasive shrub.

METHODS AND MATERIALS

Plant Collection. Plant materials were collected from an area heavily infested with tarbush on the JER. Approximately 300 plants were selected haphazardly over a large area. Leaves were stripped from leaders into plastic bags by hand using gloves, and immediately placed on ice. Leaves were frozen within 2 hr, shipped on Dry Ice to the NPURU, and stored at -20°C until extraction.

Extractions. Tarbush leaves (36.5 kg) were extracted at room temperature in approximately 3-kg batches. Extractions were carried out sequentially with 7 liters each of hexanes, diethyl ether, and 100% ethanol for 22 hr in covered 10-liter round bottom flasks placed in a circular shaker (New Brunswick Co. G10 Gyrotory

Shaker) at 150 rpm. Each fraction was filtered (Whatman No. 1) and the solvents removed under reduced pressure with a rotary evaporator. All fractions from a given solvent were combined. The hexanes fraction produced 415.5 g of a highly aromatic yellow–brown oil, the ether fraction resulted in 984.0 g of a thick green oil, and the ethanol fraction produced 2215.6 g of an extremely thick green oil. All final products were stored at -20°C until used for bioassays. Steam distillations of the extracts were conducted as previously described (Tellez et al., 2000).

Chemical Analyses. Volatile chemical analyses of extracts were performed by GC-MS (EI, 70 eV) with a DB-5 column (30 m $\times$ 0.25 mm fused silica capillary column, film thickness 0.25 μm) with He as carrier gas (1 ml/min), 1 μl injection size, and a programmed (injector temperature: 220°C, transfer line temperature: 240°C, initial column temperature: 60°C, final column temperature: 240°C, 3°C/min) run (Adams, 1995). Octane and eicosane standards were used only for determination of retention times. Identification of extract components was performed by a comparison of mass spectra with literature data (NIST/EPA/NIH, 1990; Adams, 1995), and by comparison of their relative retention times with those of authentic compounds, or by comparison of their retention indices with those in the literature (Adams, 1995). The relative amounts (RA) of individual components of the extracts are expressed as percent peak area relative to total peak area. Analyses were performed on extracts after removal of solvents, since this was representative of their composition as applied to bioassays.

Algicidal Assays. A rapid bioassay (Schrader et al., 1997) was used to determine the lowest-observed-effect concentration and the lowest-complete-inhibition concentration of *F. cernua* extracts towards isolates of the cyanobacteria *Oscillatoria perornata* and *Oscillatoria agardhii,* and the green alga *Selenastrum capricornutum*. Ether, ethanol, and hexanes extracts were dissolved in technical grade acetone, ethanol, and hexane, respectively, and placed into microplate wells by the method of Schrader et al. (1997) to achieve a final test concentration of 50 μg/ml ($N = 4$). Solvents were allowed to evaporate for 10–15 min before adding culture material. Solvents without extracts were used in control microplate wells. The bioassay was modified by using 96-well quartz microplates (Hellma Cells, Inc., Forest Hills, New York) because hexane and acetone were used as loading solvents and are not compatible with polystyrene microplates.

Fungicidal Assays. Pathogen production and inoculum preparation for *Colletotrichum fragariae* Brooks, *C. gloeosporioides* Penz. & Sacc., and *C. accutatum* Simmonds were performed according to published procedures (Wedge and Kuhajek, 1998). Conidia concentrations were determined photometrically (Espinel-Ingroff and Kerkering, 1991; Wedge and Kuhajek, 1998) from a standard curve, and suspensions were adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/ml. Inhibition of fungal growth on chromatographic plates was evaluated by modifications of thin-layer chromotography (TLC) bioautographic assays (Homans and Fuchs, 1970).

Extracts were spotted on a TLC plate with a disposable glass micropipet, loading 400, 100, 10, 1, and 0.1 μg of each extract and of each essential oil derived from each extract. Loading solvents were used as controls. Each experiment was repeated three times. To detect biological activity directly on the TLC plate, silica gel plates (250 μm, Silica Gel GF Uniplate, Analtech, Inc., Newark, Delaware) were sprayed with a spore suspension as previously described (Wedge and Nagle, 2000). Inhibition of fungal growth for each test fungus was measured four days after treatment. Concentration-dependent sensitivity of the fungal species to each extract or oil was determined by comparing size of inhibitory zones.

Termite Assays. Extracts were tested at different concentrations, proportional to extract recovery. Hexanes, ether, and ethanol extracts (189, 405, and 958 mg, respectively) were each suspended with vigorous shaking in 45 ml of acetone. A 3-ml aliquot of the suspension was placed on each of three absorbent cellulose pads (47 mm diam.) for each of the three extracts ($N = 9$). Pads of cellulose without extract ($N = 3$) were used as a control. All pads remained under a fume hood for 24 hr to allow evaporation of acetone. Plastic containers (5 cm diam. $\times$ 3.5 cm high) served as test containers. A sterile sand–vermiculite (1:1) mixture (50 g) moistened with distilled water was placed in each of 12 test containers. One hundred *Reticulitermes* sp. worker termites were placed on top of the sand in each test unit. After 10 min, a treated cellulose pad that had been moistened with distilled water was placed in each container. Each container was covered and placed in an incubator maintained at 25°C and 53% relative humidity. All test units were observed weekly for five weeks to note any dead termites or unusual termite behavior. After five weeks, test units were disassembled, and termite survival and amount of feeding on treated and untreated cellulose pads was recorded.

RESULTS AND DISCUSSION

Table 1 shows the identity, retention index, retention time, and percent composition of the volatile compounds in the hexanes, ether, and ethanol extracts. The volatile profiles for the three crude fractions were markedly different. Forty-one volatile compounds were identified in the hexanes fraction, accounting for over 91.3% of the composition of the volatiles in the extract. Forty-seven volatile compounds were identified in the ether fraction, accounting for over 84.7% of the composition of the volatiles in the extract; δ-selinene (5.8%) was tentatively identified by MS only. Fourteen volatile compounds were identified in the ethanol fraction, accounting for over 79.3% of the composition of the volatiles in the extract; δ-selinene (8.7%) and 3,7,11,15-tetramethyl-2-hexadecene-1-ol (4.5%) were tentatively identified by MS only. The volatiles in the hexanes extract consisted of a high proportion of monoterpenes, with the 27 identified monoterpenes accounting for 74.7% of the total area. The hexanes fraction contained a low proportion

TABLE 1. VOLATILE CONSTITUENTS OF HEXANES, ETHER, AND ETHANOL SEQUENTIAL EXTRACTIONS OF TARBUSH LEAVES[a]

			RA		
	R_t (min)	RI	Hexanes	Ether	Ethanol
santolina triene	282	907	t	—	—
tricyclene	306	927	t	—	—
α-thujene	311	931	0.1	0.1	—
α-pinene	322	939	2.0	1.4	—
camphene	345	954	0.9	0.1	—
sabinene	383	977	1.9	0.4	—
β-pinene	390	980	0.8	0.3	—
myrcene	413	992	19.9	6.7	—
α-phellandrene	436	1004	0.1	t	—
3-δ-carene	449	1012	12.6	6.1	—
α-terpinene	460	1018	t	t	—
o-cymene	470	1023	t	—	—
p-cymene	474	1026	0.1	t	—
limonene	486	1032	27.7	8.4	—
1,8-cineole	495	1033	t	t	—
z-β-ocimene	500	1040	t	—	—
e-β-ocimene	522	1050	0.4	0.1	—
γ-terpinene	545	1061	t	t	—
artemisia ketone	547	1062	t	—	—
artemisia alcohol	598	1084	4.3	0.6	—
p-mentha-2,4(8)diene	604	1086	t	t	—
terpinolene	610	1089	0.1	t	—
trans-pinocarveol	723	1139	t	—	—
camphor	736	1145	0.1	—	—
cis-chrysanthenol	782	1163	1.1	0.2	—
borneol	789	1166	2.4	0.5	0.2
terpin-4-ol	817	1177	t	—	—
δ-elemene	1222	1340	t	0.4	0.9
α-cubebene	1251	1352	—	0.2	—
cyclosativene	1292	1367	—	0.1	—
α-ylangene	1305	1372	—	0.1	—
α-copaene	1316	1376	t	0.2	—
β-bourbonene	1337	1384	t	t	—
β-cubebene	1351	1389	—	0.2	—
cis-jasmone	1366	1394	0.1	—	—
α-cedrene	1406	1409	—	0.2	0.2
β-caryophyllene	1421	1417	0.9	16.0	14.3
trans-α-bergamotene	1463	1435	—	0.1	—
α-guaiene or aromadendrene	1468	1437	—	t	—
α-humulene	1503	1452	0.4	5.0	6.9
γ-muurolene	1561	1476	—	1.0	0.1
γ-curcumene	1563	1477	—	t	—
germacrene D	1569	1479	1.6	24.0	43.3

TABLE 1. CONTINUED

	R_t (min)	RI	RA		
			Hexanes	Ether	Ethanol
β-selinene	1581	1484	—	0.9	1.1
viridiflorene	1595	1489	—	t	—
cis-β-guaiene	1601	1492	0.1	—	—
bicyclogermacrene	1606	1494	0.1	1.1	1.6
δ-selinene (MS only)[b]	1633	1505	0.3	5.8	8.7
trans-γ-cadinene	1650	1513	0.1	0.9	0.6
δ-cadinene	1670	1522	0.1	1.2	0.9
cadina-1,4-diene	1689	1531	—	0.1	—
α-cadinene	1702	1537	—	0.1	—
germacrene B	1745	1555	—	0.2	0.3
caryophyllene oxide	1803	1580	0.2	0.1	0.2
β-eudesmol	1956	1649	11.3	2.8	4.4
flourensadiol	2403	1864	1.4	4.6	4.5
3,7,11,15-teramethyl-2-hexadecene-1-ol (MS only)	2873	2114	—	—	4.5

[a] RT = retention time; RI = retention index as determined on DB-5 using the homologous series of *n*-hydrocarbons; RA = relative area (peak area relative to total peak area); t = trace (<0.05%); — = not found.
[b] Tentative identification (by MS only).

of sesquiterpenoids, with β-eudesmol accounting for 11.3%, and the remaining 13 accounting for 4.9% of the total relative area. The major monoterpenoids were myrcene (19.9%), 3-δ-carene (12.6%), and limonene (27.7%). The identified volatile compounds in the ether extract consisted of a mixture of monoterpenes (24.9%) and sesquiterpenes (57.0%), with a marked decrease in the proportion of β-eudesmol present (2.8%). The major components included myrcene (6.7%), 3-δ-carene (6.1%), limonene (8.4%), β-caryophyllene (16.0%), α-humulene (5.0%), and germacrene D (24.0%). Finally, the ethanol extract volatile profile was almost completely devoid of monoterpenes (0.2%) and consisted almost exclusively of sesquiterpenes, with the identified sesquiterpenes accounting for 79.1% of the total area. The major constituents included β-caryophyllene (14.3%), α-humulene (6.9%), and germacrene D (43.3%).

The three solvents used to extract tarbush leaves differed in polarity, from the fairly nonpolar hexanes to the fairly polar ethanol. These solvents were selected in order to obtain a crude separation of the components of the leaves. Although selective extraction was achieved, the separation appeared more a function of size (monoterpenoids versus sesquiterpenoids) than polarity, at least for volatile compounds. The observed difference is not an artifact of solvent removal since chromatograms obtained prior to and after solvent removal showed little difference in profile. In the past, we have observed selective extractions of terpenoid

classes with *Artemisia annua* (Tellez et al., 1999; Duke et al., 2000), although what mechanisms might be responsible in this present case remain unexplained.

Steam distillations of each extract afforded 337, 178, and 15 mg of essential oil (volatiles) per gram of hexanes, ether, and ethanol extract, respectively. Thus, a substantial portion of all three crude extracts represented the nonvolatile fraction. This distribution of volatile and nonvolatile components among the three extracts might explain results obtained in sheep feeding studies (Estell et al., 2001), in which all extracts decreased feed intake even though the amounts and profiles of the volatiles were different.

This study demonstrated that all three crude fractions were active in our fungal bioassays. Bioautography data (Table 2) showed that the essential oil derived from the hexanes extract retained activity against *C. gloeosporioides* and *C. fragariae* at lower application amounts than the other treatments. Antifungal activity of the essential oil derived from the hexanes crude extract was detected at the 1 μg dose. Antifungal activity of the essential oils derived from the ether and ethanol fractions was observed only at 10 μg doses and above. Antifungal activity of the ethanol crude extract fraction could be observed only at the highest amount (400 μg)

TABLE 2. FUNGAL GROWTH INHIBITION BY HEXANES, ETHER, AND ETHANOL EXTRACTS OF TARBUSH LEAVES AND THEIR ESSENTIAL OILS BY BIOAUTOGRAPHY USING *Colletotrichum gloeosporioides*, *C. fragaria*, AND *C. accutatum* AS INDICATORS

	Inhibition zone diameter (mean ± SD, mm)					
	Hexanes		Ether		Ethanol	
	Extract	Oil	Extract	Oil	Extract	Oil
C. gloeosporioides						
400 μg	7.3 ± 1.2	8.7 ± 0.9	10.0 ± 0.8	9.3 ± 0.5	9.7 ± 0.5	13.7 ± 0.9
100 μg	3.7 ± 0.5	6.3 ± 1.2	6.7 ± 0.5	8.3 ± 0.9	0.0 ± 0.0	9.3 ± 0.9
10 μg	0.0 ± 0.0	3.0 ± 2.2	1.0 ± 1.4	2.7 ± 0.5	0.0 ± 0.0	3.3 ± 0.5
1 μg	0.0 ± 0.0	0.7 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. fragariae						
400 μg	10.3 ± 1.2	11.0 ± 0.8	10.3 ± 1.2	11.3 ± 3.3	10.0 ± 0.8	12.7 ± 0.5
100 μg	8.0 ± 0.8	8.3 ± 1.2	7.3 ± 0.5	8.7 ± 0.9	0.0 ± 0.0	11.7 ± 0.9
10 μg	2.3 ± 0.5	3.7 ± 0.9	1.3 ± 1.9	3.3 ± 0.5	0.0 ± 0.0	6.7 ± 3.1
1 μg	0.0 ± 0.0	2.3 ± 1.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C. accutatum						
400 μg	7.0 ± 0.8	8.3 ± 0.5	10.7 ± 0.5	7.7 ± 0.5	5.3 ± 0.5	10.3 ± 0.5
100 μg	4.7 ± 0.5	5.7 ± 0.5	6.7 ± 0.5	6.3 ± 0.5	0.0 ± 0.0	9.0 ± 0.8
10 μg	0.0 ± 0.0	2.0 ± 1.4	1.0 ± 1.4	2.3 ± 1.7	0.0 ± 0.0	4.7 ± 0.9
1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1 μg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

tested at the end of four days. Comparison among extracts, other than comparing the presence or absence of activity, by comparing size of inhibition zones is not valid because of the different solvents used to spot each extract. Comparing the zones of inhibition within an extract indicates a clear dose response for all three extracts against *C. gloeosporioides*, *C. fragariae*, and *C. accutatum*.

The essential oil of the hexanes extract was consistently more active against fungi than the extract itself, suggesting that a good portion of the activity in the hexanes extract resides in its steam-distilled volatile components. Several major volatile components—α-pinene (2.0%), 3-δ-carene (12.6%), and limonene (27.7%) (Himejima et al., 1992), and β-eudesmol (11.3%) (Miyakado et al., 1976)—are reported to have antifungal activity. Another major component, myrcene (19.9%), is reported to enhance the activity of other antimicrobials (Onawunmi et al., 1984) . The steam-distilled volatiles of the ethanol fraction exhibited inhibitory activity to all three *Colletotrichum* species, but their extremely small presence (1.5%) in the extract makes it unclear whether the inhibitory effect observed for the extract is due mostly to these steam-distilled volatiles or to other non-steam-distilled components.

F. cernua extracts were screened against two species of cyanobacteria (blue-green algae) and one species of green algae to determine their potential as a selective cyanobactericide. An off-flavor in channel catfish (*Ictalurus punctatus*) raised in the southeastern United States creates an unpalatable and, therefore, unmarketable product that results in large economic losses to the industry. Most off-flavor episodes in catfish are attributed to their absorption of earthy/musty compounds produced by certain species of cyanobacteria that grow in catfish production ponds. In west Mississippi, the cyanobacterium *Oscillatoria perornata* (Skuja), a producer of the musty-odor compound 2-methylisoborneol (MIB), is thought to be the major cause of musty off-flavor in farm-raised catfish (van der Ploeg et al., 1995). Green algae are not associated with such undesirable metabolites and are also preferable to cyanobacteria in catfish production ponds because they are better oxygenators of the water and a better base for aquatic food chains (Paerl and Tucker, 1995). Therefore, the discovery of safe compounds that selectively kill cyanobacteria would benefit the channel catfish industry.

Complete inhibition of *O. perornata* was observed for the hexanes and ether extracts of *F. cernua* at 50 (μg/ml (Figure 1B). For *Oscillatoria agardhii* (a non-MIB-producing cyanobacterium), complete inhibition occurred with the hexanes extract, and these results were established visually, i.e., disappearance of filaments in the treatment wells. The formation of black precipitate in wells containing hexanes extracts and *O. agardhii* culture resulted in high absorbance readings that could be misinterpreted as accelerated growth rate by *O. agardhii* based solely on the graphed data (Figure 1A). The other two cultures did not produce any colored precipitate in wells containing hexanes extract. For *S. capricornutum*, complete inhibition was also established only for the hexanes extract (Figure 1C).

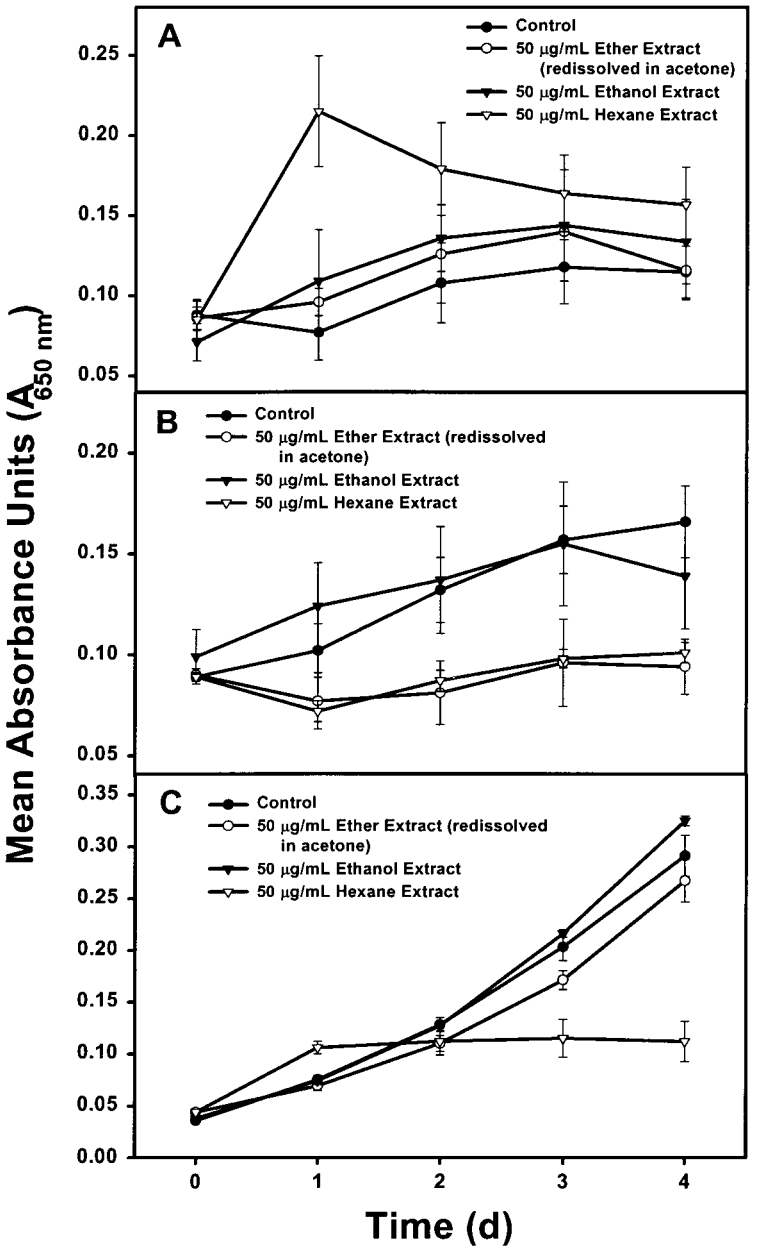


FIG. 1. Effect of tarbush extracts on growth of (**A**) *Oscillatoria agardhii*, (**B**) *O. perornata*, and (**C**) *Selenastrum capricornutum*. Error bars represent standard deviations of the mean ($n = 3$).

These results indicate selective toxicity of the ether extract of *F. cernua* towards *O. perornata* and nonselective toxicity of the hexanes extract towards all three organisms. No toxicity towards the green alga or cyanobacteria tested was observed for the ethanol extract. Additional screening of extracts and their steam-distilled oils revealed that a constituent (s) present in the extract but not the steam-distilled oil is (are) responsible for the selective toxicity of the ether extract towards *O. perornata* (data not shown).

All three crude extracts also showed a high degree of termiticidal activity after five weeks. The ethanol fraction was tested at the highest concentration and had a mean survival of 0%; there were no survivors in any of three replicates ($N = 100$ per replicate). The hexanes extract was tested at the lowest concentration but also had a nearly complete kill (mean of 2.3% survival); there were no survivors in two of the replicates and only seven survivors on the third replicate ($N = 100$ per replicate). The intermediate ether fraction had 15.7% survival (0, 35, and 12 survivors in each replicate, $N = 100$/replicate). Survival of the control group with an acetone blank was 82%. The antitermite properties of different fractions suggest the presence of more than one active compound (or set of compounds).

In conclusion, the three sequential extracts of tarbush exhibited fairly different volatile profiles. The crude organic extracts of tarbush show promise as antitermitic and antifungal agents. The ether extract of *F. cernua* holds promise as a possible selective cyanobactericide against the cyanobacterium thought to be the major cause of musty off-flavor problems in Mississippi farm-raised catfish. We are currently carrying out the bioassay-guided isolation and identification of the active components in *F. cernua.*

REFERENCES

ADAMS, R. P. 1995. Identification of Essential Oil Components by Gas Chromatography and Mass Spectroscopy. Allured Publishing, Carol Stream, Illinois.

DAYAN, F. E. and TELLEZ, M. R. 1999. Phytotoxicity of tarbush (*Flourensia cernua* DC). *Allelopathy J.* 6:1–12.

DILLON, M. O. 1984. A Systematic Study of *Flourensia* (Asteraceae, Heliantheae). Field Museum of Natural History, Chicago, Illinois.

DOLLAHITE, J. N. and ALLEN, T. J. 1975. The toxicity of the fruit of *Flourensia cernua* (tarbush) (Blackbrush). *Southwest. Vet.* 28:113–117.

DUKE, S. O., CANEL, C., RIMANDO, A. M., TELLEZ, M. R., DUKE, M. V., and PAUL, R. N. 2000. Current and potential exploitation of plant glandular trichome productivity. *Adv. Bot. Res.* 31: 121–152.

ESPINEL-INGROFF, A. and KERKERING, T. M. 1991. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. *J. Clin. Microbiol.* 29:393–394.

ESTELL, R., TELLEZ, M., FREDRICKSON, E., ANDERSON, D., HAVSTAD, K., and REMMENGA, M. 2001. Extracts of *Flourensia cernua* reduce consumption of alfalfa pellets by sheep. *J. Chem. Ecol.* 27:2277–2287.

HIMEJIMA, M. HOBSON, K., OTSUKA, T., WOOD, D., and KUBO, I. 1992. Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa*: A defense mechanism against microbial invasion. *J. Chem. Ecol.* 18:1809–1818.

HOMANS, A. L. and FUCHS, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. *J. Chromatogr.* 51:327–329.

MATHEWS, F. 1944. The toxicity of the ripe fruit of blackbrush or tarbrush (*Flourensia cernua*) for sheep and goats. Texas Agric. Exp. Stn. Bull. No. 664.

MIYAKADO, M., KATO, T., OHNO, N., and MABRY, T. J. 1976. Pinocembrin and (+)-β-eudesmol from *Hymenoclea monogyra* and *Baccharis glutinosa. Phytochemistry* 15:846.

NIST/EPA/NIH 1990. NIST/EPA/NIH 75K MS Library.

ONAWUNMI, G., YISAK, W., and OGUNLANA, E. 1984. Antibacterial constituents in the essential oil of *Cymbopogon citratus* (DC.) Stapf. *J. Ethno-Pharmacol*. 12:279–286.

PAERL, H. W. and TUCKER, C. S. 1995. Ecology of blue-green algae in aquaculture ponds. *J. World Aquacult. Soc.* 26:109.

SCHRADER, K., DE REGT, M., TUCKER, C., and DUKE, S. 1997. A rapid bioassay for selective algicides. *Weed Technol.* 11:767–774.

TELLEZ, M., ESTELL, R., FREDRICKSON, E., and HAVSTAD, K. 1997. Essential oil of *Flourensia cernua* DC. *J. Essent. Oil Res.* 9:619–624.

TELLEZ, M. R., CANEL, C., RIMANDO, A. M., and DUKE, S. O. 1999. Differential accumulation of isoprenoids in glanded and glandless *Artemisia annua L. Phytochemistry* 52:1035–1040.

TELLEZ, M. R., DAYAN, F. E., SCHRADER, K. K., WEDGE, D. E., and DUKE, S. O. 2000. Composition and some biological activities of the essential oil of *Callicarpa americana* (L.). *J. Agric. Food Chem.* 48:3008–3012.

VAN DER PLOEG, M., DENNIS, M. E., and DE REGT, M. Q. 1995. Biology of Oscillatoria Cf. Chalybea, A 2-Methylisoborneol producing blue-green alga of Mississippi catfish ponds. *Water Sci. Technol.* 31:173.

WEDGE, D. and KUHAJEK, J. 1998. A microbioassay for fungicide discovery. *SAAS Bull. Biochem. Biotechnol*. 11:1–7.

WEDGE, D. and NAGLE, D. G. 2000. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J. Nat. Prod.* 63:1050–1054.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

EXTRACTS OF *Flourensia cernua* REDUCE CONSUMPTION OF ALFALFA PELLETS BY SHEEP[1]

RICK ESTELL,[2,*] MARIO TELLEZ,[3] ED FREDRICKSON,[2] DEAN ANDERSON,[2] KRIS HAVSTAD,[2] and MARTA REMMENGA[4]

[2]*USDA/ARS Jornada Experimental Range*
Las Cruces, New Mexico 88003

[3]*USDA/ARS Natural Products Utilization Research Unit*
University, Mississippi 38677

[4]*University Statistics Center*
New Mexico State University
Las Cruces, New Mexico 88003

(Received November 8, 2000; accepted July 15, 2001)

Abstract—Effects of three extracts (hexanes, ether, and ethanol) from tarbush (*Flourensia cernua*) on intake of alfalfa pellets by lambs were examined. Forty-five ewe lambs were fed one of five treatments for five days (randomized complete block, three lambs per block on each treatment). Treatments were alfalfa pellets (CON) or alfalfa pellets plus ethanol carrier (CAR), hexanes extract (HEX), ether extract (ETH), or ethanol extract (ETOH). Extracts were applied to alfalfa pellets at the same concentration as in an equivalent amount of tarbush (as fed basis) in experiment 1 and at 10-fold dilutions of that concentration in experiment 2. Treatments were isolated from tarbush leaves by using a sequential extraction with hexanes, diethyl ether, and 100% ethanol. Lambs received 640 g of alfalfa pellets (dry matter basis) each morning and intake was monitored during a 20-min interval. Lambs were maintained and fed alfalfa pellets (4.7% of body weight) as one group except during this interval. In experiment 1, mean intake by lambs during the 20-min interval was 361, 393, 204, 212, and 228 g for CON, CAR, HEX, ETH, and ETOH, respectively (SEM = 28.9). All three extracts decreased intake ($P < 0.001$) compared to CON or CAR. Intake did not differ among the three extracts (HEX, ETH, and ETOH) or between the two controls (CON and CAR). Mean intake did not differ among treatments in experiment 2 (468, 455, 389, 381, and 431 g for CON, CAR, HEX, ETH, and ETOH, respectively; SEM = 30.5; $P = 0.187$). Several compounds are probably

*To whom correspondence should be addressed. E-mail: restell@nmsu.edu

[1]Mention of a trade name, proprietary product or vendor does not constitute a warranty of the product by the USDA or imply its approval to the exclusion of other products or vendors that may also be suitable.

0098-0331/01/1100-2275$19.50/0

responsible for the low palatability and differential use of tarbush typically exhibited by livestock.

Key Words—*Flourensia cernua*, feeding deterrent, intake, plant–animal interactions, plant extracts, sheep.

INTRODUCTION

Tarbush (*Flourensia cernua* DC) is a dominant shrub in the northern Chihuahuan Desert. It is high in crude protein and generally low in antiquality factors (except phenolics) (Estell et al., 1996). Livestock consume tarbush in limited amounts under free-ranging conditions (Nelson et al., 1970; Anderson and Holechek, 1983), depending on season and availability of other forages, but the shrub is not a preferred species. Tarbush may be toxic for some species of herbivores during flowering (Mathews, 1944; Dollahite and Allen, 1975). However, no adverse effects were observed in sheep consuming 30 or 15% tarbush leaves in mixed diets for 30 or 90 days, respectively (Fredrickson et al., 1994; King et al., 1996).

We have been using tarbush as a shrub model to examine chemical interactions between desert shrubs and mammalian herbivory. Ruminants exhibited differential use of individual plants when forced to browse tarbush (Estell et al., 1994b), and removal of surface compounds with organic solvents increased tarbush consumption by sheep (Estell et al., 1994a). Terpenes on the leaf surface of tarbush were related to its use by livestock (Estell et al., 1998a).

The objective of these experiments was to determine effects of extracts of tarbush on intake by lambs when applied to alfalfa pellets. Our hypothesis was that pellet consumption by lambs would decrease when extracts were applied.

METHODS AND MATERIALS

Experimental Protocol and Animal Management. Two experiments to examine intake of lambs consuming alfalfa pellets treated with extracts from tarbush were conducted in accordance with guidelines approved by the New Mexico State University Institutional Animal Care and Use Committee. Both experiments were part of larger studies. Experiment 1 was conducted in conjunction with a series of studies to examine the effect of specific terpenes (α-humulene, 1,8-cineole, *p*-cymene, 3-carene, and sabinene) on intake (Estell et al., 2000), and experiment 2 was part of a similar study that examined another series of terpenes (β-pinene, camphene, caryophyllene oxide, and myrcene; author's unpublished data). Both experiments were conducted as described by Estell et al. (2000). Ninety Polypay ewe lambs [45 lambs per experiment, approximately 5 months of age, mean initial body weight (BW) of 43.0 $\pm$ 0.41 kg and 36.8 $\pm$ 0.42 kg for experiments 1 and 2, respectively] without previous experience browsing tarbush were adapted to

alfalfa pellets [4.7% of BW, dry matter (DM) basis] for two weeks and a drylot pen for one week. A five-day adaptation period was conducted to familiarize lambs with handling procedures and the 20-min pen feeding (week 1), followed by a five-day period to establish baseline intake of untreated alfalfa pellets during the 20-min interval (week 2). Extracts were tested in week 8 (experiment 1) and week 7 (experiment 2). All five-day periods were separated by two-day intervals during which lambs were fed and managed as during the adaptation period.

Lambs were individually fed treated pellets each morning during a 20-min interval in an enclosed metabolism unit (1.22- $\times$ 2.44-m pens). Groups were fed in succession at 08:00, 08:30, and 09:00 hr. During the 20-min feeding, 640 g of pellets (DM basis) were offered daily to lambs, and feed refusals were measured. Except for the morning feeding period, lambs were maintained as one group in an outdoor pen with free access to water and trace-mineralized salt. Alfalfa pellets ($\geq$15% CP, 0.95 cm diam., from sun-cured alfalfa hay) were sampled randomly, composited, ground to pass a 2-mm screen in a Wiley mill, and analyzed for dry matter (93.8% and 94.9% in experiments 1 and 2, respectively) (AOAC, 1990). Lambs were weighed on day 5 each week immediately preceding the 08:00 hr feeding.

Lambs were also group-fed untreated alfalfa pellets twice daily at 13:00 hr (950 g DM/lamb) and 10:00 hr (remainder required to provide a mean intake of 4.7% of BW). An amount of untreated feed equal to the total unconsumed feed during 20-min tests was also fed at 10:00 hr. During the two-day intervals between intake measurements, lambs were fed an additional 640 g DM/lamb at 10:00 hr.

Treatments. Treatments applied to alfalfa pellets consisted of extracts from an equivalent amount of tarbush (as fed basis, 680 g) in experiment 1. These concentrations should most accurately reflect those to which livestock would be exposed when browsing tarbush. For experiment 2, 10-fold dilutions of the extracts served as treatments. Two control groups (one receiving untreated pellets and one receiving pellets sprayed with ethanol carrier) were included in each experiment. Treatment extracts were thawed at room temperature for 24 hr, dissolved in the appropriate amount of ethanol, and stored in amber glass bottles. Extracts were not completely soluble in the amount of ethanol used, and some settling occurred. Consequently, suspensions were swirled immediately before decanting and application.

Solutions were applied at 1 ml/20 g of alfalfa pellets with graduated cylinders (experiment 1) or graduated high-density polyethylene spray bottles (experiment 2). Treatments were sprayed in a stream pattern in experiment 2 to minimize volatilization. Pellets were placed in one end of feed pans, and pans were tilted and gently shaken while treatments were applied in an effort to apply treatments completely and evenly. Approximately 10 min lapsed between application and feeding, and order of application was rotated systematically (within

and across days) to minimize bias due to time between spraying and feeding. Treatments were applied in an adjacent separately ventilated room, and an exhaust fan in the metabolism unit was used to remove aromas and minimize potential drift among pens. One stainless steel pan was used for each lamb in each experiment to minimize cross-contamination, and pans were rinsed with ethanol after each feeding.

Bulk Extraction. Tarbush exhibiting lush green growth (prebloom stage) without visible evidence of insect damage was harvested from one location on the Jornada Experimental Range near Las Cruces, New Mexico. Leaves were removed by hand using gloves and placed in plastic bags on ice. Approximately 36 kg of fresh material representing several hundred plants were collected on September 10, 1997, between 07:30 and 12:00 hr. Leaf material was frozen within 1–2 hr of collection, shipped on Dry Ice to the USDA-ARS Natural Products Utilization Research Unit (Oxford, Mississippi), and stored in a -20°C cold room. Frozen leaves were extracted sequentially with hexanes, diethyl ether, and 100% ethanol. Extractions were conducted in 3-kg batches at room temperature on a circular shaker at 150 rpm for 22 hr in a covered 10-liter round-bottom flask with 7 liters of each solvent. Extracts from each solvent were filtered (Whatmann No. 1 filter paper), solvents were removed with reduced pressure in a 10-liter rotary evaporator, and 12 extracts from each solvent were combined in amber glass bottles and frozen. Oils were stored in a -20°C cold room until shipped on Dry Ice to Las Cruces, New Mexico.

Statistical Analysis. Lambs were randomly assigned to pen, group, and treatment at the beginning of each experiment in a randomized complete block design. Randomization was restricted to three lambs per treatment in each group (block). Repeated-measures analysis of variance was conducted using GLM procedures of SAS Institute (1989) for each experiment to evaluate the consistency of intake among days within experiment. The model contained group (blocking factor), day, treatment, day $\times$ treatment interaction, and experimental error (animal nested within treatment). Orthogonal polynomial contrasts were tested among days in this analysis. Analysis of variance for each experiment was also conducted by using GLM procedures of SAS Institute (1989) with intake (five-day means) during the 20-min interval as the dependent variable and treatment and group as the independent variables in the model. Although pen and animal are confounded and pen was not in the model, previous experiments that used these facilities did not reveal pen effects (Estell et al., 1998b). Means from analysis of variance were separated ($P < 0.05$) by LSD (SAS Institute, 1989) in the case of a significant F value ($P < 0.05$). Intake of control lambs ($N = 9$) was subjected to analysis of variance with time as the treatment factor to evaluate the consistency of intake of controls over time. Means were separated ($P < 0.05$) by LSD (SAS Institute, 1989) in the case of a significant F value ($P < 0.05$).

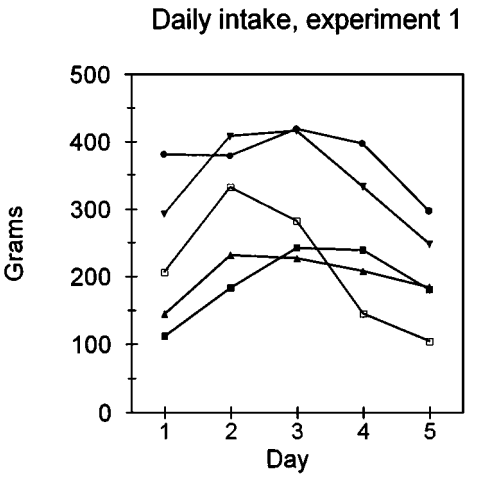


FIG. 1. Daily intake (g/day during 20 min feeding) by lambs of alfalfa pellets treated with *Flourensia cernua* extracts at the same concentration as in intact plants. Treatments were control (▼) and control with carrier (●), hexanes extract (■), ether extract (▲), or ethanol extract (□); $N = 9$ lambs/treatment; SEM = 28.9; linear treatment ($P < 0.001$) and quadratic mean ($P < 0.001$) effects were detected.

RESULTS AND DISCUSSION

When intake was examined by day with a repeated-measures analysis of variance, a linear treatment effect ($P < 0.001$) and a quadratic mean effect ($P < 0.001$) were detected in experiment 1 (Figure 1), and a quadratic ($P = 0.001$) mean effect was detected in experiment 2 (Figure 2). Quadratic mean effects in both experiments reflect the increased intake early in the week and the decrease in the latter part of the week. This shift was evident across treatments and therefore unrelated to treatments. The linear treatment effect in experiment 1 indicates changes in intake over time were not consistent among treatments (day × treatment interaction), and likely reflects the dramatic decrease in the ETOH treatment between days 2 and 5 (Figure 1).

Across days, intakes for CON (361 g) and CAR (393 g) differed ($P < 0.001$) from HEX (204 g), ETH (212 g), and ETOH (228 g) in experiment 1 (Figure 3). Although the pattern was similar in experiment 2 (Figure 4), means were not statistically different (468, 455, 389, 381, and 431 g for CON, CAR, HEX, ETH, and ETOH, respectively). All three treatments clearly reduced intake in experiment 1, supporting our hypothesis; however, the three fractions representing different crude mixtures of volatile and nonvolatile compounds extracted by increasingly polar

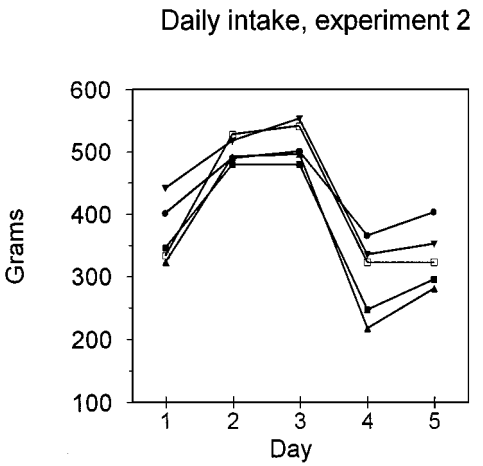


FIG. 2. Daily intake (g/day during 20 min feeding) by lambs of alfalfa pellets treated with *Flourensia cernua* extracts at a 10-fold dilution of the concentration in intact plants. Treatments were control (▼) and control with carrier (●), hexanes extract (■), ether extract (▲), or ethanol extract (□); $N = 9$ lambs/treatment; SEM = 30.5; linear ($P = 0.010$) and quadratic mean ($P < 0.001$) effects were detected.

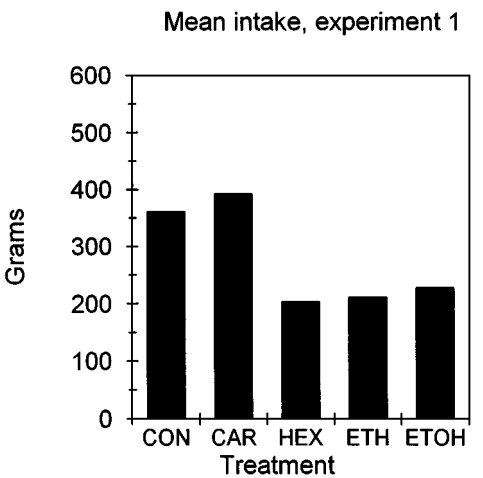


FIG. 3. Mean intake (g/day during 20 min feeding) by lambs of alfalfa pellets treated with *Flourensia cernua* extracts at the same concentration as in intact plants. Treatments were control (CON) and control with carrier (CAR), hexanes extract (HEX), ether extract (ETH), or ethanol extract (ETOH); $N = 9$ lambs/treatment; SEM = 28.9; CON and CAR differed from HEX, ETH, and ETOH ($P < 0.001$).

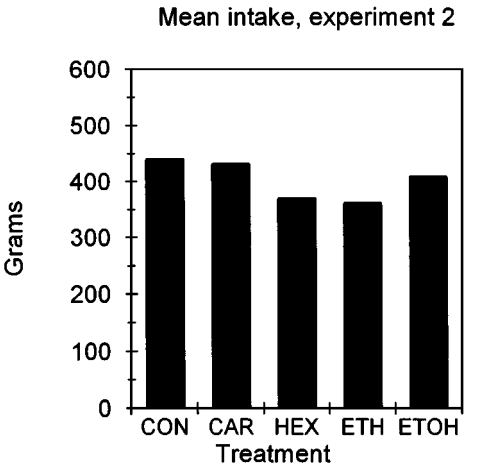


FIG. 4. Mean intake (g/day during 20 min feeding) by lambs of alfalfa pellets treated with *Flourensia cernua* extracts at a 10-fold dilution of the concentration in intact plants. Treatments were control (CON) and control with carrier (CAR), hexanes extract (HEX), ether extract (ETH), or ethanol extract (ETOH); $N = 9$ lambs/treatment; SEM = 30.5; treatment means did not differ ($P > 0.05$).

solvents were equally effective in deterring intake at concentrations equivalent to those encountered in intact plants.

Treatments CON and CAR were not different in experiments 1 or 2, indicating no effect of the ethanol carrier on intake of alfalfa pellets. No effect of group was detected in either experiment. Intake for the CON lambs during the 20-min interval was 361 g (experiment 1) and 468 g (experiment 2). Intake for the same nine lambs during the adaptation period (week 2) was 403 g and 536 g for experiments 1 and 2, respectively. Intake of control lambs during adaptation and week of treatment did not differ in either experiment ($P > 0.05$; SEM = 38.7 and 32.8, respectively). Because intake was adjusted weekly and because prior treatments did not greatly affect intake, we did not expect CON intake to differ over time.

Previously at this location, when a mixture of cattle, sheep, and goats were forced to use tarbush, they preferentially browsed certain plants to the exclusion of others, and this differential use was negatively related to epicuticular wax concentration (Estell et al., 1994b). Lambs consumed more tarbush when surface compounds were removed with organic solvents (Estell et al., 1994a). Concentrations of specific mono- and sesquiterpenes on tarbush leaves were related to this differential use (e.g., α-pinene and flourensadiol) (Estell et al., 1998a). To date, 15 volatile compounds (camphor, limonene, *cis*-jasmone, β-caryophyllene, borneol, α-pinene, sabinene, 3-carene, *p*-cymene, α-humulene, 1,8-cineole, camphene, myrcene, caryophyllene oxide, and β-pinene) have been tested individually

for effects on intake (Estell et al., 1998b, 2000, unpublished data). Results from these studies have generally shown minor or no effects on intake by lambs when individual compounds were applied to alfalfa pellets.

Because all treatments significantly reduced intake in experiment 1, a 10-fold dilution of the extracts was applied in experiment 2 in an attempt to identify the most potent fraction. None of the diluted extracts significantly influenced intake; however, the amount of residue recovered (415, 984, and 2215 g for hexanes, ether, and ethanol fractions, respectively) during extraction suggests the hexanes fraction was most potent, based on the fact that it was recovered in the lowest amount and, therefore, contributed the least mass to its respective treatment solution.

Volatile profiles for each extract were characterized by using gas chromatography–mass spectrometry (Tellez et al., 2001). Volatile profiles in the extracts were related more to molecule size than to any obvious polarity differences. The hexanes fraction contained most of the monoterpenes (and few sesquiterpenes), the ether fraction contained primarily sesquiterpenes (with some monoterpenes), and the ethanol fraction was almost exclusively sesquiterpenes. The volatile compounds contributing greater than 10% of the total peak area in the hexane fraction were limonene, myrcene, 3-carene, and β-eudesmol. For both the ether and ethanol fractions, only germacrene D and β-caryophyllene were present in excess of 10% of the total peak area (Tellez et al., 2001). Limonene, myrcene, 3-carene, and β-caryophyllene did not affect intake when examined individually (Estell et al., 1998b, 2000, unpublished data), although, to our knowledge, effects of germacrene D and β-eudesmol on mammalian herbivory have not been examined . These crude fractions likely also contain numerous nonvolatile compounds, particularly in the more polar ether and ethanol fractions (e.g., alkaloids, flavonoids, etc.). Flavonoids (Rao et al., 1970), 4-hydroxyacetophenone derivatives (Bohlmann and Grenz, 1977), and benzofurans and benzopyrans (Aregullin-Gallardo, 1985) have been characterized in tarbush. Certainly, these or other nonvolatile compounds in the extracts may be responsible for the reduced intake of alfalfa pellets by lambs in this study.

The decreased intake observed in experiment 1 may be due to reduced palatability, given that many compounds in the crude terpenoid fraction are bitter. Bitterness is thought to be a primary aversive stimulus influencing forage preferences (Krueger et al., 1974), although compounds considered to be bitter are not necessarily deterrent to herbivores (Nolte et al., 1994). Hanks et al. (1975) reported that methanolic extracts containing phenolics from a number of subspecies of rubber rabbitbrush, varying in palatability and use by mule deer, resulted in different patterns when subjected to paper chromatography. Dohi et al. (1996) found methanolic extracts of perennial ryegrass sprayed on low-quality hay stimulated intake by goats, whereas pentane extracts either had no effect or reduced intake. Pass et al. (1998) examined the deterrent properties of eucalyptus for

common ringtail possums, using plants that were herbivore deterrent and herbivore susceptible. Several fractions from low-use plants differed from controls, including a methanolic fraction, a chloroform subfraction, and the steam distillate (Pass et al., 1998).

Intake reductions could be attributed to reduced digestibility and or passage rate due to microbial toxicities. Terpenes have been reported to decrease *in vitro* digestibility in ruminants, suggesting microbial toxicities (Oh et al., 1967; Schwartz et al., 1980), particularly if rumen microflora are not adapted to the compounds (Oh et al., 1967). Sinclair et al. (1988) indicated both dry matter and protein digestibility in snowshoe hares were reduced when ether or methanol extracts of white spruce or bog birch were applied to rabbit chow at levels found in those species normally. However, it is doubtful that reduced digestibility was responsible for decreased intake in this study, given the short duration of the study and the fact that lambs maintained an average total intake of 4.7% of BW.

There was little indication that intake reduction was due to negative feedback in this study, even though aversions can be formed within a five-day period (Provenza et al., 1990). The only evidence of aversion to extracts occurred for the ETOH treatment (experiment 1; Figure 1), in which intake decreased substantially during the latter part of the week (linear treatment effect, suggesting a day $\times$ treatment interaction, $P < 0.001$).

In conclusion, compounds in these extracts are probably partly responsible for the low palatability and differential use of tarbush by ruminants. Knowledge of specific chemical interactions with feed intake may ultimately lead to mechanisms to alter feeding behavior and diet selection. Potential benefits of increased shrub consumption would depend on the balance of nutritional benefits and metabolic impacts of phytotoxins.

Acknowledgments—The authors are grateful for the assistance of Rob Dunlap, Roy Libeau, Larry Shupe, and John Smith.

REFERENCES

ANDERSON, D. M. and HOLECHEK, J. L. 1983. Diets obtained from esophageally fistulated heifers and steers simultaneously grazing semidesert tobosa rangeland. *Proc. West. Sect. Am. Soc. Anim. Sci.* 34:161–164.

AOAC. 1990. Official Methods of Analysis. Association of Official Analytical Chemists, Arlington, Virginia.

AREGULLIN-GALLARDO, M. 1985. Chemical and biological significance of benzofurans and benzopyrans in the Asteraceae. PhD dissertation. University of California, Irvine, California.

BOHLMANN, F. and GRENZ, M. 1977. Über Inhaltsstoffe der Gattung *Flourensia Chem. Ber.* 110:295–300.

DOHI, H., YAMADA, A., and FUKUKAWA, T. 1996. Effects of organic solvent extracts from herbage on feeding behavior in goats. *J. Chem. Ecol.* 22:425–430.

DOLLAHITE, J. M. and ALLEN, T. J. 1975. The toxicity of the fruit of *Flourensia cernua* (tarbush) (blackbrush). *Southwest. Vet.* 28:113–117.

ESTELL, R. E., ANDERSON, D. M., and HAVSTAD, K. M. 1994a. Effects of organic solvents on use of tarbush by sheep. *J. Chem. Ecol.* 20:1137–1142.

ESTELL, R. E., FREDRICKSON, E. L., ANDERSON, D. M., MUELLER, W. F., and REMMENGA, M. D. 1994b. Relationship of tarbush leaf surface secondary chemistry to livestock herbivory. *J. Range Manage.* 47:424–428.

ESTELL, R. E., FREDRICKSON, E. L., and HAVSTAD, K. M. 1996. Chemical composition of *Flourensia cernua* at four growth stages. *Grass Forage Sci.* 51:434–441.

ESTELL, R. E., FREDRICKSON, E. L., ANDERSON, D. M., HAVSTAD, K. M., and REMMENGA, M. D. 1998a. Relationship of leaf surface terpene profile of tarbush with livestock herbivory. *J. Chem. Ecol.* 24:1–12.

ESTELL, R. E., FREDRICKSON, E. L., TELLEZ, M. R., HAVSTAD, K. M., SHUPE, W. L., ANDERSON, D. M., and REMMENGA, M. D. 1998b. Effect of volatile compounds on consumption of alfalfa pellets by sheep. *J. Anim. Sci.* 76:228–233.

ESTELL, R. E., FREDRICKSON, E. L., ANDERSON, D. M., HAVSTAD, K. M., and REMMENGA, M. D. 2000. Effects of individual terpenes on consumption of alfalfa pellets by sheep. *J. Anim. Sci.* 78:1636–1640.

FREDRICKSON, E., THILSTED, J., ESTELL, R., and HAVSTAD, K. 1994. Effects of chronic ingestion of tarbush (*Flourensia cernua*) on ewe lambs. *Vet. Hum. Toxicol.* 36:409-415.

HANKS, D. L., MCARTHUR, E. D., PLUMMER, A. P., GIUNTA, B. C., and BLAUER, A. C. 1975. Chromatographic recognition of some palatable and unpalatable subspecies of rubber rabbitbrush in and around Utah. *J. Range Manage.* 28:144–148.

KING, D. W., ESTELL, R. E., FREDRICKSON, E. L., HAVSTAD, K. M., WALLACE, J. D., and MURRAY, L. W. 1996. Effects of *Flourensia cernua* ingestion on intake, digestion, and ruminal fermentation of sheep consuming tobosa. *J. Range Manage.* 49:325–330.

KRUEGER, C. W., LAYCOCK, W. A., and PRICE, D. A. 1974. Relationships of taste, smell, sight and touch to forage selection. *J. Range Manage.* 27:258–262.

MATHEWS, F. P. 1944. The toxicity of the ripe fruit of blackbrush tarbush (*Flourensia cernua*) for sheep and goats. *Texas Agric. Exp. Stn. Bull.* No. 664.

NELSON, A. B., HERBEL, C. H., and JACKSON, H. M. 1970. Chemical composition of forage species grazed by cattle on an arid New Mexico range. *New Mexico Agric. Exp. Stn. Bull.* No. 561.

NOLTE, D. L., MASON, J. R., and LEWIS, S. L. 1994. Tolerance of bitter compounds by an herbivore, *Cavia porcellus. J. Chem. Ecol.* 20:303–308.

OH, H. K., SAKAI, T., JONES, M. B., and LONGHURST, W. M. 1967. Effect of various essential oils isolated from Douglas fir needles upon sheep and deer rumen microbial activity. *Appl. Microbiol.* 15:777–784.

PASS, D. M., FOLEY, W. J., and BOWDEN, B. 1998. Vertebrate herbivory on *Eucalyptus*—identification of specific feeding deterrents for common ringtail possums (*Pseudocheirus peregrinus*) by bioassay-guided fractionation of *Eucalyptus* ovata foliage. *J. Chem. Ecol.* 24: 1513–1527.

PROVENZA, F. D., BURRITT, E. A., CLAUSEN, T. P., BRYANT, J. P., REICHARDT, P. B., and DISTEL, R. A. 1990. Conditioned flavor aversion: A mechanism for goats to avoid condensed tannins in blackbrush. *Am. Nat.* 136:810–828.

RAO, M. M., KINGSTON, D. G. I., and SPITTLER, T. D. 1970. Flavonoids from *Flourensia cernua. Phytochemistry* 9:227–228.

SAS Institute. 1989. SAS/STAT User's Guide, Version 6, 4th ed., Vol. 2. SAS Institute, Inc., Cary, North Carolina.

SCHWARTZ, C. C., NAGY, J. G., and REGELIN, W. L. 1980. Juniper oil yield, terpenoid concentration, and antimicrobial effects on deer. *J. Wildl. Manage.* 44:107–113.

SINCLAIR, A. R. E., KREBS, C. J., SMITH, J. N. M., and BOUTIN, S. 1988. Population biology of snowshoe hares. III. Nutrition, plant secondary compounds and food limitation. *J. Anim. Ecol.* 57:787–806.

TELLEZ, M., ESTELL, R., FREDRICKSON, E., POWELL, J., WEDGE, D., SCHRADER, K., and KOBAISY, M. 2001. Extracts of *Flourensia cernua* (L): Volatile constituents and antifungal, antialgal, and antitermite bioactivities. *J. Chem. Ecol.* 27:2265–2277.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

CHEMICAL DEFENSES OF THE SACOGLOSSAN MOLLUSK *Elysia rufescens* AND ITS HOST ALGA *Bryopsis* sp.

MIKEL A. BECERRO,[1,2,*] GILLES GOETZ,[1,3] VALERIE J. PAUL,[2] and PAUL J. SCHEUER[1]

[1]*Department of Chemistry*
University of Hawaii at Manoa
2545 The Mall, Honolulu, Hawaii 96822

[2]*University of Guam Marine Laboratory, UOG Station*
Mangilao, Guam 96923

(Received September 21, 2000; accepted July 15, 2001)

Abstract— Sacoglossans are a group of opisthobranch mollusks that have been the source of numerous secondary metabolites; however, there are few examples where a defensive ecological role for these compounds has been demonstrated experimentally. We investigated the deterrent properties of the sacoglossan *Elysia rufescens* and its food alga *Bryopsis* sp. against natural fish predators. *Bryopsis* sp. produces kahalalide F, a major depsipeptide that is accumulated by the sacoglossan and that shows in vitro cytotoxicity against several cancer cell lines. Our data show that both *Bryopsis* sp. and *Elysia rufescens* are chemically protected against fish predators, as indicated by the deterrent properties of their extracts at naturally occurring concentrations. Following bioassay-guided fractionation, we observed that the antipredatory compounds of *Bryopsis* sp. were present in the butanol and chloroform fractions, both containing the depsipeptide kahalalide F. Antipredatory compounds of *Elysia rufescens* were exclusively present in the dichloromethane fraction. Further bioassay-guided fractionation led to the isolation of kahalalide F as the only compound responsible for the deterrent properties of the sacoglossan. Our data show that kahalalide F protects both *Bryopsis* sp. and *Elysia rufescens* from fish predation. This is the first report of a diet-derived depsipeptide used as a chemical defense in a sacoglossan.

Key Words—Antipredatory role, herbivore–prey relationship, depsipeptides, kahalalide F, sacoglossan mollusks, green algae, *Elysia rufescens*, *Bryopsis* sp.

*To whom correspondence should be addressed at Center for Advanced Studies (CEAB, CSIC), Cami de Sta Barbara s/n, E-17300 Blanes (GI), Spain. E-mail: mikel@ceab.csic.es

[3]Present address: Pharmacia, Searle Discovery Research, Mail Zone GG3D, 700 Chesterfield Parkway North, Chesterfield, Missouri 63198.

0098-0331/01/1100-2287$19.50/0

INTRODUCTION

Sacoglossans (=ascoglossans) are a group of opisthobranch mollusks that feed primarily on siphonaceous green algae (Williams and Walker, 1999). They are highly specialized herbivores that can sequester functional chloroplasts from their diet and use them as a source of photosynthetic energy (Clark et al., 1990; Williams and Walker, 1999). Sacoglossans can also sequester secondary metabolites from their diets or use the sequestered chloroplasts to convert bicarbonate into a variety of carbohydrates that can be used for the synthesis of secondary metabolites (Ireland and Scheuer, 1979; Ireland and Faulkner, 1981; Paul and Van Alstyne, 1988; Gavagnin et al., 2000). It has been hypothesized that these compounds defend sacoglossans against predators. These putative chemical defenses are throught to provide ecological advantages and may function as a driving force in the evolution of this group (Cimino and Ghiselin, 1998).

Although marine natural products chemists have isolated numerous secondary metabolites from sacoglossans (Faulkner, 1992; Avila, 1995), their ecological roles remain largely uninvestigated. Many species synthesize polypropionates (Ireland and Scheuer, 1979; Ireland and Faulkner, 1981; Ksebati and Schmitz, 1985; Dawe and Wright, 1986; Roussis et al., 1990; Di Marzo et al., 1991; Vardaro et al., 1991; Gavagnin et al., 1996). However, these compounds do not show an antipredatory role when they have been experimentally tested (Hay et al., 1989; Roussis et al., 1990). Both the chemically defended sacoglossan *Cyerce nigricans* and its chemically defended host alga *Chlorodesmis fastigiata* contain chlorodesmin (Hay et al., 1989). Although chlorodesmin significantly deters feeding by herbivorous fishes (Paul, 1987; Wylie and Paul, 1988), it does not account for the antipredatory properties of the mollusk (Hay et al., 1989). Two propionate-derived metabolites isolated from the same mollusk species also lacked the deterrent properties of the extracts (Roussis et al., 1990).

Our study focused on the sacoglossan *Elysia rufescens* and its food alga *Bryopsis* sp. Kahalalide F is the major metabolite of a series of amino and fatty acid-derived depsipeptides produced by the green alga *Bryopsis* sp. that are accumalated by *Elysia* spp. (Hamman and Scheuer, 1993; Hamman et al., 1996). Kahalalide F shows a series of in vitro activities against tumor cell lines, viruses, and fungi (Hamman and Scheuer, 1993), but no ecological roles have been investigated. In our study, we investigated whether or not *Bryopsis* sp. and *Elysia rufescens* are chemically defended against generalist fish predators and, if so, whether or not kahalalide F is the compound responsible for the deterrent properties.

METHODS AND MATERIALS

Extraction and Isolation. We collected 5.4 kg wet mass of *Elysia rufescens* and 4.67 kg wet mass of *Bryopsis* sp. by snorkeling at low tide near Black Point,

Oahu, Hawaii, during February 1995. Marilyn Dunlap and Alison Kay identified the animals. A voucher specimen is deposited at the Bernice P. Bishop Museum, Honolulu, BPBM 247679. *Bryopsis* sp. is an undescribed *Bryopsis* species highly abundant in the reef flat at Black Point. In contrast to *Bryopsis* sp. at other sites (Kan et al., 1999, personal observation), the specimens at Black Point lacked macroepophytes. We used bioassay-guided fractionation to isolate and identify ecologically active compounds. We used various solvents to extract and fractionate the extracts from both species along gradients of polarity (Figures 1 and 2). The general goal and approach was the same for the two species, but the specifics of the process varied between *E. rufecens* and *Bryopsis* sp. The sacoglossans were extracted with ethanol (5×3 liters) and dichloromethane (CH_2Cl_2, 2 liters). Ethanol and CH_2Cl_2 extracts from *E. rufescens* were combined (282.3 g) and partitioned between CH_2Cl_2 and water (Figure 1). The aqueous layer was extracted with *n*-BuOH, leaving 216.4 g of aqueous extract after evaporation. The *n*-BuOH layer was combined with the CH_2Cl_2 layer, concentrated (65.9 g), and partitioned between hexanes (30.5 g) and MeOH–H_2O (9 : 1) (35.4 g). The methanol layer was collected and water was added to adjust the MeOH concentration to 60%. Extraction with CH_2Cl_2 and concentration yielded 23 g of CH_2Cl_2 extract and 12.4 g of the aqueous methanol extract. The CH_2Cl_2 fraction was subjected to ODS flash column chromatography, by using a stepwise aqueous methanol gradient (50% MeOH = ODS1, 70% MeOH = ODS2, 90% MeOH = ODS3, 100% MeOH = ODS4, $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) = ODS5). Fraction ODS3 (12.2 g), was subjected to ODS flash column chromatography by using a stepwise aqueous acetonitrile gradient [50% MeCN, 60% MeCN, 70% MeCN, 80% MeCN, 100% MeOH, $CHCl_3$–MeOH–H_2O (CMW, 7 : 3 : 0.5)]. The peptide-containing fractions (monitored by TLC) were combined (8.3 g) and purified on an ODS column by using a stepwise aqueous MeCN gradient solvent system (61%, 62%, 70% aqueous MeCN and 100% MeOH). The kahalalide F-containing fraction eluted with 61% MeCN (4.1 g), and it was passed through an ODS BondElut short column. A reverse-phase HPLC separation (Ultracarb 10 ODS PO; MeOH–H_2O–TFA, 65 : 35 : 0.05) led to 2 g of pure kahalalide F. All the non-kahalalide F-containing fractions and the HPLC side fractions were recombined to reconstitute what was called fraction ODS6 (Figure 1).

The methanol (2×3 liters) and $CHCl_3$–MeOH (1 : 1) (2×3 liters) extracts from *Bryopsis* sp. were combined (159.82 g) and partitioned between $CHCl_3$ and water (Figure 2). The aqueous layer was extracted with *n*-BuOH (4.23 g). The $CHCl_3$ layer was concentrated (11.09 g) and partitioned between hexanes (4.71 g) and MeOH–H_2O (9 : 1) (6.87 g). The methanol layer was collected (2.67 g), and water was added to adjust the MeOH concentration to 60%. Extraction with $CHCl_3$ and concentration yielded 4.2 g of chloroform fraction.

We used TLC and proton nuclear magnetic resonance (NMR spectra measured on a General Electric QE-300 or GN Omega 500 instrument) to detect kahalalide

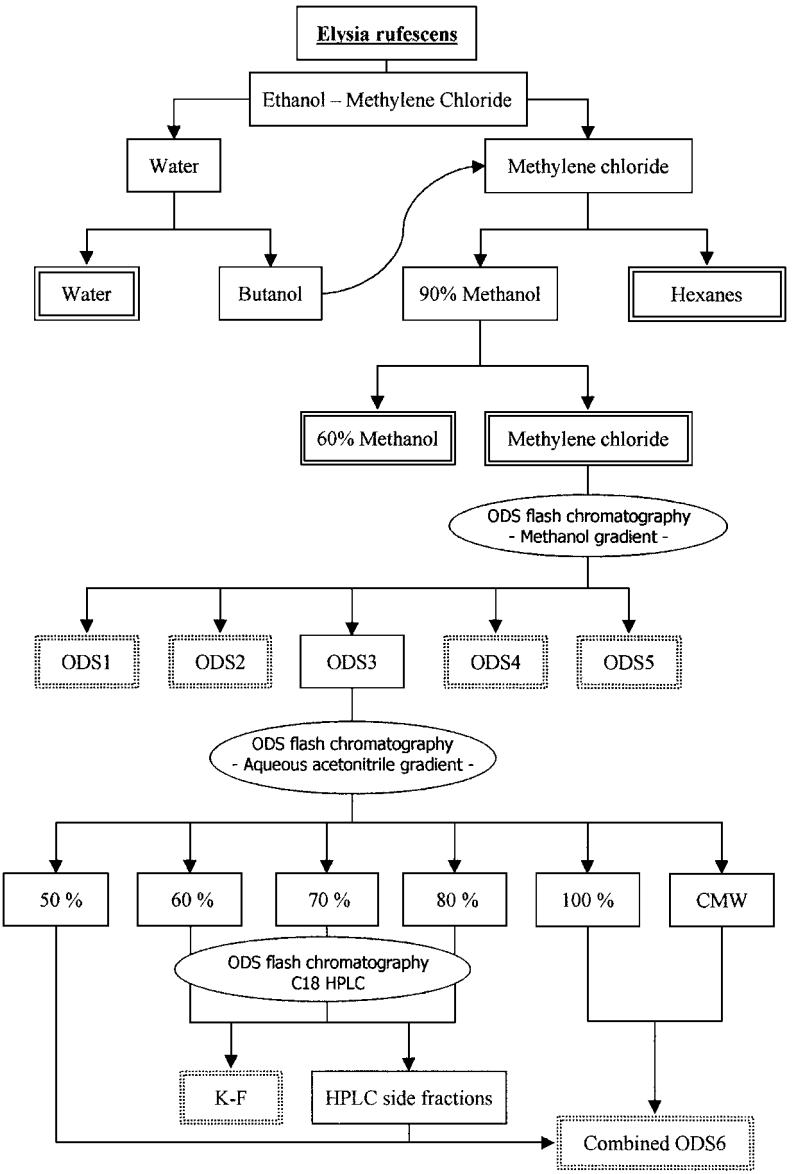


FIG. 1. Scheme of the extraction and partitioning procedure used to obtain secondary metabolites from the sacoglossan *Elysia rufescens*. Fractions enclosed in a double frame were tested at their naturally occurring concentrations against natural fish predators in the field. Fractions sharing the same double frame were tested in the same feeding experiment. See text for more information on the isolation procedure and assay techniques.

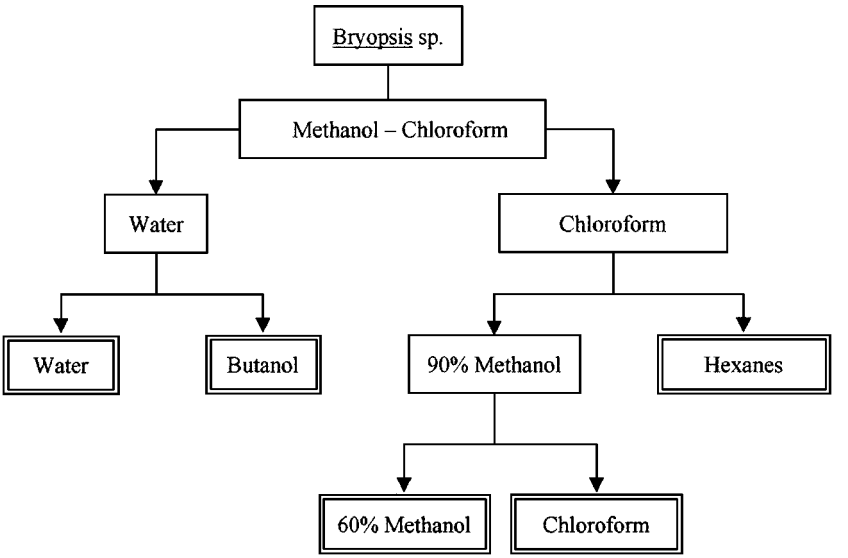


FIG. 2. Scheme of the extraction procedure used to obtain secondary metabolites from the green alga *Bryopsis* sp. Fractions sharing the same double frame were tested at their naturally occurring concentrations against natural fish predators in the same field feeding experiment. See text for more information on the extraction procedure and assay techniques.

F in the fractions and in the mucus produced by *Elysia*. The presence of secondary metabolites in the mucus has been traditionally considered as indirect evidence of the ecological role of the compound (Faulkner, 1992). To collect mucus, we put slugs with seawater in zip-lock bags during collection and then transferred the bags with slugs to containers with ice for transportation to the laboratory. Once in the laboratory we took the slugs out of the seawater, which had taken on a mucilaginous consistency due to the production of mucus by the slugs. We freeze-dried the water–mucus solution, extracted with DCM, and then partitioned using the *Elysia* scheme. Presence of kahalalide F was monitored in the fractions by proton NMR analysis.

Antipredatory Experiments. We used field experiments to determine whether *Bryopsis* sp. and *Elysia rufescens* are chemically defended against a natural coral reef fish assemblage. Methods were similar to those of Becerro et al. (1998). We added extracts from *Bryopsis* sp. and *E. rufescens* to an artificial diet consisting of 5 g of ground food, 2.5 g of carrageenan, and 80 ml of water. We used ground material of the green alga *Enteromorpha* sp. or ground catfish pellets (Kruse's Perfection Brand) in an attempt to better mimic the nutritional characteristics of *Bryopsis* sp. and *E. rufescens* in our artificial diets. We added the necessary amount of extracts, fractions, or compounds relative to wet mass of the food to

match the natural concentration of extracts, fractions, or compounds relative to the wet mass of *Bryopsis* or *E. rufescens*. The actual amount of extract (dissolved in 2 ml of dichloromethane–methanol 1 : 1) added to the mixture varied according to the percent yield (per wet mass) of the particular extract or fraction tested. Control foods were prepared by adding 2 ml of solvent to the carrageenan-food diet. The mixture was poured into 1-cm^3 molds containing a rubber O-ring, so that safety pins could be used to attach cubes to ropes (40 cm long). Each rope contained either four control or four treated food cubes. We placed 20 pairs of control and treated ropes on the reef of Western Shoals, Apra Harbor, Guam. Pairs were removed when approximately half of the cubes were eaten in any of the treatments. We used Wilcoxon signed-ranks test for paired comparisons to test for significant differences in the number of control and treatment cubes eaten.

RESULTS

In field experiments, extracts from *Bryopsis* sp. and *Elysia rufescens* significantly deterred fish predators at naturally occurring concentrations. The antipredatory properties of *Bryopsis* sp. are associated with the butanol and $CHCl_3$ fractions ($P < 0.001$ and $P = 0.01$ respectively, Figure 3). TLC analysis showed that kahalalide F is exclusively present in the active fractions.

The antipredatory properties of *Elysia rufescens* were associated with the methylene chloride fraction ($P = 0.002$, Figure 4), a combination of the butanol and methylene chloride fractions from the first fractionation procedure (Figure 1). Kahalalide F was the only compound responsible for the antipredatory properties of the extract ($P = 0.02$, Figure 5). Kahalalide F was also present in the mucus, as observed by proton NMR, although the concentration at which it occured is unknown.

DISCUSSION

Secondary chemistry seems to play a major role in the biology, ecology, and evolution of mollusks. Sea hares, cephalaspideans, and nudibranchs either sequester secondary metabolites from their diet or synthesize them de novo to use them as chemical defenses (Faulkner, 1992; Avila, 1995). Although the data available for sacoglossan mollusks seem to support a defensive role for these compounds (Gavagnin et al., 1994a), experimental evidence is still scarce and does not always support this hypothesis. Our study provides evidence that the sacoglossan *Elysia rufescens* and its host alga *Bryopsis* sp. are chemically defended against generalist fish predators. Kahalalide F, a major depsipeptide sequestered by *E. rufescens* from the green alga *Bryopsis* sp., is the compound responsible for

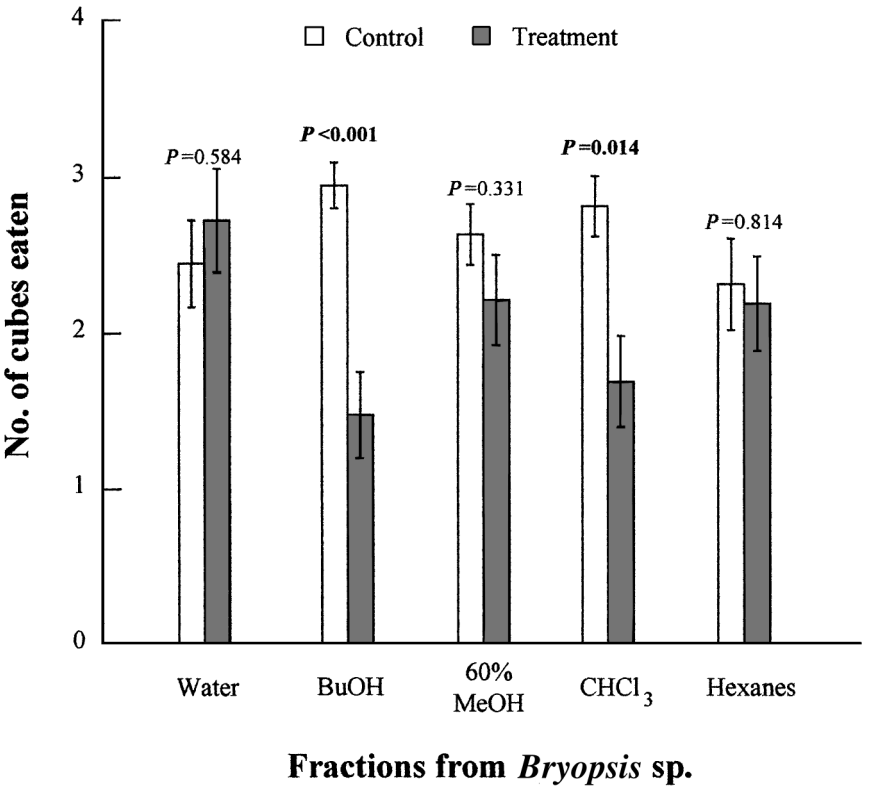


FIG. 3. Feeding deterrence of fractions from the green alga *Bryopsis* sp. towards natural fish consumers in the field. Bars represent average (mean ± 1 SE) number of control (empty bars) and treated (filled bars) cubes eaten in the field by natural fish predators. Mean differences between treatments and their respective controls were tested with Wilcoxon signed-ranks test for paired comparisons. The aqueous (water), butanol (BuOH), 60% methanol (MeOH), chloroform ($CHCl_3$), and hexanes fractions were all tested at their naturally occurring concentrations.

this activity. To our knowledge, this is the first report of a diet-derived depsipeptide used as a chemical defense by sacoglossans.

Sequestration of secondary metabolites is widespread among opisthobranch mollusks. There is ample evidence that sea hares and nudibranchs incorporate biologically active compounds in their tissues and use them for their own defense (Faulkner, 1992; Avila, 1995). Similarly, sacoglossans also accumulate or modify secondary metabolites from their diets. *Elysiella pusilla* (=*Elysia halimedae*) sequesters diterpenoids from its food alga *Halimeda macroloba* and uses them as defense against fish predators (Paul and Van Alstyne, 1988). The sacoglossan

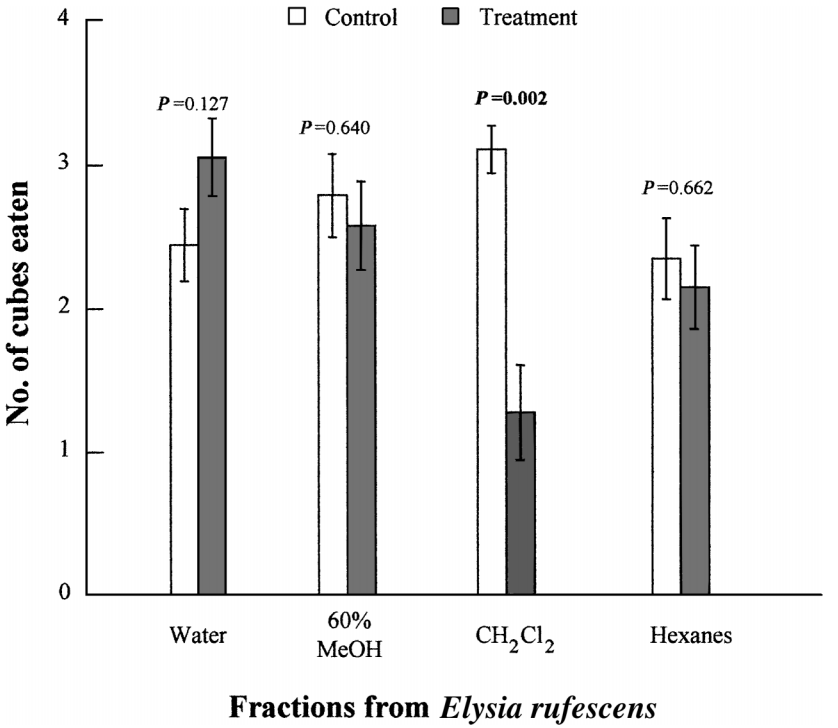


FIG. 4. Feeding deterrence of fractions from the sacoglossan *Elysia rufescens* towards natural fish predators in the field. Bars and statistical values as in Figure 3. The aqueous (water), 60% methanol (MeOH), methylene chloride (CH_2Cl_2), and hexanes fractions were all tested at their naturally occurring concentrations.

Oxynoe panamensis feeds on the chemically defended alga *Caulerpa sertularoides*, from which it incorporates caulerpin and caulerpicin (Doty and Aguilar-Santos, 1970). Chlorodesmin, a diterpenoid from the green alga *Chlorodesmis fastigiata*, significantly deters feeding by some herbivorous fishes (Paul, 1987; Wylie and Paul, 1988). However, the sacoglossan *Cyerce nigricans* specializes on feeding on *Chlorodesmis fastigiata* (Hay et al., 1989), from which it incorporates chlorodesmin. Although the sacoglossan is chemically defended, chlorodesmin does not account for the deterrent properties of the mollusk (Hay et al., 1989). Our data support a deterrent role for the metabolites ingested by *Elysia rufescens* from *Bryopsis* sp. Similarly, the sacoglossan *Costasiella ocellifera* sequesters the brominated compound avrainvilleol from its diet alga *Avrainvillea longicaulis* and uses it as a defense against fish predators (Hay et al., 1990).

The production of polypropionate metabolites by marine mollusks is well documented (Cimino and Sodano, 1993), including that by many sacoglossan

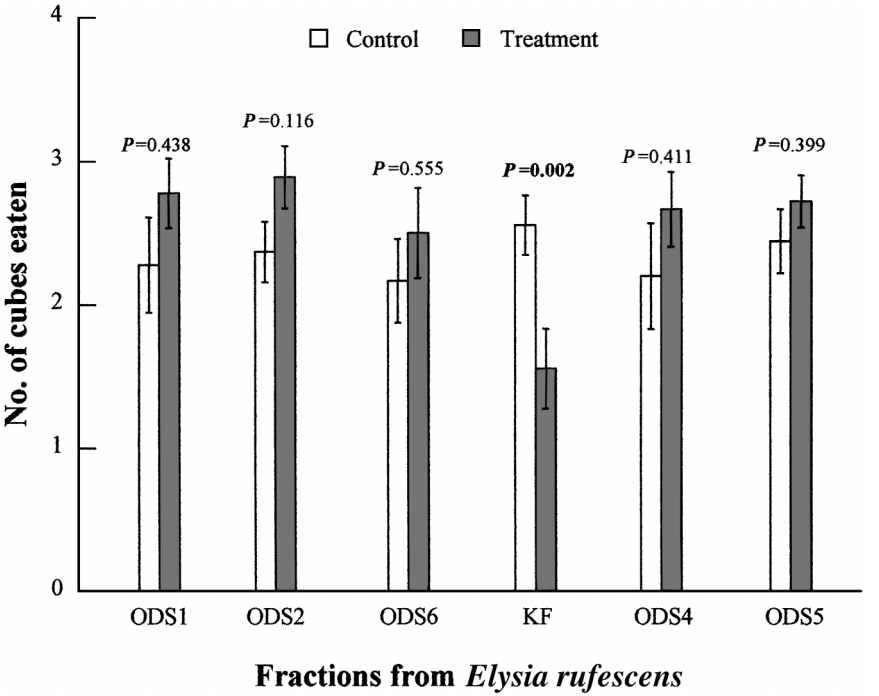


FIG. 5. Feeding deterrence of methylene chloride subfractions from the sacoglossan *Elysia rufescens* towards natural fish predators in the field. Bars and statistical values as in Figure 3. All subfractions were tested at their naturally occurring concentrations. Subfraction labels: ODS1 = 50% methanol, ODS2 = 70% methanol, ODS6 = all compounds (except kahalalide F) obtained from the 90% methanol subfraction, KF = kahalalide F, ODS4 = 100% methanol, ODS5 = chloroform–methanol–water (7 : 3 : 0.5), methylene chloride (CH_2Cl_2), See text and Figure 1 for more information on the partitioning procedure and isolation of kahalalide F).

species (Ireland and Scheuer, 1979; Ireland and Faulkner, 1981; Ksebati and Schmitz, 1985; Dawe and Wright, 1986; Roussis et al., 1990; Di Marzo et al., 1991; Vardaro et al., 1991; Gavagnin et al., 1996). Many polypropionates isolated from mollusks have strong antibacterial (Biskupiak and Ireland, 1983; Dawe and Wright, 1986), cytotoxic (Ksebati and Schmitz, 1985), and ichthyotoxic (Gavagnin et al., 1994b; Vardaro et al., 1991) properties, and crude extracts from species containing polypropionates significantly inhibit feeding by fish predators (Hay et al., 1989). Polypropionates are also present in the mucus of sacoglossans (Di Marzo et al., 1991; Vardaro et al., 1991). Deterrent secretions have been described in many opisthobranchs including sacoglossans (see review by Avila, 1995), and the presence of secondary metabolites in the mucus may be considered as indirect

evidence for the role of these compounds as a defensive mechanism against predators (Faulkner, 1992). The sacoglossan *Elysiella pusilla* (= *Elysia halimedae*) incorporates from its diet *Halimeda macroloba* the aldehyde halimedatetraacetate, which the sacoglossan reduces to its corresponding alcohol and incorporates in high concentrations in its body, mucus, and egg masses (Paul and Van Alstyne, 1988). The alcohol deters feeding by fish predators at natural concentrations (Paul and Van Alstyne, 1988). Polypropionates might function similarly, although experimental data on the sacoglossan *Cyerce nigricans* failed to support a deterrent role for polypropionates (Hay et al., 1989; Roussis et al., 1990), and further research is necessary to establish their role against predation. Whether polypropionates play other biological or ecological roles is uncertain. There is evidence supporting the involvement of cyercenes in the regenerative processes after autotomy of body parts in the sacoglossan *Cyerce cristallina* (Di Marzo et al., 1991), a process widely distributed among sacoglossans that has received little attention (Lewin, 1970; Di Marzo et al., 1991; Trowbridge, 1994).

Our bioassay-guided fractionation procedure shows that kahalalide F is the only compound responsible for the antipredatory properties of *Elysia rufescens*. In the mollusk, kahalalide F is the major metabolite out of a group of several compounds isolated from the mollusk and its dietary alga (kahalalides A–J) (Hamman and Scheuer, 1993; Hamman et al., 1996; Goetz et al., 1997). Kahalalide F is found in *E. rufescens* at concentrations between 0.4% (this study) to 1% (Hamann et al, 1996), which are several orders of magnitude higher than the concentration found in the alga (0.0005%, this study). Kahalalide F shows remarkable clinical bioactivity while the rest of the kahalalides lack significant cytotoxicity (Hamman et al., 1996; Goetz et al., 1997). Although toxicity and deterrent activity are not necessarily related (Pawlik et al., 1995), the diverse biological activity of kahalalide F shows that the same compound may exhibit both clinically oriented and ecologically oriented activities. Since we only performed bioassay-guided fractionation with the extracts from *E. rufescens*, the possibility that the minor, nontoxic kahalalides may help deter predators in *Bryopsis* sp. cannot be completely ruled out. Detailed TLC analyses of every fraction showed that all of the active algal fractions contained kahalalide F, while we found no traces of kahalalide F in the nonactive fractions.

It is worth noting that the concentration at which kahalalide F deters predators in the alga is very low (0.0005% of the algal wet mass). Even if all of the kahalalides contribute to this effect, their total percentage of the algal biomass is 0.0032%. Halimedatetraacetate, the major metabolite in *Halimeda macroloba*, is about 0.2% of the algal wet mass (Paul and Van Alstyne, 1988). Both the sacoglossan *Cyerce nigricans* and its host alga *Chlorodesmis fastigata* contain the cytotoxic diterpenoid chlorodesmin (Hay et al., 1989). Although the sacoglossan is chemically defended against fish predators, chlorodesmin is not responsible for the antipredatory properties of the crude extract when tested at natural

concentrations (less than 1% of the mollusk dry mass) (Hay et al., 1989). Two pyrones from the same sacoglossan species accounted for 0.9% and 0.45% of the dry mass of the mollusk and also failed to account for the repellent properties of the crude extract (Roussis et al., 1990). Because of their low concentrations, minor compounds may be easily overlooked in marine chemical ecology, yet they may play a determinant role in the biology and ecology of benthic organisms. The killer sponge *Dysidea* sp. is chemically defended against fish predators (Thacker et al., 1998). However, the major compound, 7-olepupuane, does not account for the total activity of the extract, suggesting that either addition or synergism of other minor compounds enhances the activity of the major compound (Thacker et al., 1998). Our study may be an example of how minor compounds (kahalalide F, 0.0005% of the algal mass) account for the activities detected in the whole extract.

Elysia rufescens is a highly cryptic but chemically defended species. In fact, *E. rufescens* may have evolved a variety of defensive mechanisms to reduce the chances of predation. We showed that *Bryopsis* sp. is a chemically defended alga, that may provide the sacoglossan an associational refuge (Hay et al., 1990; Hay, 1992; Duffy and Hay, 1994). By feeding on *Bryopsis* sp., *E. rufescens* sequesters algal chloroplasts and makes itself highly cryptic. However, predation may be high on cryptic organisms (Trowbridge, 1994), so the acquisition of other defensive strategies may expand the benefits of crypsis. *E. rufescens* sequesters the antipredatory compound from *Bryopsis*, accumulates the compound up to several times above the concentration in the alga, and becomes chemically defended itself. Moreover, *E. rufescens* releases the antipredatory compound into its mucus, which may be considered as a defensive mechanism to deter predators (Lewin, 1970; Jensen, 1984; Trowbridge, 1994). Predation is an important factor influencing mortality in benthic systems (Lubchenco and Gaines, 1981), and several mechanisms may work together with the same goal. Predator–prey relationships are important processes in marine benthic communities. Many of these relationships are chemically mediated interactions between predators and their prey. By investigating these associations, we will broaden our understanding of the biology and ecology of benthic organisms and the factors that affect the evolution of these predator–prey interactions.

Acknowledgments—We thank D. Galario, D. Ginsburg, C. Lacy, T. Mau, Y. Nakao, C. Nguyen, J. Starmer, and W. Yoshida for their field and laboratory assistance and M. Dunlap and A. Kay for the sacoglossan identification. We also thank staff members of The University of Guam Marine Laboratory and The University of Hawaii Chemistry Department for providing not only their facilities but also a nice environment in which to work. Comments from two anonymous reviewers greatly improved this manuscript and are sincerely appreciated. This research was supported by a Basque Government Postdoctoral Fellowship to M.A.B. and funded by NIH grant GM38624 to V.J.P. and Sea Grant College Program and PharmaMar project to G.G. and P.J.S. This is contribution 454 of the University of Guam Marine Laboratory.

REFERENCES

AVILA, C. 1995. Natural products from opisthobranch mollusks: A biological review. *Oceanogr. Mar. Biol. Annu. Rev.* 33:487–559.

BECERRO, M. A., PAUL, V. J., and STARMER, J. 1998. Intraspecimen variation in chemical defenses of the sponge *Cacospongia* sp. and its consequences on generalist fish predators and the specialist nudibranch predator *Glossodoris pallida*. *Mar. Ecol. Prog. Ser.* 168:187–196.

BISKUPIAK, J. E. and IRELAND, C. M. 1983. Pectinatone, a new antibiotic from the mollusk *Siphonaria pectinata*. *Tetrahedron Lett.* 24:3055–3058.

CIMINO, G. and GHISELIN, M. T. 1998. Chemical defence and evolution in the Sacoglossa (Mollusca: Gastropoda: Opisthobranchia). *Chemoecology* 8:51–60.

CIMINO, G. and SODANO, G. 1993. Biosynthesis of secondary metabolites in marine mollusks, pp. 78–115, *in* P. J. Scheuer (ed.). Topics in Current Chemistry 167. Springer, Berlin, Germany.

CLARK, K. B., JENSEN, K. R., and STIRTS, H. M. 1990. Survey for functional kleptoplasy among Atlantic Ascoglossa (=Sacoglossa) (Mollusca: Opisthobranchia). *The Veliger* 33:339–345.

DAWE, R. D. and WRIGHT, J. L. C. 1986. The major polypropionate metabolites from the sacoglossan mollusk, *Elysia chlorotica*. *Tetrahedron Lett.* 27:2559–2562.

DI MARZO, V., VARDARO, R. R., DE PETROCELLIS, L., VILLANI, G., MINEI, R., and CIMINO, G. 1991. Cyercenes, novel pyrones from the ascoglossan mollusk *Cyerce cristallina*. Tissue distribution, biosynthesis and possible involvement in defense and regenerative processes. *Experientia* 47:1221–1227.

DOTY, M. S. and AGUILAR-SANTOS, G. 1970. Transfer of toxic algal substances in marine food chains. *Pac. Sci.* 24:351–355.

DUFFY, J. E. and HAY, M. E. 1994. Herbivore resistance to seaweed chemical defense: The roles of mobility and predator risk. *Ecology* 75:1304–1319.

FAULKNER, D. J. 1992. Chemical defenses of marine mollusks, pp. 119–163, *in* V. J. Paul (ed.). Ecological Roles of Marine Natural Products. Comstock Publishing Associates, Ithaca, New York.

GAVAGNIN, M., MARIN, A., CASTELLUCCIO, F., VILLANI, G., and CIMINO, G. 1994a. Defensive relationships between *Caulerpa prolifera* and its shelled sacoglossan predators. *J. Exp. Mar. Biol. Ecol.* 175:197–210.

GAVAGNIN, M., SPINELLA, A., CASTELLUCCIO, F., CIMINO, G., and MARIN, A. 1994b. Polypropionates from the Mediterranean mollusk *Elysia timida*. *J. Nat. Prod.* 57:298–304.

GAVAGNIN, M., MOLLO, E., CIMINO, G., and ORTEA, J. 1996. A new γ-Dihydropyrone propionate from the Caribbean sacoglossan *Tridachia crispata*. *Tetrahedron Lett*. 37:4259–4262.

GAVAGNIN, M., MOLLO, E., MONTARANO, D., ORTEA, J., and CIMINO, G. 2000. Chemical studies of Caribbean sacoglossans: Dietary relationships with green algae and ecological implications. *J. Chem. Ecol*. 26:1563–1578.

GOETZ, G., NAKAO, Y., and SCHEUER, P. J. 1997. Two acyclic kahalalides from the sacoglossan mollusk *Elysia rufescens*. *J. Nat. Prod.* 60:562–567.

HAMMAN, M. T. and SCHEUER, P. J. 1993. Kahalalide F: A bioactive depsipeptide from the sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J. Am Chem. Soc*. 115:5825–5826.

HAMMAN, M. T., OTTO, C. S., SCHEUER, P. J., and DUNBAR, D. C. 1996. Kahalalides: Bioactive peptides from a marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. *J. Org. Chem.* 61:6594–6600.

HAY, M. E. 1992. The role of seaweed chemical defenses in the evolution of feeding specialization and in the mediation of complex interactions, pp. 93–118, *in* V. J. Paul (ed.). Ecological Roles of Marine Natural Products. Comstock Publishing Associates, Ithaca, New York.

HAY, M. E., PAWLIK, J. R., DUFFY, J. E., and FENICAL, W. 1989. Seaweed-herbivore-predator interactions: Host–plant specialization reduces predation on small herbivores. *Oecologia* 81:418–427.

HAY, M. E., DUFFY, J. E., PAUL, V. J., RENAUD, P. E., and FENICAL, W. 1990. Specialist herbivores reduce their susceptibility to predation by feeding on the chemically defended seaweed *Avrainvillea longicaulis*. *Limnol. Oceanogr.* 35:1734–1743.

IRELAND, C. and FAULKNER, D. J. 1981. The metabolites of the marine mollusks *Tridachiella diomedea and Tridachnia crispata*. *Tetrahedron* 37(suppl. 1):233–240.

IRELAND, C. and SCHEUER, P. J. 1979. Photosynthetic marine mollusks: In vivo ^{14}C incorporation into metabolites of the sacoglossan *Placobranchus ocellatus*. *Science* 205:922–923.

JENSEN, K. R. 1984. Defensive behavior and toxicity if ascoglossan opisthobranch *Mourgana germaineae* Marcus. *J. Chem. Ecol.* 10:475–486.

KAN, Y., FUJITA, T., SAKAMOTO, B., HOKAMA, Y., and NAGAI, H. 1999. Kahalalide K. A new cyclic depsipeptide from the Hawaiian green alga *Bryopsis* species. *J. Nat. Prod.* 68:1169–1172.

KSEBATI, M. B. and SCHMITZ, F. J. 1985. Tridachiapyrones: Propionate-derived metabolites from the sacoglossan mollusk *Tridachia crispata*. *J. Org. Chem.* 50:5637–5642.

LEWIN, R. A. 1970. Toxin secretion and tail autonomy by irritated *Oxynoe panamensis* (Opisthobranchiata; Sacoglossa). *Pac. Sci.* 24:356–358.

LUBCHENCO, J. and GAINES, S. D. 1981. A unified approach to marine plant–herbivore interactions. I. Populations and communities. *Annu. Rev. Ecol. Syst.* 12:405–437.

PAUL, V. J. 1987. Feeding deterrent effects of algal natural products. *Bull. Mar. Sci*. 41:514–522.

PAUL, V. J. and VAN ALSTYNE, K. L. 1988. Use of ingested algal diterpenoids by *Elysia halimeade* Macnae (Opisthobranchia: Ascoglossa) as antipredator defenses. *J. Exp. Mar. Biol. Ecol.* 119: 15–29.

PAWLIK, J. R, CHANAS, B., TOONEN, R. J., and FENICAL, W. 1995. Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. *Mar. Ecol. Prog. Ser.* 127:183–194.

ROUSSIS, V., PAWLIK, J. R., HAY, M. E., and FENICAL, W. 1990. Secondary metabolites of the chemically rich ascoglossan *Cyerce nigricans*. *Experientia* 49:327–329.

THACKER, R. W., BECERRO, M. A., LUMBANG, W. A., and PAUL, V. J. 1998. Allelopathic interactions between sponges in a tropical reef community. *Ecology* 79:1740–1750.

TROWBRIDGE, C. D. 1994. Defensive responses and palatability of specialist herbivores: Predation on NE Pacific ascoglossan gastropods. *Mar. Ecol. Prog. Ser.* 105:61–70.

VARDARO, R. R., DI MARZO, V., CRISPINO, A., and CIMINO, G. 1991.Cyercenes, novel polypropionate pryrones from the autotomizing Mediterranean mollusk *Cyerce cristallina*. *Tetrahedron* 47:5569–5576.

WILLIAMS, S. I. and WALKER, D. I. 1999. Mesoherbivore–macroalgal interactions: Feeding ecology of sacoglossan sea slugs (Mollusca, Opisthobranchia) and their effects on their food algae. *Oceanogr. Mar. Biol. Annu. Rev.* 37:87–128.

WYLIE, C. R. and PAUL, V. J. 1988. Feeding preferences of the surgeonfish *Zebrasoma flavenses* in relation to chemical defenses of tropical algae. *Mar. Ecol. Prog. Ser.* 45:23–32.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

CHEMICAL ORIENTATION OF BROWN BULLHEADS, *Ameiurus nebulosus*, UNDER DIFFERENT FLOW CONDITIONS

MARILYN L. SHERMAN and PAUL A. MOORE*

Laboratory for Sensory Ecology
J.P. Scott Center for Neuroscience
Mind and Behavior Department of Biological Sciences
Bowling Green State University
Bowling Green, Ohio 43403

(Received November 7, 2000; accepted July 15, 2001)

Abstract—The spatiotemporal information in chemical signals provides critical information for organisms during chemical orientation. Information in chemical signals is influenced by the hydrodynamic conditions of the environment. Hydrodynamically distinct environments will contain different types of information, which will influence how organisms orient. This study was designed to examine how the orientation behavior of the brown bullhead (*Ameiurus nebulosus*) is influenced by flow regime. The experiment was conducted in a flume under two different flow conditions. Treatments consisted of control (no odor) and plain gelatin (odor). Percent success, swimming speed, turning angle, heading angle, heading angle upstream, and net-to-gross ratio were analyzed. Brown bullheads were 100% successful in finding the odor source under no flow and 57% successful in flow. Bullheads swam differently in the no-flow condition when compared to the flow condition. Since, these fish did not orient the same under different flow conditions, it appears that hydrodynamics plays a role in shaping their behavior.

Key Words—Chemical orientation, catfish, swimming behavior, hydrodynamics, chemical signal.

INTRODUCTION

For many organisms, chemical signals are important sources of information that influence behaviors such as orienting toward food, attracting mates, avoiding predators, or determining social status (Lewis and Gower, 1980; Croll and Chase, 1980; Carr, 1988; Tierney and Atema, 1988; Breithaupt and Atema, 1993; Chivers and Smith, 1993; Sorensen and Scott, 1994; Hazlett, 1994; Chivers et al., 1996; Zippel et al., 1997; Bradbury and Vehrencamp, 1998). For instance, starfish are able to

*To whom correspondence should be addressed. E-mail: pmoore@bgnet.bgsu.edu

0098-0331/01/1100-2301$19.50/0

respond to a chemical stimulus and orient toward a food source in no-flow conditions (Moore and Lepper, 1997). Ovaries from female frillfin gobies develop a chemical that elicits courtship behavior in males, even in the absence of females (Tavolga, 1956). Western toad tadpoles (*Bufo boreas*) are able to recognize predator odors. They will display antipredator behavior when exposed to chemical cues from familiar predators, but will not display this behavior toward unfamiliar predators (Kiesecker et al., 1996). Yellow bullheads have been found to use chemical signals in recognizing individuals and associating odors with rank in hierarchies. Subordinate fish will avoid an area of an aquarium where water from the tank of a dominant fish has been introduced, even though the dominant fish is not present (Bardach and Todd, 1970).

One of the better-studied aspects of chemical signals is chemical orientation. Chemical orientation is defined as the directional movement of an organism toward an odor source (van der Steen and Maat, 1979). This orientation behavior is also observed in a variety of terrestrial organisms (Kennedy, 1986; Belanger and Arbas, 1998; Willis and Arbas, 1998; Takken et al., 1997). Aquatic organisms, in particular, have the ability to orient toward food, mates, home streams, parents, or offspring (Bardach et al., 1967; Tosi and Sola, 1993; Atema et al., 1980; Stabell, 1992; Weissburg and Zimmer-Faust, 1994). During orientation, fish exposed to chemical stimuli, such as food, display characteristic maneuvers that help them locate the odor source. Dogfish use a figure-eight pattern when searching for food (Parker, 1912), whereas sea robins use an s-shaped or zigzag search pattern when swimming up an odor gradient (Bardach and Case, 1965).

In order for chemical orientation to occur, an organism must be stimulated by a putative cue and must extract directional and distance information from it. Distance and direction information can be gained from a variety of cues, such as wind, flow direction, electroreception, and chemicals (Able, 1991). In the case of a chemical signal, there are two main characteristics that can provide information: quality and quantity of the signal. The quality is the type of chemical compounds that are stimulatory for that organism. It provides the organism with information on source type, i.e., food, predator, and reproductive condition (Sorensen et al., 1989; Schwenk, 1995; Carr et al., 1996; Rasmussen and Schulte, 1998; Swaisgood et al., 1999). For example, gravid garter snake females release nonvolatile methyl ketones from cutaneous glands along the ground as they travel, providing information to males, which follow the females (Mason et al., 1989). Cichlids secrete proteinaceous mucus from their bodies, which maintains contact between the brood and parents, as well as provides food for the young (Barlow, 1974). Several common metabolites of low molecular weight have been found to be feeding stimulants (Carr, 1988). Other types of metabolites such as polyketides or alkaloids are feeding deterrents (Carr, 1988).

The quantity of chemical signals and their distribution in time and space can provide distance and direction information (Atema, 1988; Moore and Atema,

1991; Moore et al., 1994; Murlis, 1986, 1987; Murlis et al., 1992; Vickers, 2000; Zimmer-Faust et al., 1995). At macroscopic size scales, the spatial and temporal information in a chemical signal is structured primarily by fluid flow (Moore et al., 1994; Vickers, 2000). When an odor is released, the concentration fluctuates, creating a signal that is patchy in space and time. This patchiness consists of intermittent plumes in the water column with patches of high, low, or zero odor concentration. As the odor plume moves away from the source it spreads horizontally and vertically. (Murlis and Jones, 1981; Zimmer-Faust et al., 1995; Finelli et al., 1999; Moore et al., 2000). The degree of patchiness is influenced by the hydrodynamic conditions of the particular flow environment (Moore et al., 1994; Weissburg, 1997). Environments with different flow characteristics will consequently have different signal characteristics (Moore et al., 2000). In order to orient efficiently, animals need olfactory behaviors and physiological properties of sensory systems that can extract information from odor fluctuations (Moore et al., 1994; Atema, 1995).

Catfish provide an excellent opportunity to address questions concerning the influence of flow on orientation behavior. Ictalurid catfish are a model system for examining the role of chemical signals in orientation. Many of the species are nocturnal in habit and found in habitats of slow-moving or still waters with poor visibility, whereas others are found in turbulent flow conditions. For example, brown bullheads (*Ameiurus nebulosus*) are typically found in no-flow or low-flow conditions (backwater areas of rivers, ponds, and lakes). These fish are morphologically suited for environments dominated by chemical information (Bardach and Todd, 1970). Their bodies are covered with thousands of external taste buds, as well as dense concentrations on the barbels (Bardach and Todd, 1970; Atema, 1971). The physiological aspects of olfaction and gustation in channel catfish and bullheads have been extensively studied (Caprio and Raderman-Little, 1978; Caprio and Byrd Jr., 1984; Caprio et al., 1989; Miyamoto et al., 1990; Kang and Caprio, 1991; Restrepo et al., 1990; Kanwal and Finger, 1997). However, the behavioral aspects of orientation have not been thoroughly studied. The purpose of this study was to test the hypothesis that catfish, which are adapted to a particular hydrodynamic condition, orient more successfully to an odor source in their normal hydrodynamic environment than in one to which they are not adapted.

METHODS AND MATERIALS

Animals. Brown bullheads (*Ameiurus nebulosus*) were bought from Northeast Aquatics and placed in community tanks for observation over several months. This observation time allowed the fish to become accustomed to their new environment. The mean size for bullheads was 21 ± 0.72 cm. Fish were kept on a reversed 12L : 12D schedule. Fish from the community tank were randomly caught and

isolated in 10-gal tanks (25 × 31 × 51 cm) for at least two weeks before experimentation. Temperature ranged from 55 to 60°F in the winter and to 65°F in the summer. Both community and isolation tanks were routinely cleaned. Diet consisted of goldfish food (Wardley Pond Ten), fish (whitefish or pollack), and catfish food (Net Profit fish food). Fish were fed with the main light on either late morning or early afternoon. Past research has shown that brown bullheads have the ability to exhibit diurnal phasing, showing no or less-pronounced negative phototactic behavior (Eriksson 1978; Eriksson and van Veen, 1980). It has also been shown that, in nature, when there is a rise in water level, catfish will forage during the day (Pflieger, 1975). Valentinčič et al. (1994) reported that channel catfish fed uninhibitedly in a lighted room within a week of being moved to a test tank.

Test Arena. All orientation experiments were conducted in a plywood flow tank (244 × 57.3 × 62 cm; Figure 1). The test section of the flume was 212 cm long. The start gate was placed at the downstream section of the tank. This holding area was 26.5 × 58 cm. The odor source was placed at the upstream section of

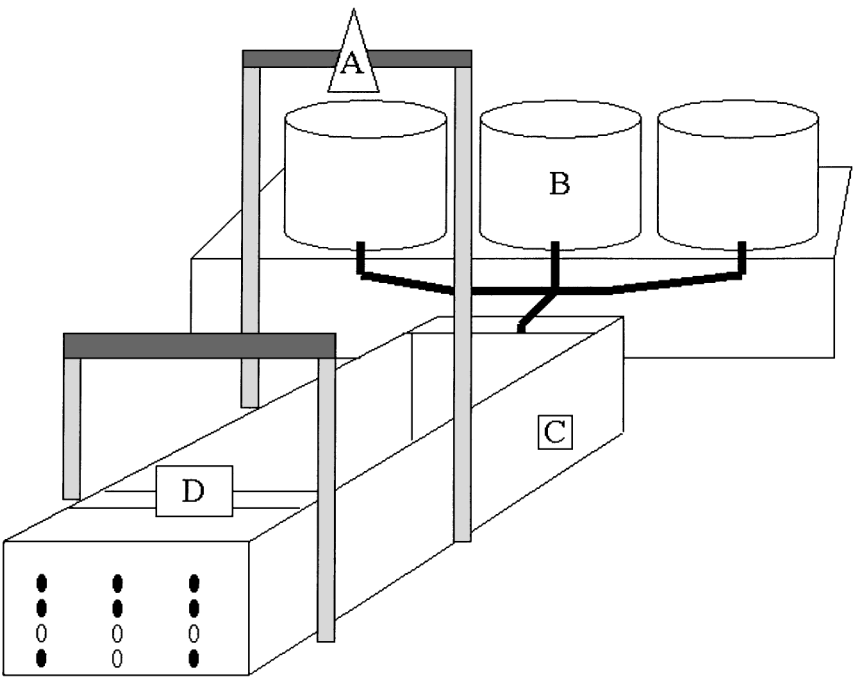


FIG. 1. Diagram of the experimental tank. The triangle with A shows camera location; B represents the head tanks. The flow tank is labeled C and the start gate is labeled D.

the tank, which was placed at least 15 cm away from the upstream collimator. The source was 29 cm from each side of the tank and 22 cm from the head collimator. The odor source was 197 cm away from start gate. This allowed test subjects to move beyond the odor source. Water was kept at a constant depth of 16 cm. Flow velocities were calculated by visually timing a neutrally buoyant object's movement over a given distance five times and taking the average time in seconds. Flow velocity during the flow trials was 4.2 ± 0.88 cm/sec and 0.0 ± 0.0 cm/sec during the no-flow trials. Flume water was stored in head tanks above the flow tank and was allowed to age for two days before use. Head tanks held a total of 115 gal for refilling the flow tank during no-flow trials and providing the water for the gravity feed in the flow trials. After each trial, the tank was drained completely and fresh and distilled water was used to rinse down the tank before the next trial.

Odor Source. Treatments consisted of a control (empty mesh bag), plain gelatin, or fish gelatin. Gelatin has been used as a source of food and a method of delivery for specific amino acid supplementation to test subjects (Keen, 1982; Sarwar and Ratnayake 2000; Buentello and Gatlin III, 2000). Preliminary data showed that plain gelatin produced a stronger response from the research subjects than fish gelatin. Those that did respond to the fish gelatin did not show any overall difference in swimming parameters, and so the data with the fish odor were dropped from further analysis. The odor sources were placed into a mesh bag, which was tied to the head collimator by a 16-cm piece of clear fish line. Weights (6 g) were used to hold down the bag. Four Knox gelatin packages were added to four cups of hot water and placed into a plastic container (30×16 cm). This was stirred together until everything was mixed. It was then placed into the refrigerator. Gel blocks ($2.5 \times 4 \times 2$ cm) were cut from the larger dish of prepared gelatin. The odor treatment was placed into the tank at least 30 sec before the start gate was opened.

Dye Trials. Dye trials were carried out for the no-flow treatment to quantify how fast the odor moved in the flume. 1-$\times$ 2-cm squares were measured, drawn, and numbered on the floor of the tank with a permanent marker. Grid sheets were used for visual marking of the odor trail during a 5-min trial. The dye gel block was made following the same recipe as the odor gel blocks (Moore and Lepper, 1997). The dye gelatin was cut into 1- $\times$ 2-cm squares instead of the size used for the experiment because the dye blocks kept breaking in half. Once placed into the flume, dye filaments would come off the gel block before timing and dissipate quickly, moving about the tank. Once the block was sitting on the bottom of the tank, timing started and the number of 1- $\times$ 2-cm squares covered in dye were marked out on the grid sheet until the 5-min experimental period was up. The number of squares was counted and the average dye movement in no flow was calculated to be 0.056 cm^2/sec.

Experimental Design. Fish were starved for two days before they were used in a trial, and each was given a week between tests to avoid any learning biases. Separate groups were tested for flow ($N = 7$) and no flow ($N = 7$); however, a single fish was randomly tested under all odor conditions within a single flow condition.

Presentation of odor was randomized and each fish was given a week between trials. A randomly chosen fish was placed into the start gate section of tank and given 20 min to acclimate. After 20 min fish were resting on the bottom of tank in the start section. The start gate was slowly opened and left open so fish had the option to stay in this section. The acclimation time was chosen based on other fish research (Bardach et al., 1967; Ellingsen and Døving, 1986; Nevitt, 1991; Giaquinto and Volpato, 1997; Smith, 2000), which examined a variety of different acclimation times and fish behaviors. A camera (Quazar model VM-52 SVHSC CCD) was set 3 ft above the flow tank and connected to a Hi-Fi Stereo Hi 8 recorder (NTSC EV-C100). Recording was carried out under red lights. The no-flow trials were taped for 20 min, and the flow trials were taped for 4.5 min or until the catfish first hit the odor source. Behavioral trials were conducted until an animal successfully located the odor source. A trial was defined successful when the test subject touched the odor source with its mouth.

Data Analysis. The Peak Motus Motion Analysis System was used to digitize orientation paths under both flow conditions. A fish's position was digitized every second. Animals took an average of 30 sec in no flow and 32 sec in flow to locate the odor source. Therefore, control groups were digitized for 30 sec for the no flow and 32 sec for the flow. The coordinate (x, y) data were then put into an in-house standard orientation template to calculate speed, turning angle, heading angle, heading angle upstream, and net-to-gross ratio (Moore et al., 1991) (Figure 2). When calculating the angles, the right and left angles were not differentiated; hence all angles fall between 0 and 180°. This type of analysis allows us to perform statistical analysis with standard linear statistics (e.g., MANOVAs). Circular statistics are slightly more conservative than MANOVAs. Performing an analysis using linear statistics allows us to analyze the fine details of fish movement patterns. The consequences for a linear analysis is that it is possible for a zig-zag pattern typically found in moths to have a mean heading angle of 60°. For statistical analysis, a single mean for each parameter was calculated from the total orientation path. The total path means were subsequently used for all statistical analysis as the data points that were put into the two-way MANOVA. A two-way MANOVA was used to analyze speed, speed towards source, turning angle, heading angle, heading angle upstream, and net-to-gross ratio for bullheads. All significant P values were set at 0.05. A Fisher's exact test was used to analyze percent success.

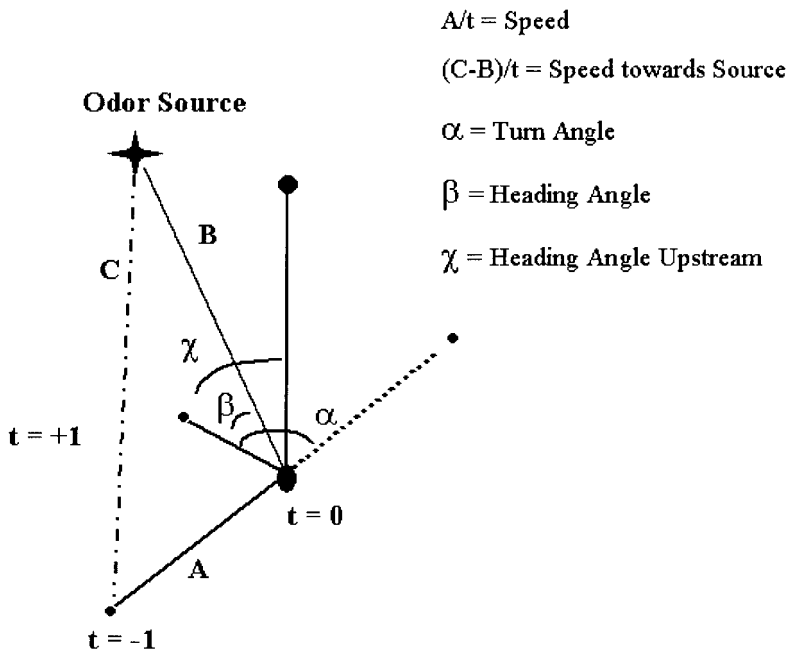


FIG. 2. Diagram shows three successive positions ($t = -1$, 0, and $+1$:•) on a hypothetical orientation path used to define behavioral parameters. The dashed line represents the catfish's projected path (if it continued in a straight line), from which a turn angle value (α) at point $t = 0$ is calculated. The solid line (B) represents the straight line to the odor source (four point star) from which heading angle relative to the odor source (β) is calculated. The unlabeled line represents the path that is straight upstream, from which the heading angle relative to upstream (χ) is calculated. Swimming speed (A) is the distance moved from time point $t = -1$ to $t = 0$. The difference between distance to the odor source at time $t = -1$ (dash–dot line, C) and distance to the odor source at time $t = 0$ (solid line, B) is the swimming speed toward the source.

RESULTS

Success at Finding Odor Source. Brown bullheads were successful in locating the odor source under both flow and no flow conditions. Seven of seven brown bullheads (100%) found the odor source under no flow. Four of seven bullheads (57%) were successful in finding the odor source under flow. The Fisher exact test showed no significant difference between the two odor treatments (Fisher exact, one-tailed; $P > 0.09$).

Qualitative Description of Orientation Behavior. In no flow, catfish swam in a more linear fashion when odor was present. In general, fish made fewer turns

while swimming toward the odor source. Fish were seen mouthing the mesh bag once they found the odor source. During the control trials, fish swam around the tank and did not investigate the mesh bag. They could be seen swimming along the edges more than individuals in the odor trials. The catfish pathway was more circuitous under flow for both treatments. An example of swimming paths for each treatment is illustrated in Figure 3.

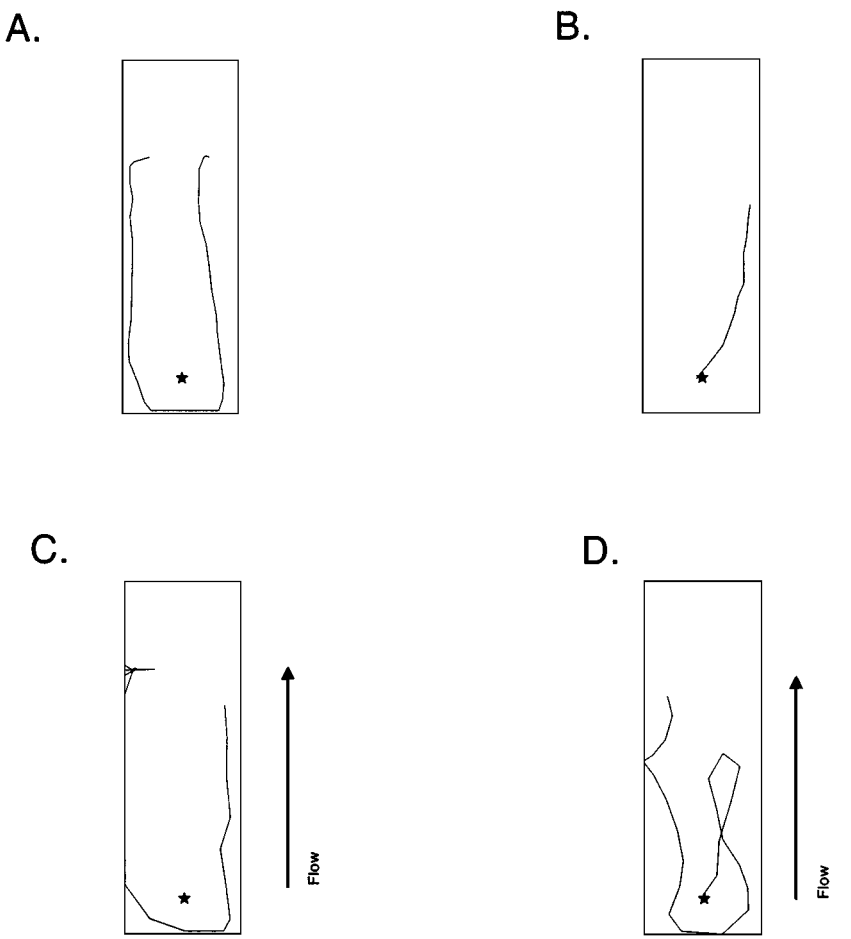


FIG. 3. (**A**) A representation of the path made by a bullhead under no flow and no odor source. (**B**) A typical path made by bullheads with an odor source in no flow. (**C**) A representation of the path made by a bullhead under flow without an odor source. (**D**) A typical path made by bullheads with an odor source under flow. The star represents the odor source (or contial), and the arrows illustrate the direction of the flow.

General Effect. Brown bullheads oriented differently when an odor was present (MANOVA, Rao's $R = 7.04$, $P < 0.01$). They also oriented differently under flow and no flow (MANOVA, Rao's $R = 5.59$, $P < 0.02$). This effect was seen in the net-to-gross ratio, swimming speed, heading angle upstream, heading angle toward source, and turning angle toward source. The post-hoc analysis provided a more detailed view of this behavior.

Effect of Flow and Odor on Swimming Speed. Brown bullheads swam significantly faster under flow (6 ± 2 sec) conditions than during no flow (14 ± 2 sec) in the control trials (Figure 4; Newman-Keuls post-hoc, $P < 0.02$). In addition, brown bullheads also swam faster when stimulated with an odor source in flow (13 ± 1 sec) than no flow (19 ± 1 sec) (Newman-Keuls post-hoc, $P < 0.005$).

Orientation Paths. Brown bullheads swam a straighter path when an odor was present (indicated by a higher net-to-gross ratio) in the no flow conditions (0.85 ± 0.1) (Figure 4; Newman-Keuls post-hoc, $P < 0.001$) as compared to the flow condition (0.19 ± 0.04). Brown bullheads swam more randomly in no flow (0.22 ± 0.09) and flow (0.10 ± 0.01) controls (blanks) (indicated by the lower net-to-gross ratio) (Newman-Keuls post-hoc, $P > 0.05$). Turning angle toward source was lower for brown bullheads during odor trials in no flow ($16 \pm 3^\circ$) condition than flow ($35 \pm 2^\circ$) (Figure 5; Newman-Keuls post-hoc, $P < 0.002$). However, there was no difference in turning angle for the control trials in no flow ($24 \pm 8^\circ$) or flow ($38 \pm 7^\circ$) (Newman-Keuls post-hoc, $P > 0.05$). The heading angle relative to an odor source measures the accuracy of an organism's movement toward an odor source. Brown bullheads had a lower heading angle while orienting in no flow ($23 \pm 9^\circ$) than flow ($68 \pm 4^\circ$) condition (Figure 5, Newman-Keuls post-hoc, $P < 0.001$). The control group in no flow ($55 \pm 15^\circ$) did not differ from the control group in flow ($76 \pm 4^\circ$) for heading angle toward source (Newman-Keuls post-hoc, $P > 0.05$). Brown bullheads had a lower heading angle upstream when orienting to an odor source in no flow ($23 \pm 10^\circ$) condition (Figure 5, Newman-Keuls post-hoc, $P < 0.02$) as compared to flow ($61 \pm 10^\circ$). There was no difference in heading angle upstream between the control group in no flow ($57 \pm 16^\circ$) and flow ($75 \pm 5^\circ$) (Newman-Keuls post-hoc, $P > 0.05$).

DISCUSSION

The results of this experiment indicated two important conclusions for catfish orientation. First, it confirmed our expectations that brown bullheads would orient more successfully under no-flow conditions compared to flow conditions. Although not significantly different, brown bullheads appeared to have a higher success rate in finding the odor source under no flow (100%) than flow (57%). These trends are in the direction of our hypothesis, but the conservative nature of the Fisher exact test does not show significant differences between these two

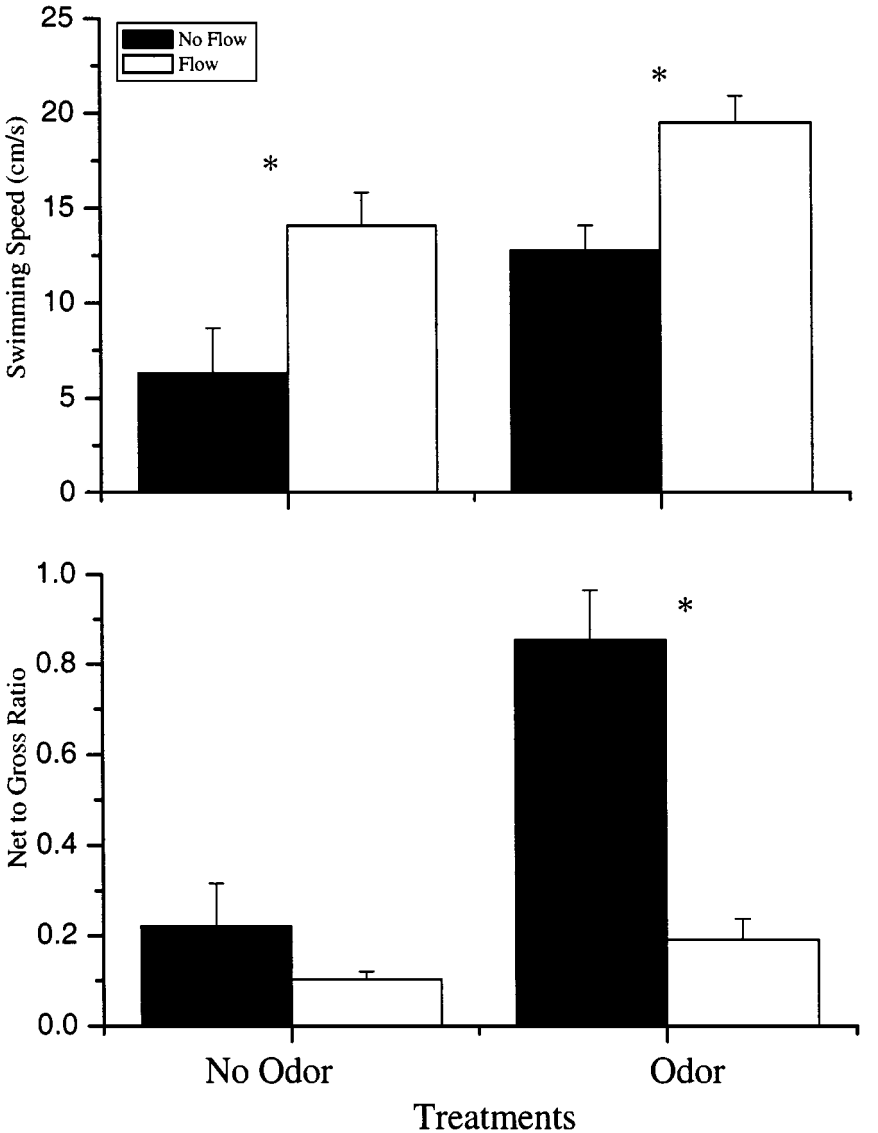


FIG. 4. The upper graph illustrates the swimming speed of bullheads in no-flow (black) and flow (white) conditions. The bottom graph is the net-to-gross ratio of bullheads in no flow (black) and flow (white). An asterisk indicates a significant difference using MANOVA followed by post-hoc test. $N = 7$ for each flow treatment.

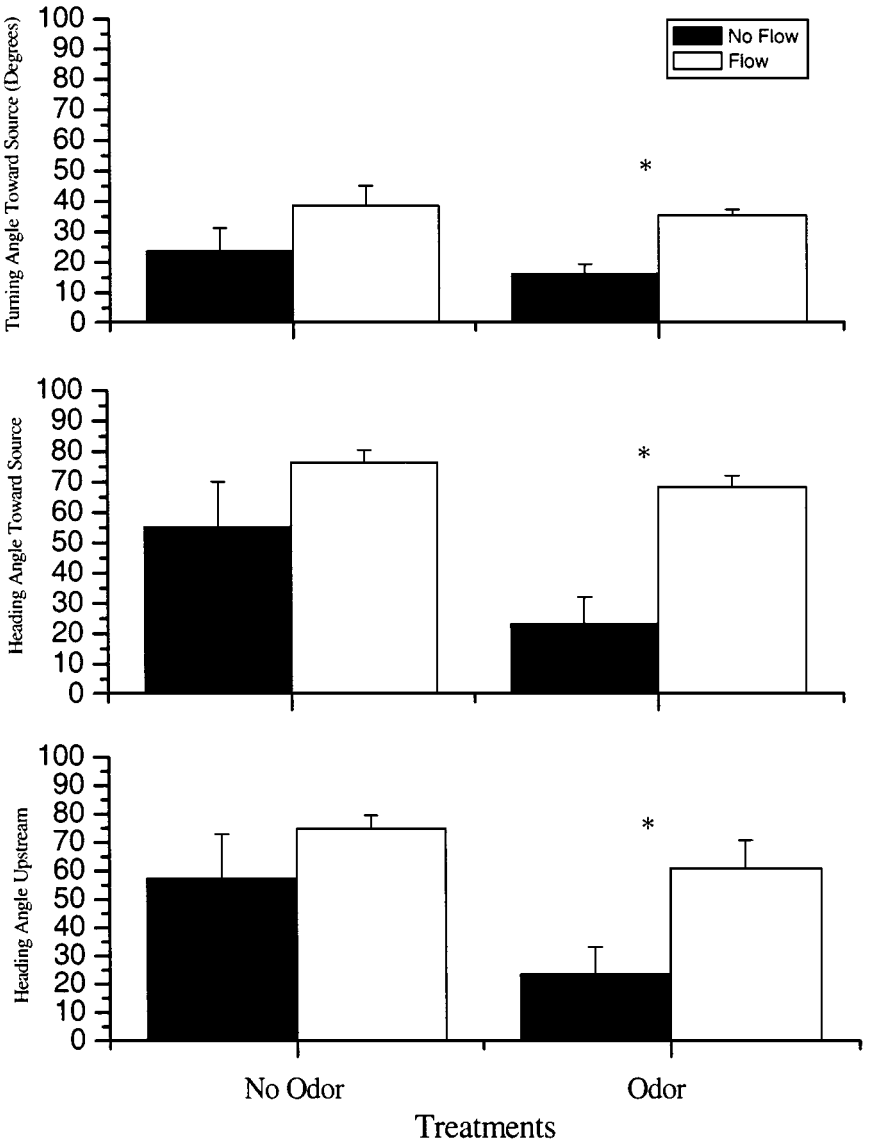


FIG. 5. The upper graph illustrates the turning angle of bullheads in no-flow (black) and flow (white) conditions. The middle graph is the heading angle toward the source of bullheads in no flow (black) and flow (white). The bottom graph shows the heading angle relative to upstream of bullheads in no-flow (black) and flow (white) conditions. The asterisk indicates a significant difference using a MANOVA followed by a post-hoc test. $N = 7$ for each flow treatment.

treatments. We expect either a less conservative test (chi squared) or a larger number of replicates would show significance. Second, it showed that bullhead orientation was influenced by changes in the flow conditions. A statistical interaction was found between the flow/no-flow treatment and the odor treatment with regard to swimming speed. During odor stimulation, bullheads swam faster in the flow condition when compared to the no-flow condition. Since both flow and odor modulated the bullhead's orientation response, this statistical effect suggests that bullheads may use some type of an odor mediated rheotactic response.

The orientation paths exhibited by bullheads during chemical stimulation were different for the flow and no-flow conditions. Bullheads had more linear orientation paths under no-flow conditions than in flow conditions. Post-hoc analysis showed that bullheads did not swim differently in no-flow or flow in the absence of odor. Other spatial aspects of orientation, such as turning angle, heading angle, and heading angle upstream were smaller under no flow when compared to flow conditions. This indicates that bullheads made smaller course corrections when orienting to the odor source under no flow than under the flow condition. Larger angles in the control trials indicate greater course correction, therefore illustrating a less directed swimming. There was no difference between flows in any of these parameters when odor was absent from the trials. Overall, the orientation angles and the net-to-gross ratio indicated that bullheads swam directly to the odor source under no flow. These results suggest that bullheads may be performing a gradient type of search in no-flow conditions, which supports past research on catfish (Bardach et al., 1967). The no-odor results also indicate that the shape of the tank did not influence the swimming behavior of the fish. If the tank were to constrain orientation behavior, we would expect that both no-odor controls would show similar spatial effects.

In the odor/flow treatment, catfish could be gathering information by the distribution of the odor patches and possibly some information on flow direction. Bullheads have been found to be sensitive to odor concentration differences (Johnsen and Teeter, 1980) and have also been found to orient successfully under no-flow to a liquid odor source (Bardach et al., 1967). The odor source used in this study diffused slowly based on dye trials, but patches of odor source came off while placing the source in the tank. These patches could have diffused out in the water column, setting up patches of odor for the bullheads to encounter as they come out of the start gate. Gelatin has several amino acids (Bohidar, 1997; Lüpke and Brückner, 1998) [such as arginine and alanine (Caprio et al., 1993; Ivanova and Caprio 1993; Valentinčič and Caprio, 1994)] that have been found to be stimulatory to catfish.

In flow conditions, turbulence directly impacts odor distribution and indirectly influences an animal to modify its locomotor behavior during orientation (Weissburg and Zimmer-Faust, 1993). Eddies that are formed by turbulence interact

with the odor plume, influencing the spatial and temporal fluctuations of the odor concentration (Moore and Atema, 1991). In addition to flow speed, substrate can also influence the hydrodynamics of a habitat. In a study detailing the influence of habitat on chemical signal structure, Moore et al. (2000) found that chemical signals have different spatial and temporal information on different substrates such as sand, cobbles, or gravel. Presumably, the level of turbulence in a system directly impacts the frequency of odor pulses that are measured in an odor plume (Moore et al., 2000). The modifications in locomotor behavior seen in the brown bullheads between the flow, and no-flow, could be due to either differences in the odor plumes or the flow affecting their swimming ability. Still, the results seem to indicate that the swimming behavior pattern seen is related to the distribution of the odor and not the flow regime. There were no significant differences between the controls in any of the orientation parameters, except speed.

An important hypothesis that has been put forth and is being supported by several studies states that organisms should have sensory systems and behaviors that are adapted to the specific sensory conditions of their environment (Wehner, 1987; Atema, 1988; Moore et al., 1991; Endler, 1995; Huber et al., 1997; Weissburg, 1997; Endler and Basolo, 1998; Kotrschal et al., 1998; Schneider et al., 1998; Land et al., 1999; Persons et al., 1999; Poling and Fuiman, 1999; Schneider and Moore, 2000). From this hypothesis we could predict that organisms should perform behavioral tasks more efficiently or more successfully when presented with conditions that are similar to their natural habitats. If we alter environmental conditions that structure sensory information, we would expect to find either changes in behavior or decreased success in performing a specific behavioral task. We found both of these results with brown bullheads.

This phenomenon has also been found with regard to orientation behavior in other species. For example, blue crabs (*Callinectes sapidus*) traveled upstream and at higher speeds toward a food source (clams) in smooth-turbulent flows (Weissburg and Zimmer-Faust, 1994). These behaviors changed when turbulence shifted from smooth to rough. In this case, crabs were slower and made more frequent stops. It was also found that rheotactic and chemical information are necessary for crab orientation (Weissburg and Zimmer-Faust, 1994). Crayfish (*Orconectes rusticus*) are able to use information from the temporal and spatial distribution of chemicals to make directional decisions. Differences were found in how fast they located a food source, time spent moving, and how fast they moved on different substrates. These differences in orientation behavior were based on the hydrodynamics associated with chemical signal structure (Moore and Grills, 1999). In moths, pheromone pulse frequency determines flight form of males in two phylogenetically distant moths, a noctuid (*Heliothis virescens*) and a pyralid (*Cadra cautella*) (Vickers and Baker, 1994; Mafra-Neto and Cardé, 1994, 1998). These moths fly faster and straighter to sources of high-pulse frequencies and slower and more zigzag to sources of low-pulse frequencies. Finally, tsetse flies (*Glossina pallidipes*) show a

stepwise orientation approach, which allows them to assess wind direction between flight bursts (Bursell, 1984).

In summary, this study demonstrates that bullheads can orient successfully to an odor source and are successful under flow conditions that are similar to their natural environment. It also suggests that bullheads may be generalists in their orientation behavior. Since the spatial pattern of orientation was different in the two flow conditions tested, they may be able to switch search strategies depending on the flow condition and signal structure. This suggests that the hydrodynamic environment may be an important factor in the shaping of an organism's chemosensory behavior. However, further studies need to be done to distinguish if bullheads are really using different search strategies under different flow conditions. More studies are also required in order to understand how bullheads are finding the odor source under no flow.

Acknowledgments—The authors would like to thank the Laboratory for Sensory Ecology and other reviewers for their critique of this manuscript. This work was supported by grant IBN-9614492. The research was also conducted according to animal research protocols (Protocol # 98-001).

REFERENCES

ABLE, K. P. 1991. Common themes and variations in animal orientation systems. *Am. Zool.* 31:157–167.

ATEMA, J. 1971. Structures and functions of the sense of taste in the catfish (*Ictalurus natalis*). *Brain Behav. Evol.* 4:273–294.

ATEMA, J. 1988. Distribution of chemical stimuli, pp. 3–28, *in* Sensory Biology of Aquatic Animals. J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Springer-Verlag, New York.

ATEMA, J. 1995. Chemical signals in the marine environment: Dispersal, detection, and temporal signal analysis. *Proc. Natl. Acad. Sci. USA* 92:62–66.

ATEMA, J., HOLLAND, K., and IKEHARA, W. 1980. Olfactory Responses of yellowfin tuna (*Thunnus albacares*) to prey odors: chemical search image. *J. Chem. Ecol.* 6:457–465.

BARDACH, J. E. and CASE, J. 1965. Sensory capabilities of the modified fins of squirrel hake (*Urophycis chuss*) and sea robins (*Prionotus carolinus* and *P. evolans*). *Copiea* 2:194–206.

BARDACH, J. E. and TODD, J. H. 1970. Chemical communication in fish, pp. 205–240, *in* J. Johnsten, Jr., D. G. Moulton, and A. Turk (eds.). Advances in Chemoreception, Vol. 1. Appleton Century Crofts, New York.

BARDACH, J. E., TODD, J. H., and CRICKMER, R. 1967. Orientation by taste in fish of the genus *Ictalurus*. *Science* 155:1276–1278.

BARLOW, G. W. 1974. Contrasts in social behavior between Central American cichlid fishes and coral-reef surgeon fishes. *Am. Zool.* 14:9–34.

BELANGER, J. H. and ARBAS, E. A. 1998. Behavioral strategies underlying pheromone-modulated flight in moths: Lessons from simulation studies. *J. Comp. Physiol. A.* 183:345–360.

BOHIDAR, H. B. 1998. Hydrodynamic properties of gelatin in dilute solutions. *Int. J. Biol. Macromolec.* 23:1–6.

BRADBURY, J. W. and VEHRENCAMP, S. L. 1998. Principles of Animal Communication. Sinauer Associates, Sunderland, Massachusetts.

BREITHAUPT, T. and ATEMA, J. 1993. Evidence for the use of urine signals in agnostic interactions of the American lobster. *Biol. Bull.* 185:318.

BUENTELLO, J. A. and GATLIN, D. M., III. 2000. The dietary arginine requirements of channel catfish (*Ictalurus punctatus*) is influenced by endogenous synthesis of arginine from glutamic acid. *Aquaculture* 188:311–321.

BURSELL, E. 1984. Observations on the orientation of tsetse flies (*Glossina pallidipes*) to wind-borne odours. *Physiol. Entomol.* 9:133–137.

CAPRIO, J. and BYRD, R. P., JR. 1984. Electrophysiological evidence for acidic, basic, and neutral amino acid olfactory receptor sites in the catfish. *J. Gen. Physiol.* 84:403-422.

CAPRIO, J. and RADERMAN-LITTLE, R. 1978. Scanning electron microscopy of the channel catfish olfactory lamellae. *Tissue Cell* 10:1–9.

CAPRIO, J., DUDEK, J., and ROBINSON, J. J., II. 1989. Electro-olfactogram and multiunit olfactory receptor responses to binary and trinary mixtures of amino acids in the channel catfish, *Ictalurus punctatus. J. Gen. Physiol.* 93:245–262.

CAPRIO, J., BRAND, J. G., TEETER, J. H., VALENTINCIC, T., KALINOSKI, D. L., KOHBARA, J., KUMAZAWA, T., and WEGERT, S. 1993. The taste system of the channel catfish: From biophysics to behavior. *TINS* 16:192–196.

CARR, W. E. S. 1988. The molecular nature of chemical stimuli in the aquatic environment, pp. 3–28, *in* J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Sensory Biology of Aquatic Animals. Springer-Verlag, New York.

CARR, W. E. S., NETHERTON III, J. C., GLEESON, R. A., and DERBY, C. D. 1996. Stimulants of feeding behavior in fish: Analyses of tissues of diverse marine organisms. *Biol. Bull.* 190:149–160.

CHIVERS, D. P. and SMITH, R. J. F. 1993. The role of olfaction in chemosensory-based predator recognition in the fathead minnow, *Pimephales promelas*. *J. Chem. Ecol.* 19:623–633.

CHIVERS, D. P., BROWN, G. E., and SMITH, R. J. F. 1996. The evolution of chemical alarm signals: Attracting predators benefits alarm signal senders. *Am. Nat.* 148:649–659.

CROLL, R. P. and CHASE, R. 1980. Plasticity of olfactory orientation to foods in the snail *Achatina fulica. J. Comp. Physiol.* 136A:267–277.

ELLINGSEN, O. F. and DØVING, K. B. 1986. Chemical fractionation of shrimp extracts inducing bottom food search behavior in cod (*Gadus morhua* L.). *J. Chem. Ecol.* 12:155–168.

ENDLER, J. A. 1995. Multiple-trait coevolution and environmental gradients in guppies. *TREE* 10: 22–29.

ENDLER, J. A. and BASOLO, A. L. 1998. Sensory ecology, receiver biases and sexual selection. *TREE* 13:415–420.

ERIKSSON, L.-O. 1978. Diurnalism versus nocturnalism-dualism within fish individuals, pp. 69–89, *in* J. E. Thorpe (ed.). Rhythmic Activity of Fishes. Academic Press, New York.

ERIKSSON, L.-O. and VAN VEEN, T. 1980. Circadian rhythms in the brown bullhead, *Ictalurus nebulosus* (Teleosti). Evidence for an endogenous rhythm in feeding, locomotor, and reaction time behavior. *Can. J. Zool.* 58:1899–1907.

FINELLI, C. M., PENTCHEFF, N. D., ZIMMER-FAUST, R. K., and WETHEY, D. S. 1999. Odor transport in turbulent flows: Constraints on animal navigation. *Limnol. Oceanogr.* 44:1056–1071.

GIAQUINTO, P. C. and VOLPATO, G. L. 1997. Chemical communication, aggression, and conspecific recognition in the fish Nile tilapia. *Physiol. Behav.* 62:1333–1338.

HAZLETT, B. 1994. Crayfish feeding responses to zebra mussels depend on microorganisms and learning. *J. Chem. Ecol.* 20:2623–2630.

HUBER, R., VAN STAADEN, M. J., KAUFMAN, L. S., and LIEM, K. F. 1997. Microhabitat use, trophic patterns, and the evolution of brain structure in African cichlids. *Brain Behav. Evol.* 50:167–182.

IVANOVA, T. T. and CAPRIO, J. 1993. Odorant receptors activated by amino acids in sensory neurons of the channel catfish *Ictalurus punctatus. J. Gen. Physiol.* 102:1085–1105.

JOHNSEN, P. B. and TEETER, J. H. 1980. Spatial gradient of chemical cues by catfish. *J. Comp. Physiol.* 140:95–99.

KANG, J. and CAPRIO, J. 1991. Electro-olfactogram and multiunit olfactory receptor responses to complex mixtures of amino acids in the channel catfish *Ictalurus punctatus*. *J. Gen. Physiol.* 98:699–721.

KANWAL, J. S. and FINGER, T. E. 1997. Parallel medullary gustatospinal pathways in a catfish: Possible neural substrates for taste-mediated food search. *J. Neurosci.* 17:4873–4885.

KEEN, W. H. 1982. Behavioral interactions and body size differences in competition for food among juvenile brown bullhead (*Ictalurus nebulosus*). *Can. J. Fish. Aquat. Sci.* 39:316–320.

KENNEDY, J. S. 1986. Some current issues in orientation to odour sources, pp. 11–25, *in* T. L. Payne, M. C. Birch, and C. E. J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

KIESECKER, J. M., CHIVERS, D. P., and BLAUSTEIN, A. R. 1996. The use of chemical cues in predator recognition by western toad tadpoles. *Anim. Behav.* 52:1237–1245.

KOTRSCHAL, K., VAN STAADEN, M. J., and HUBER, R. 1998. Fish brains: Evolution and environmental relationships. *Rev. Fish Biol. Fish.* 8:373–408.

LAND, M. F., GIBSON, G., HORWOOD, J., and ZEIL, J. 1999. Fundamental differences in the optical structure of the eyes of nocturnal and diurnal mosquitoes. *J. Comp. Physiol. A.* 185:91–103.

LEWIS, D. B. and GOWER, D. M. 1980. Biology of Communication. John Wiley & Sons, New York.

LÜPKE, M. and BRÜCKNER, H. 1998. Gas chromatographic evaluation of amino acid epimerisation in the course of gelatin manufacturing and processing. *Z Lebensm. Unters. Forsch. A* 206:323–328.

MAFRA-NETO, A. and CARDÉ, R. T. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144.

MAFRA-NETO, A. and CARDÉ, R. T. 1998. Rate of realized interception of pheromone pulses in different wind speeds. modulates almond moth orientation. *J. Comp. Physiol. A* 18:563–572.

MASON, R. T., FALES, H. M., JONES, T. H., PANNEL, L. K, CHINN, J. W., and CREWS, D. 1989. Sex pheromones in snakes. *Science* 245:290–293.

MIYAMOTO, T., RESTREPO, D., and TEETER, J. H. 1990. Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science* 249:1167–1168.

MOORE, P. A. and ATEMA, J. 1991. Spatial information in the three-dimensional fine structure of an aquatic odor plume. *Biol. Bull*. 181:408–418.

MOORE, P. A. and GRILLS, J. 1999. Chemical orientation to food by the crayfish, *Orconectes rusticus*: Influence by hydrodynamics. *Anim. Behav.* 58:953–963.

MOORE, P. A. and LEPPER, D. 1997. Role of chemical signals in the orientation behavior of the sea star *Asterias forbesi*. *Biol. Bull.* 192:410–417.

MOORE, P. A., ATEMA, J., and GERHARDT, G. A. 1991. Fluid dynamics and microscale chemical movement in the chemosensory appendages of the lobster, *Homarus americanus*. *Chem. Senses* 16:663–674.

MOORE, P. A., WEISSBURG, M. J., PARRISH, J. M., ZIMMER-FAUST, R. K., and GERHARDT, G. A. 1994. Spatial distribution of odors in simulated benthic boundary layer flows. *J. Chem. Ecol.* 20:255–279.

MOORE, P. A., GRILLS, J. L., and SCHNEIDER, R. W. S. 2000. Habitat-specific signal structure for olfaction: An example from artificial streams. *J. Chem. Ecol.* 26:565–584.

MURLIS, J. 1986. The structure of odor plumes, pp. 27–38, *in* T. L. Payne, M. C. Birch, and C. E. J. Kennedy (eds.). Mechanisms in Insect Olfaction. Clarendon Press, Oxford.

MURLIS, J. 1987. Odor plumes and the signals they provide, pp. 221–231, *in* R. T. Cardé and A. K. Minks (eds.). Pheromone Research: New Directions. Chapman and Hall, New York.

MURLIS, J. and JONES, C. D. 1981. Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

MURLIS, J., ELKINTON, J. S., and CARDÉ, R. T. 1992. Odor plumes and how insects use them. *Annu. Rev. Entomol.* 37:505–532.

NEVITT, G. A. 1991. Do fish sniff? A new mechanism of olfactory sampling in pleuronectid flounders. *J. Exp. Biol.* 157:1–18.

PARKER, G. H. 1912. The directive influence of the sense of smell in the dogfish. *Bull. US Bur. Fish.* 32:35–46.

PERSONS, M. H., FLEISHMAN, L. J., FRYE, M. A., and STIMPHIL, M. E. 1999. Sensory response patterns and the evolution of visual signal design in anoline lizards. *J. Comp. Physiol. A* 184:585–607.

PFLIEGER, W. L. 1975. The Fishes of Missouri. Missouri Department of Conservation, Missouri.

POLING, K. R. and FUIMAN, L. A. 1999. Behavioral specialization in developing sciaenids and its relationship to morphology and habitat. *Environ. Biol. Fish.* 54:119–133.

RASMUSSEN, L. E. L. and SCHULTE, B. A. 1998. Chemical signals in the reproduction of Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. *Anim. Reprod. Sci.* 53:19–34.

RESTREPO, D., MIYAMOTO, T., BRYANT, B. P., and TEETER, J. H. 1990. Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science* 249:1167–1168.

SARWAR, G. and RATNAYAKE, W. M. N. 2000. Effects of amino acid supplementation of dietary proteins on serum cholesterol and fatty acids in rats. *Nutr. Res.* 20:665–674.

SCHNEIDER, R. W. S. and MOORE, P. A. 2000. The physics of chemoreception revisted: How the environment influences chemically mediated behavior, pp. 159–176, *in* P. Domenici and R. W. Blake (eds.). Biomechanics in Animal Behavior. BIOS Scientific Publishers Ltd., Oxford.

SCHNEIDER, R. W. S., PRICE, B. A., and MOORE, P. A. 1998. Antennal morphology as a physical filter of olfaction: Temporal tuning of the antennae of the honeybee, *Apis mellifera. J. Insect. Physiol.* 44:677–684.

SCHWENK, K. 1995. Of tongues and noses: Chemoreception in lizards and snakes. *TREE* 10:7–12.

SMITH, M. E. 2000. Alarm response of *Arius felis* to chemical stimuli from injured conspecifics. *J. Chem. Ecol.* 26:1635–1647.

SORENSEN, P. W., STACEY, N. E., and CHAMBERLAIN, K. J. 1989. Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. *Horm. Behav.* 23:317–332.

SORENSEN, P. W. and SCOTT, A. P. 1994. The evolution of hormonal sex pheromones in teleost fish: Poor correlation between the pattern of steroid release by goldfish and olfactory sensitivity suggests that these cues evolved as a result of chemical spying rather than signal specialization. *Acta Phys. Scand.* 152:191–205.

STABELL, O. B. 1992. Olfactory control of homing behavior in Salmonids, pp. 249–270, *in* T. J. Hara (ed.). Fish Chemoreception. Chapman & Hall, New York.

SWAISGOOD, R. R., LINDBURG, D. G., and ZHOU, X. 1999. Giant pandas discriminate individual differences in conspecific scent. *Anim. Behav.* 57:1045–1053.

TAKKEN, W., DEKKER, T., and WIJNHOLDS, Y. G. 1997. Odor-mediated flight behavior of *Anopheles gambiae* Giles *sensu stricto* and *An. stephensi* Liston in responses to CO_2, acetone, and 1-octen-3-ol (Diptera: Culicidae). *J. Insect. Behav.* 10:395–407.

TAVOLGA, W. A. 1956. Visual, chemical, and sound stimulus as cues in the sex discriminatory behavior of the gobiid fish, *Bathygobius soporator. Physiol. Zool.* 31:259–271.

TIERNEY, A. J. and ATEMA, J. 1988. Behavioral responses of crayfish (*Orconectes virilis* and *Orconectes rusticus*) to chemical feeding stimulants. *J. Chem. Ecol.* 14:123–133.

TOSI, L. and SOLA, C. 1993. Bile salts and taurine as chemical stimuli for glass eels, *Anguilla anguilla:* A behavioral study. *Environ. Biol. Fish* 37:197–204.

VALENTIŃČIČ, T. and CAPRIO, J. 1994. Consummatory feeding behavior to amino acids in intact and anosmic channel catfish *Ictalurus punctatus*. *Physiol. Behav.* 55:857–863.

VALENTIŃČIČ, T., WEGERT, S., and CAPRIO, J. 1994. Learned olfactory discrimination versus innate taste responses to amino acids in channel catfish (*Ictalurus punctatus*). *Physiol. Behav.* 55:865–873.

VAN DER STEEN, W. J. and TER MAAT, A. 1979. Theoretical studies on animal orientation I. Methodological appraisal of classifications. *J. Theor. Biol.* 79:223–234.

VICKERS, N. J. 2000. Mechanisms of animal navigation in odor plumes. *Biol. Bull.* 198:203–212.

VICKERS, N. J. and BAKER, T. C. 1994. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Natl. Acad. Sci. USA* 91:5756–5760.

WEHNER, R. 1987. Matched filters—neural models of models of the external world. *J. Comp. Physiol. A* 161:511–531.

WEISSBURG, M. J. 1997. Chemo- and mechanosensory orientation by crustaceans in laminar and turbulent flows: From odor trails to vortex streets, pp. 215–246, *in* M. Lehrer (ed.). Orientation and Communication in Arthropods. Birkhäuser Verlag, Basel, Switzerland.

WEISSBURG, M. J. and ZIMMER-FAUST, R. K. 1993. Life and death in moving fluids: Hydrodynamic effects on chemosensory-mediated predation. *Ecology* 74:1428–1443.

WEISSBURG, M. J. and ZIMMER-FAUST, R. K. 1994. Odor plumes and how blue crabs use them in finding prey. *J. Exp. Biol.* 197:349–375.

WILLIS, M. A. and ARBAS, E. A. 1998. Variability in odor-modulated flight by moths. *J. Comp. Physiol. A* 182:191–202.

ZIMMER-FAUST, R. K., FINELLI, C. M., PENTCHEFF, N. D., and WETHEY, D. S. 1995. Odor plumes and animal navigation in turbulent water flow: A field study. *Biol. Bull.* 188:111–116.

ZIPPEL, H. P., SORENSON, P. W., and HANSEN, A. 1997. High correlation between microvillous olfactory receptor cell abundance and sensitivity to pheromones in olfactory nerve-sectioned goldfish. *J. Comp. Physiol. A* 180:39–52.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

EVIDENCE FOR USE OF CHEMICAL CUES BY MALE HORSESHOE CRABS WHEN LOCATING NESTING FEMALES (*Limulus polyphemus*)

CYNTHIA HASSLER and H. JANE BROCKMANN*

Department of Zoology
University of Florida
Gainesville, Florida 32611-8525

(Received March 21, 2001; accepted July 15, 2001)

Abstract—Horseshoe crabs come ashore in attached pairs during spring high tides to mate and nest on beaches of the Atlantic and Gulf coasts. Unattached males also come ashore and crowd around the nesting pairs as satellites and engage in sperm competition with the attached male. Females with no satellites and females with large numbers of satellites nest next to one another on the same tide. When females are removed and replaced by a cement model, satellite males continue to be attracted to the same location. Models over sites where females with many satellites had nested are more attractive to males than sites from which a female with no satellites had been removed or a site where no crab had been nesting recently. A second experiment demonstrated that males are responding to chemical cues. A sponge filled with seawater taken from below a female with many satellites and placed under a model female was more attractive to males than a sponge filled with seawater. This is the first demonstration that horseshoe crabs use chemical cues, in addition to visual cues, to locate mates.

Key Words—*Limulus polyphemus*, horseshoe crab, Merostomata, Xiphosura, pheromone, chemical cues.

INTRODUCTION

Chemical cues are used by a wide range of aquatic invertebrates to find food (Ache, 1972; Dunham et al., 1997; Zhou and Rebach, 1999), locate hosts (Mackie and Grant, 1974), identify predators and habitat (Krapf, 1986; Wudkevich et al., 1997; Hazlett, 2000), and recognize conspecifics (Caldwell, 1985; Karavanich and Atema, 1998). Identifying and locating mates with chemical cues is well known

*To whom correspondence should be addressed. E-mail: hjb@zoology.ufl.edu

0098-0331/01/1100-2319$19.50/0

among arthropods (Ache, 1987; Krieger and Breer, 1999), including crustaceans (Atema and Engstrom, 1971; Dunham, 1978; Borowsky, 1985, 1991; Gleeson, 1991; Oh and Dunham, 1991; Stanhope et al., 1992; Hazlett, 1996; Frey et al., 1998; Bushmann and Atema, 2000), spiders (Kaston, 1936; Tietjen and Rovner, 1982; Pollard et al., 1987; Evans and Main, 1993) and scorpions (Gaffin and Brownell, 1992). Although horseshoe crabs (*Limulus polyphemus*) are known to use chemical cues to identify food (Barber, 1956, 1961), no study has demonstrated a role for chemical signals in mate recognition.

Pairs of horseshoe crabs come ashore on spring high tides to lay and fertilize eggs (Shuster, 1982), while lone males roam the beach forming groups around the nesting pairs. A pair is formed when a male grasps a female's terminal spines with his claws (Botton et al., 1996) and rides to the nesting beach with her. A group forms when an unattached male contacts a nesting pair (Rudloe, 1980; Barlow et al., 1986) and becomes a "satellite" of that pair (Figure 1). Satellites push on attached males but rarely displace them (Brockmann, 1990). Paternity analyses demonstrate that the satellite males are engaging in sperm competition with the attached male and with other satellite males that may be present (fertilization is

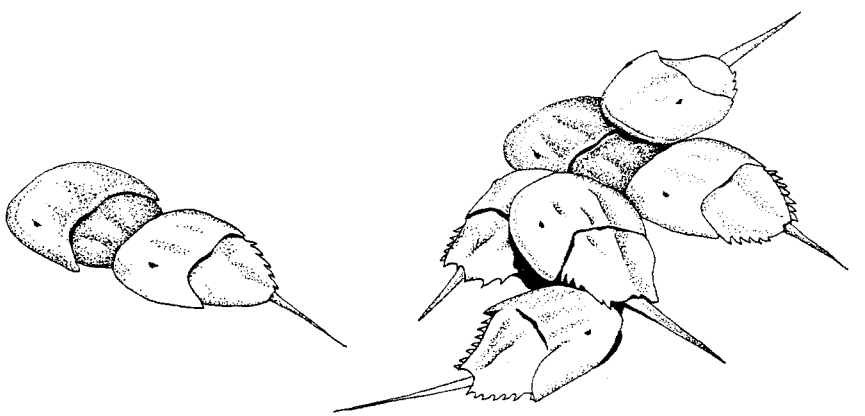


FIG. 1. Pairs of horseshoe crabs with many satellites (right) nest next to pairs with no satellites (left) (drawing by Jennifer Piascik from slide taken at Seahorse Key, Florida). In the experiment to test the role of chemical cues in attracting satellite males, we removed a pair with many satellites and a pair with no satellites and replaced them with cement castings of females (models). Satellite males approached and remained with a concrete model that was pushed into the sand over the spot where a nesting female with many satellites had been removed (along with her associated satellites). The newly arriving males were individuals that had been searching the beach for nesting pairs and had not nested with this female previously.

external and occurs underneath the female as she is laying eggs) (Brockmann et al., 1994). Satellites are remarkably successful at fertilizing eggs if they are in the right position and even those in large groups may have some success (Brockmann et al., 2000). Satellite male groups are distributed unevenly: some nesting pairs have many satellites, whereas others have none (Brockmann, 1996). Larger females and those in better condition have more satellites than smaller, older individuals (Brockmann, 1996; Agresti, 1996). In order to understand the formation of male mating groups, we examined the proximate cues used by males to locate nesting females on the beach.

What are the likely cues used by unattached males to find nesting pairs? By using cement castings of females, Barlow et al. (1982, 1984, 1987, 1988) demonstrated that males use visual cues when locating unattached females offshore. Using a camera-tracking system, he showed that males veer toward other crabs when within visual range (Powers et al., 1991). The crabs can see other crabs or objects resembling crabs under a wide range of conditions including at night (Herzog et al., 1996) and when contrast is low (Passaglia et al., 1997a,b). Although unattached males are, on average, older than attached males and their surfaces, including their eyes, are in poorer condition (Brockmann and Penn, 1992), all show visual activity (Wasserman and Cheng, 1996). Clearly, visual cues are used by unattached males to locate females, and they are probably also used to locate pairs nesting on the beach, just as females use visual cues to avoid the nesting sites of other females (Barlow et al., 1982; Powers et al., 1991).

Several pieces of evidence suggest that chemical cues may also be involved in locating mates. Satellite males walk in circles around the depression left on the beach by recently departed nesting females (Cohen and Brockmann, 1983; Hassler, 1999). Some of these are males that were involved in the satellite group, but some are not (authors' personal observation). Furthermore, when all satellites are removed from a nesting pair, newly arriving unattached males (not those that were removed) are more attracted to those pairs that had many satellites than to those that had few (Brockmann, 1996). Horseshoe crabs have chemoreceptors on all limbs, on the spines of the gnathobases and chilicerae, and on the flabellum (Patten, 1894; Barber, 1961; Hayes, 1966, 1971, 1985; Hayes and Waterman, 1969; Wyse, 1971). Strong and highly specific chemoreceptive responses toward amino acids from prey items have been recorded from these structures (Barber, 1951, 1953, 1956; Wyse, 1971; Hayes and Barber, 1982). Taken together, then, it seems reasonable to suggest that male horseshoe crabs may be using chemical cues, in addition to visual cues, to locate nesting pairs. Patten (1894), Hanström (1926), Pomerat (1933), and Kaplan (1988) speculated that olfactory cues may be used by male horseshoe crabs to find females, but no data were collected. By using cement castings of females in the field, we provide the first experimental evidence that unattached male horseshoe crabs use chemical cues to locate mates.

METHODS AND MATERIALS

Study Sites. Two beaches were used in this study: Seahorse Key (SHK), an island on the west coast of Florida in the northern Gulf of Mexico, approximately 4 km from Cedar Key, Levy County, Florida (29°06′N, 83°04′W) and Breakwater Harbor (BWH) at the mouth of the Delaware Bay in Cape Henlopen State Park (38°45′N, 75°05′W), Lewes, Delaware (Penn and Brockmann, 1994). Horseshoe crabs at SHK nest each spring along a 2-km stretch of low-energy, fine sand beach on the south shore of the island. At BWH they nest along a 4-km section of low-energy, sand and gravel beach on the south side of the harbor. We collected data on the distribution of pairs and group sizes during alternate weeks (new and full moon): at SHK from March 16 to May 8, 1996, and at BWH from June 17 to 30, 1996. We conducted experiments on the responses of unattached males to models of nesting females during alternate weeks from June 6 to July 26, 1997, at BWH.

Location of Nesting Pairs. During four tides in Florida and six tides in Delaware, we surveyed a 100-m stretch of beach (divided into ten, 10-m sections) continually from the peak of the high tide (as predicted by NOAA tide tables) until no crabs remained on the beach. When we encountered a nesting pair, we marked the female's location with a prelabeled vinyl flag and recorded the time, direction the female was facing, and number of satellites in physical contact with the female or her attached male, i.e., group size. During the following daytime low tide, we measured distances from each flag to reference stakes that were placed on the beach at the start of the season. These distances were transformed into X–Y coordinates and plotted on a map of the beach.

Number of Satellites per Female. Many pairs of horseshoe crabs return repeatedly across consecutive tides and nest in different locations on the beach. If unattached males are using cues from individuals, then particular nesting pairs might show some consistency in the number of satellites they attract from one tide to the next. We walked along a 1-km section of beach at Seahorse Key that had been marked off in 100-m sections and counted the number of individually marked nesting pairs and the sizes of the groups of satellites surrounding them. The crabs had been marked as part of another study using methods previously described (Brockmann and Penn, 1992; Brockmann, 1996). Our census was conducted once per tide between the time of the maximum high tide and 1 hr after on alternate weeks (new and full moons) from March 17, to May 20, 1996. For females that returned to nest across two or three different tides (not necessarily consecutive nights), we looked for consistent patterns in the number of satellites they attracted. A complication of this analysis is that there may be different operational sex ratios (OSR) from one night to the next and, thus, different expected numbers of satellites. For this reason, we also examined whether the operational sex ratio affected the numbers of satellites around females.

Chemical Cues Experiments. We examined cues used by unattached males to locate nesting pairs with two experimental manipulations conducted in the field at BWH. A pilot study revealed that unattached males would approach a cement casting of a female (model) and even come out of the water and crawl up the beach to a casting if it were placed over a spot where a female had been nesting recently. In the first experiment, we compared responses of newly arriving, unattached males toward three cement castings (models) of females presented simultaneously: (1) a casting placed over a location from which a pair with many satellites had been removed (many-satellites model); (2) a casting placed over a nearby location where a pair that had no satellites had been removed (no-satellites model); and (3) a casting placed over a nearby location where no horseshoe crabs had been nesting during this tide (no-pair model). If satellites were using chemical cues to locate nesting pairs, then they should approach the many-satellites model more than the no-satellites or no-pair models. In this experiment visual cues were eliminated as a way for crabs to differentiate between models since all three models were alike (and were rinsed off and switched from one trial to the next). Furthermore, no auditory or tactile cues differentiated the models (as may occur with live, nesting females). In the second experiment, we compared the response of satellites to two casts of a female, both placed at sites where no crabs had been nesting (although nesting pairs were nearby). Under one we placed a sponge filled with water collected from underneath a female with many satellites (many-satellites sponge), and under the other model we placed a sponge filled with seawater (seawater sponge). If satellites were responding to chemical cues, then they should approach the first model more than the second.

We prepared 10 cement castings (models) using the undamaged carapaces from similar-sized dead females found on the beach. We rinsed, dried, and filled the cavities of the shells with a mixture of equal parts concrete, sand, and mortar. A finished cast consisted of a female carapace with a hardened concrete filling so the model female would remain in place when placed at the shoreline. A different combination of the 10 models was used for each trial of the two experiments.

We began the first experiment by walking along the beach until we encountered a nesting pair with more than four satellites (where the many-satellites model would be placed) that was located within a meter of a pair with no satellites (where the no-satellites model would be placed) and that were at approximately the same height on the beach relative to the water line (Figure 1). We also chose a nearby patch of sand (1 m away) on which no pair was nesting (where the no-pair model would be placed). After the three locations had been chosen, we began the experiment by quickly removing the nesting crabs and exchanging them for the concrete models. We lifted females out of sand and carefully brought live pairs several meters up the beach, well above the waves, and placed them upside down where they remained for the duration of the trial. We pushed each cement casting into the

depression left in the sand by the female that had just been removed and scraped sand over the front edge of the casting so that it closely resembled a live nesting female. We used the same procedure for the no-pair model by pushing it into the sand so that it resembled the others. We treated satellites in one of two ways: either (1) all satellites were allowed to stay with the model after the live pair was removed (experiment 1 stay) or (2) all satellites were removed from the live nesting pair and placed upside down high on the beach for the duration of the trial, and all new satellites investigating the models were removed (experiment 1 remove). Observation posts were located between models and removed pairs about half a meter above the central model. We alternated the order in which we exchanged pairs for models, and we alternated the use of the cement models among trials (so that all casts were used equally for the three treatments). We recorded the number of new satellite males that approached each model at 30-sec intervals over 10 min by using scan sampling. We defined approach as an unattached male moving within at least 5 cm of the model for at least 10 sec with the anterior portion of the prosoma directed toward the model. For the analyses presented here, we used data from the 30-sec interval preceding 1, 4, 6, and 10 min. For each tide on which this experiment was conducted ($N = 7$), we ran as many trials as possible (two to nine trials per tide, total trials = 36 for experiment 1 stay and 18 trials for experiment 1 remove). If unattached males were using chemical cues to locate nesting pairs, they should approach the many-satellites models more often than the no-satellites or no-pair models.

In the second experiment, we placed the cement models over sand where no pairs had been nesting, placed sponges under the models, and recorded the behavior of approaching males as in the preceding experiment (experiment 1 stay), except that we continued each trial for only 5 min. To collect water from under the female with many satellites, we squeezed out the sponge, lifted the female's carapace slightly, and pushed the sponge underneath, where it remained for 30 sec. To collect water from a nearby site with no female, we squeezed out a sponge and inserted it into the sand to the same depth as the sponge placed under the live female where it remained for 30 sec. Each sponge was then placed under the posterior one third of its assigned model so that only a small portion of the end was visible. We used cellulose sponges (7.5 cm thick $\times$ 11.25 cm wide $\times$ 17.5 cm long) that we squeezed out several times and then soaked in a bucket of fresh seawater before and between trials. Although we alternated the sponges between nights, a sponge was used for only one treatment per night. If satellites are more attracted to the model with the many-satellites sponge than to the model with the seawater sponge, then it would support the hypothesis that unattached males are using chemical cues when choosing to join nesting pairs. We conducted the maximum number of trials possible during each tide ($N = 6$ tides; four to nine trials per tide; total trials = 50).

RESULTS

In both Florida (SHK; $N = 143$ nests) and Delaware (BWH; $N = 192$ nests), pairs with groups of satellites around them were interspersed on the beach with pairs that had no satellites (Figure 2). We found no differences in the distribution of groups and lone pairs, but nesting crabs were aggregated. At SHK, most females nested in a narrow band of beach at the top of the tide line, from 3.5 to 5.5 m above low tide (Figure 2) whereas in BWH females nested across a broader section of beach (1 to 5.5 m above the low tide line; Figure 2). Although we searched for nests over a 100-m section of beach, crabs nested in only 30 m of beach on the 10 tides that we measured locations at SHK and BWH.

When individual females returned repeatedly to nest on the beach, those that had larger numbers of satellites on their first visit were likely to have larger numbers of satellites on subsequent visits ($\chi^2 = 12.6$, $df = 4$, $N = 99$, $P = 0.01$ between the first and second visits). We also found an increase in the number of satellites across three returns by a female (Friedman test: $\chi^2 = 24.8$, $df = 2$, $N = 104$, $P < 0.001$). The numbers of satellites that a pair attracted was correlated with the overall OSR on that tide ($r_s = 0.34$, $P < 0.0001$ for visit 2) and the OSR increased across the three visits. However, OSR cannot be the full explanation because there was an increase in the number of satellites per pair over the first two visits (visit 1: mean 0.6 ± 0.8 satellites; visit 2: mean 1.1 ± 0.1; Wilcoxon test, $Z = -3.7$, $P = 0.0001$), but there was no increase in OSR between those two visits (OSR for visit 1: mean $= 1.2 \pm 0.1$; visit 2: mean = 1.1 ± 0.08; Wilcoxon test, $Z = -1.5$, $P = 0.15$).

The experimental manipulations showed strong effects. When a live nesting pair was removed from a site and replaced with a cement casting of a female, one to three satellite males approached the model each minute. The males left the water and crawled up the beach 1–5 cm and then either returned to the water immediately (26/36) or remained with the model (10/36) grasping the carapace with their claws for 0.5–10 min. When satellites were allowed to remain in the vicinity of the model (experiment 1 stay), there was a difference in the numbers of satellites attracted to the three models (0 min indicates the number of satellites that initially remained with the model; Friedman ANOVA: $N = 36$, $df = 2$; $P = 0.0088$ at 0 min; $P = 0.0022$ at 1 min, $P = 0.0418$ at 4 min, $P = 0.0381$ at 6 min, and $P = 0.0035$ at 10 min; Figure 3a). A difference existed between the responses to the no-satellites and the no-pair models at 1 min ($N = 17$, $P = 0.0184$) and 10 min ($N = 22$, $P = 0.0093$). Experiment 1 stay also revealed a correlation between the number of satellites present around the original nesting pair and the numbers around the models (e.g., $r_s = 0.472$, $P = 0.0066$ for the many-satellites model at 10 min) and a correlation between the number of satellites remaining around the model at the start of a trial and the numbers subsequently attracted to the models

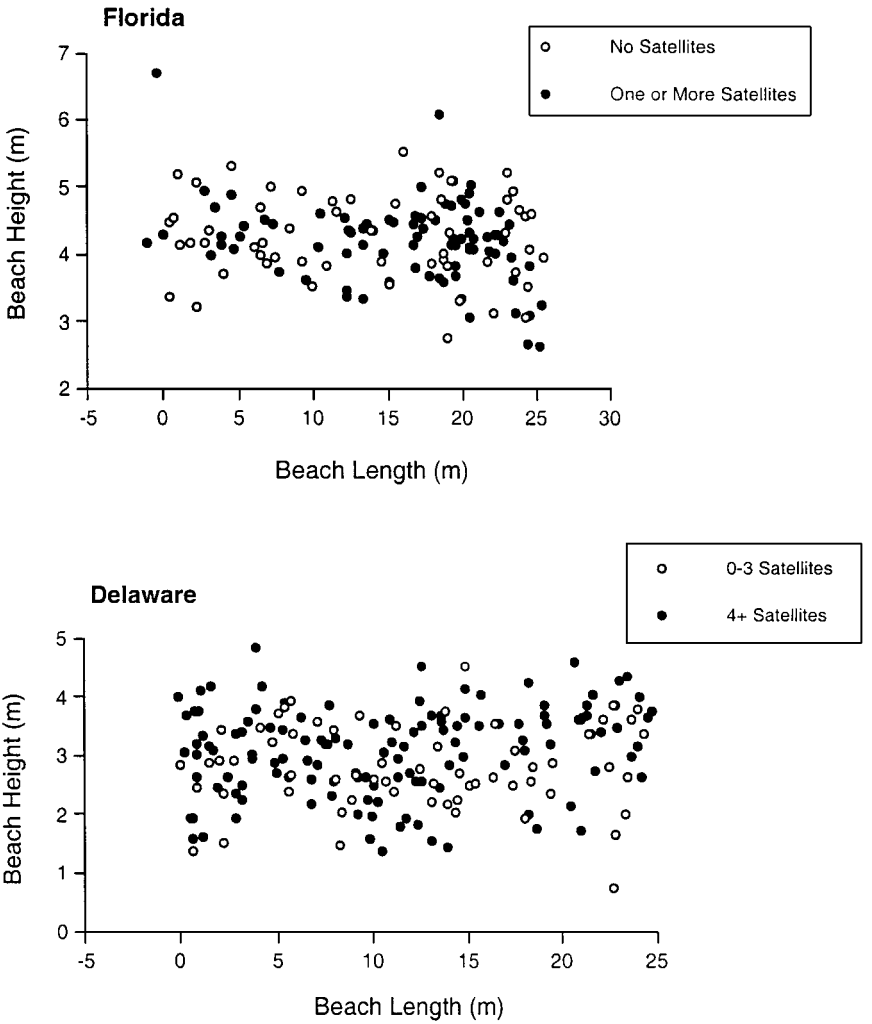


FIG. 2. A comparison of nesting locations of females over four consecutive tides (each point represents one pair) at SHK, when many satellites were present (closed circles, many satellites means ≥ 1; $N = 85$) and with no satellites (open circles, $N = 57$) and at BWH, with many satellites present (closed circles, many satellites means ≥ 4; $N = 130$) and with no satellites (open circles, $N = 62$).

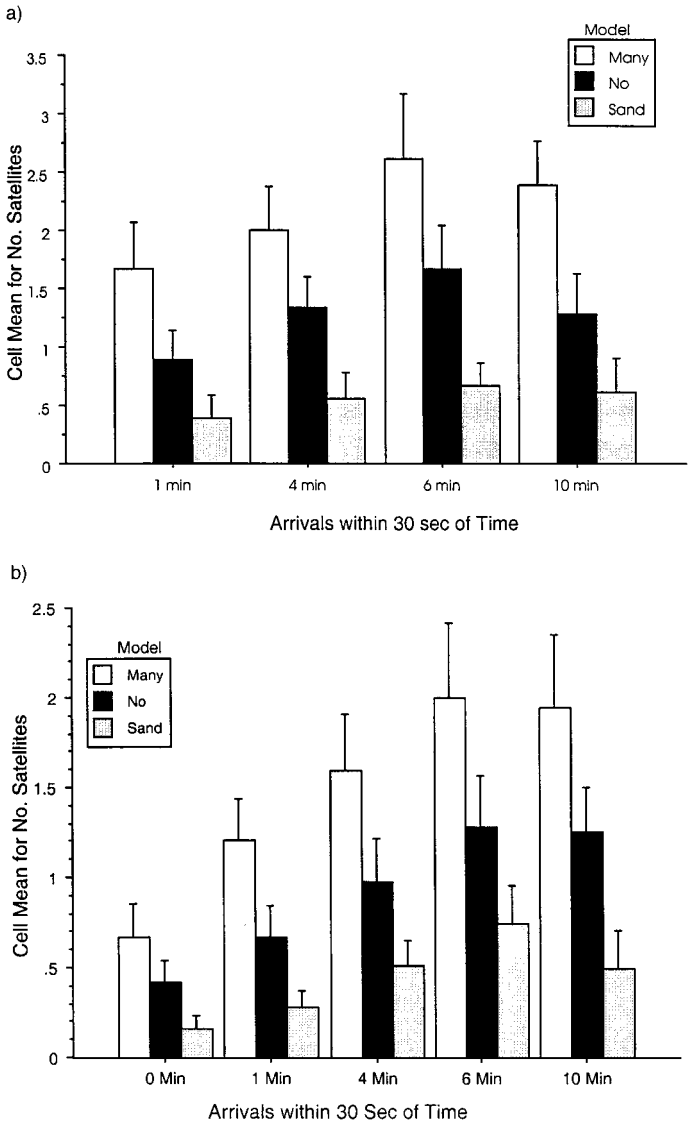


FIG. 3. Total number of satellite males approaching models that were placed over locations where nesting pairs with many satellites had been removed (open bars), where nesting pairs with no satellites had been removed (black bars), and a patch of sand where no pair had been nesting (stippled bars). a) In experiment 1 stay, satellites were not removed, b) but in experiment 1 remove, all satellites were removed when the many satellites pair was picked up and all newly arriving satellites were also removed. The bars show standard errors across all individuals that were tested.

(e.g., $r_s = 0.40$, $P = 0.07$ for number of satellites at start and at 6 min for many-satellites model; $r_s = 0.54$, $P = 0.0155$ for no-satellites model; $r_s = 0.73$, $P = 0.0014$ for no-pair model). When all satellites were removed from around the nesting pairs and all newly arriving satellites were removed (experiment 1 remove), a difference continued to exist in the number of unattached males attracted to the three models (Friedman ANOVA: $N = 18$, $df = 2$; $P = 0.0183$ at 1 min, $P = 0.0105$ at 4 min, $P = 0.0047$ at 6 min, and $P = 0.0037$ at 10 min; Figure 3b). Satellite males were more likely to approach the many-satellites than the no-satellites model at 6 min (Wilcoxon test $N = 14$, $P = 0.0259$) and 10 min ($N = 16$, $P = 0.019$) and were more likely to approach no-satellites than no-pair models at 4 min ($N = 10$, $P = 0.0122$) and 6 min ($N = 12$, $P = 0.0174$).

More unattached males approached models with sponges containing seawater from a live nesting female with satellites (many-satellites sponge: mean 2.6, $N = 50$) than to models with sponges filled with seawater (seawater sponge: mean 1.8, $N = 50$) (Wilcoxon signed-ranks test: $W = 256.5$, $N = 41$, $P = 0.0226$; Figure 4). Satellites also remained longer with the model with the many-satellites sponge than with the model that had the seawater sponge (Wilcoxon signed-ranks

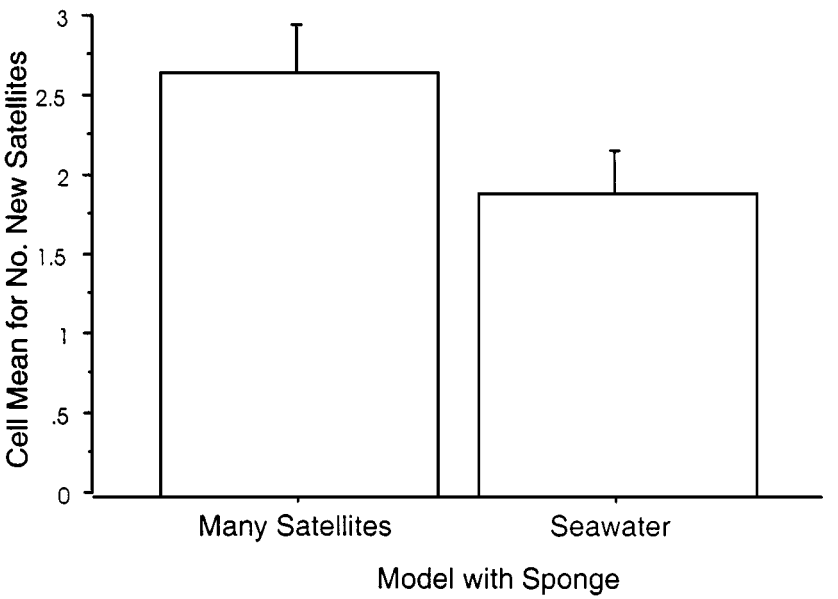


FIG. 4. Satellite male responsiveness (average number of males approaching the two models in 5 min; bars show standard error of the mean) to models with sponges placed underneath; one model had a sponge containing water from underneath a female with many satellites and the other model had a sponge containing seawater.

test: $W = 282.5$, $N = 43$, $P = 0.0216$). Furthermore, there was a correlation between the number of satellites with the original pair (the pair from which the many-satellites sponge water was collected) and the number of satellites that were attracted to the model with the many-satellites sponge ($r_s = 0.379$, $P = 0.0412$).

DISCUSSION

This study provides evidence that unattached males are using chemical cues when choosing to join a nesting pair. Once attracted to a pair, the satellite engages in sperm competition with the attached male and other satellites that have formed a group around the nesting pair (Brockmann et al., 2000). Females with many satellites and females with no satellites often nest side by side (Figures 1 and 2). We took advantage of this behavior when we conducted experimental manipulations in which we removed females from the beach and replaced them with a cement cast (experiment conducted with a paired design). Models that were placed over locations where females with many satellites had been removed were significantly more likely to attract new unattached males than models placed over locations where pairs with no satellites had been removed or where no pair had recently nested (Figure 3). The effect remained whether or not all newly arriving satellites were removed (Figure 3a,b). In a second experiment, we showed that the attractive effect could be transferred to a model by filling a sponge with water taken from underneath a female with many satellites (Figure 4). By replacing the live nesting pair with standardized female models, we removed possible tactile and auditory signals. Although we have clear evidence for chemical cues, visual cues were also important: the cement-filled concrete casts of a female were necessary to produce a consistent and well-oriented response by males. In the experiments that we conducted, however, visual cues could not be responsible for the differences in response among the three models. This study provides a nice example of an increasingly apparent feature of many chemosensory systems (Dunham and Oh, 1996)—hierarchical or multimodal communication (Passaro and Marler, 1999) in which different signals (e.g., visual and olfactory) may be used at different scales (Atema, 1995).

The fact that lone males preferred the many-satellites model supports the hypothesis that chemical cues are being used. However, in the initial model experiment, we did not fully remove cues that may be emitted by the local environment around the nesting pair. To do this we must remove the cues given off by a particular microlocation while trying to maintain cues that are given off by the nesting female. The female model with sponge experiment does this: the sponge filled with water taken from a female with satellites attracted significantly more males than a sponge filled with seawater (Figure 4). In addition, satellites remained longer with the many-satellites sponge model than with the seawater sponge model (Figure 4).

This suggests that satellite males use chemical cues to choose among nesting pairs and that those chemicals are coming primarily from the pair rather than the nesting site. This chemical cue may be a pheromone from the nesting female, the nesting pair and their satellites, or perhaps from the eggs or oviducal fluids.

The models we used were simply concrete-filled shells of females and did not include the attached male that is nearly always present with a nesting female on the beach. Despite this, the lone males treated the model female as though she were an attached female: a newly arriving male moved to a position over the female's incurrent canal, just as he would do with a live, nesting female (Barlow et al., 1988; Brockmann, 1990). This behavior is consistent with previous observations of lone females that sometimes arrive on the beach, probably having lost their attached male on the way to shore (Brockmann and Penn, 1992).

Individual females show a consistent pattern in the number of satellites they attract when they return to the beach to nest over several tides. A female that had more satellites than average on her first visit to the beach was likely to have more satellites than average on subsequent visits. When a female returned to the beach several times, she was likely to gain additional satellites on each subsequent visit so that a female on her third visit had, on average, more satellites than on her first visit. There are several possible explanations for the individual differences among females in their attractiveness to males and for the increased attractiveness of females to satellites over the breeding period (generally one week associated with the new or full moon). In most cases, the first visit was the visit on which the female was marked, measured, and separated from her original attached male. Subsequently, males usually remain attached to the same female. This means that as she returns repeatedly to breed (Brockmann and Penn, 1992), her attached male may be less able to defend against satellite males. If the attached male becomes depleted of sperm, this may mean that satellites are more attracted to the pair because of the reduced sperm competition. Alternatively, females may be increasing their attractiveness by releasing more chemical cues over the week of breeding to ensure that satellites are present to fertilize all their eggs. It is also possible that females may be depositing the majority of their eggs on their third visit and are, thus, more attractive to satellites by releasing more attractant or because more eggs are present.

If chemical cues attract satellites, we must ask who is producing those cues. It seems unlikely that the attached male produces cues that attract satellites since the attached male competes for fertilizations with satellite males (Brockmann et al., 1994). The data collected in this study are consistent with the hypothesis that the satellite males produce a chemical cue that attracts other males or that in combination with female odors attracts males. We think this is unlikely because satellites lose fertilizations when other males are present (Brockmann et al., 2000) and because this hypothesis does not explain consistent differences among females in the numbers of males they attract. The most likely candidate for producing the

chemical signal is the female. It is possible that the female attracts more males to ensure that her eggs are fertilized (Pennington, 1985; Levitan and Petersen, 1995), particularly if the attached male becomes depleted over the week of nesting. Females may also gain from the intensely competitive interactions among males (Brockmann, 1990) if the male that fertilizes her eggs is in some way superior to the individuals he outcompetes. If females are gaining reproductive success, however, why is it that some females apparently fail to produce the chemical attractant (those with no satellites)? Perhaps the most striking result of this study is the consistency of the response by unattached males: in both experiments, the number of males attracted to a model (when only chemical cues were available) is correlated with the numbers that were originally present around the nesting female (from which water was taken or over which the model was placed). It could be that some females are unable to produce the chemical, perhaps due to age or condition, or perhaps some females produce more of the chemical. Brockmann (1996) found that female condition is correlated with the number of satellites, but it is not a strong effect. A far stronger correlation was found between female size and the number of satellites. Perhaps larger females produce more of this chemical cue or perhaps larger females lay more eggs (we have no evidence of this) or have more oviducal fluid associated with their eggs that contains the chemical. Future studies should assay the effects of chemicals produced by females, eggs, attached and satellite males, and their sperm.

The chemical compound to which the satellites are attracted is not known, but this study provides some evidence as to its properties. The chemical continues to attract males for at least 10 min after the nesting pair has left the beach, despite repeated washing by the waves. Horseshoe crab receptors are particularly sensitive to amino acids and steroids from their prey items (Barber, 1956), and many aquatic organisms have been shown to use amino acids and steroids for mate attraction (Liley, 1972). For example, fluid taken from the ovaries of gravid females in some species of fish elicited mating responses in conspecific males (Tavolga, 1956; Partridge et al., 1976; Little, 1983). It seems reasonable that female horseshoe crabs should produce a chemical cue along with the eggs to stimulate spawning by males, as occurs in other externally fertilizing species (Mackie and Grant, 1974), but no such chemical is known from horseshoe crabs. Other chemicals, however, are known. The flatworm *Bdelloura candida*, a parasite that lives in the gills of *Limulus*, is attracted by a substance that is stable in seawater for at least five days and is destroyed by boiling (Chevalier and Steinbach, 1969). Horseshoe crab eggs are known to produce a substance that attracts sperm, but this is known to work only at close range (Shoger and Bishop, 1967). Female horseshoe crabs and their eggs are used as a highly effective bait in the eel and conch fishery, and Ferrari and Targett (2000) have identified a high-molecular-weight protein chemoattractant in the eggs and hemolymph. Clearly, a chemical analysis of the seawater from beneath females with and without satellites is needed to identify the nature of the chemicals involved.

Satellite male horseshoe crabs form groups of highly variable size around some nesting females while ignoring others (Figure 1). The formation of these groups is puzzling since satellite males compete for fertilizations, and increasing group size increases competition (Brockmann et al., 2000). This study demonstrates that satellite males are using chemical signals to choose among nesting pairs when they come ashore to mate. How the chemical is produced and the selective advantages for producing and responding to the chemical are not yet known.

Acknowledgments—Field assistants provided exceptional help in collecting the data reported here: Sandra Nichemias, Mary Hart, Chris Hess, and Patrick McKann at SHK and Kim Abplanalp at BWH. Henry Coulter and Al Dinsmore provided logistic support. We thank the University of Florida Marine Laboratory at Seahorse Key and its director, Frank Maturo, and the Suwannee River National Wildlife Refuge and its manager, Ken Litzenberger, for their support of this project (permit No. 35899). The University of Delaware, College of Marine Studies, Lewes, Delaware, provided research facilities and accommodation, and the Cape Henlopen State Park gave permission to work on the Breakwater Harbor Beach. Dan Brazeau and Nancy Targett provided advice and logistic support. The research presented here was supported in part by a National Science Foundation grant (OCE90-06392) and by the Florida Foundation.

REFERENCES

ACHE, B. 1972. Amino acid receptors in antennae of *Homarus americanus*. *Comp. Biochem. Physiol.* 41:807–811.

ACHE, B. 1987. Chemoreception in invertebrates, pp. 39–64, *in* T. E. Finger and W. L. Silver (eds.). Neurobiology of Taste and Smell. John Wiley, New York.

AGRESTI, A. 1996. An Introduction to Categorical Data Analysis. John Wiley, New York.

ATEMA, J. 1995. Chemical signals in the marine environment: Dispersal, detection and temporal signal analysis. *Proc. Natl. Acad. Sci. USA* 92:62–66.

ATEMA, J. and ENGSTROM, D. G. 1971. Sex pheromone in the lobster, *Homarus americanus*. *Nature* 232:261–263.

BARBER, S. B. 1951. Contact chemoreception in *Limulus*. *Anat. Rec.* 111:561–562.

BARBER, S. B. 1953. Action potential activity of *Limulus* chemoreceptor nerve fibers. *Anat. Rec.* 117:587.

BARBER, S. B. 1956. Chemoreception and proprioception in *Limulus*. *J. Exp. Zool.* 131:51–73.

BARBER, S. B. 1961. Responses of *Limulus* chemoreceptors to amino acid stimulus. *Am. Zool.* 1:435.

BARLOW, R. B., IRELAND, L. C., and KASS, L. 1982. Vision has a role in *Limulus* mating behavior. *Nature* 296:65–66.

BARLOW, R. B., POWERS, M. K., KASS, L., FIORDALICE, R. W., Camara, M. D., and Howard, H. 1984. Vision in *Limulus* mating behavior during the day and at night. *Biol. Bull.* 167:522–523.

BARLOW, R. B., POWERS, M. K., HOWARD, H., and KASS, L. 1986. Migration of *Limulus* for mating: Relation to lunar phase, tide height, and sunlight. *Biol. Bull.* 171:310–329.

BARLOW, R. B., POWERS, M. K., HOWARD, H., and Kass, L. 1987. Vision in *Limulus* mating and migration, pp. 69–84, *in* W. F. Herrnkind and A. B. Thistle (eds.). Signposts in the Sea. Florida State University Press, Tallahassee, Florida.

BARLOW, R. B., POWERS, M. K., and KASS, L. 1988. Vision and mating behavior in *Limulus*, pp. 419–434, *in* J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Sensory Biology of Aquatic Animals. Springer-Verlag, New York.

BOROWSKY, B. 1985. Responses of the amphipod crustacean *Gammarus palustris* to waterborne secretions of conspecifics and congenerics. *J. Chem. Ecol.* 11:1545–1552.

BOROWSKY, B. 1991. Patterns of reproduction of some amphipod crustaceans and insights into the nature of their stimuli, pp. 33–49, *in* R. T. Bauer and J. W. Martin (eds.). Crustacean Sexual Biology. Columbia University Press, New York.

BOTTON, M. L., SHUSTER, C. N., SEKIGUCHI, K., and SUGITA, H. 1996. Amplexus and mating behavior in the Japanese horseshoe crab, *Tachypleus tridentatus. Zool. Sci.* 13:151–159.

BROCKMANN, H. J. 1990. Mating behavior of horseshoe crabs, *Limulus polyphemus. Behaviour* 114:206–220.

BROCKMANN, H. J. 1996. Satellite male groups in horseshoe crabs, *Limulus polyphemus*. *Ethology* 102:1–21.

BROCKMANN, H. J. and PENN, D. 1992. Male mating tactics in the horseshoe crab, *Limulus polyphemus. Anim. Behav.* 44:653–665.

BROCKMANN, H. J., COLSON, T., and POTTS, W. 1994. Sperm competition in horseshoe crabs (*Limulus polyphemus*). *Behav. Ecol. Sociobiol.* 35:153–160.

BROCKMANN, H. J., NGUYEN, C., and POTTS, W. 2000. Paternity in horseshoe crabs when spawning in multiple-male groups. *Anim. Behav*. 60:837–849.

BUSHMANN, P. J. and ATEMA, J. 2000. Chemically mediated mate location and evaluation in the lobster, *Homarus americanus. J. Chem. Ecol.* 26:883–899.

CALDWELL, R. L. 1985. A test of individual recognition in the stomatopod *Gonodactylus festae. Anim. Behav.* 33:101–106.

CHEVALIER, R. L. and STEINBACH, H. B. 1969. A chemical signal attracting the flatworm *Bdelloura candida* to its host, *Limulus polyphemus. Biol. Bull.* 137:394.

COHEN, J. A. and BROCKMANN, H. J. 1983. Breeding activity and mate selection in the horseshoe crab, *Limulus polyphemus*. *Bull. Mar. Sci.* 33:274–281.

DUNHAM, D. W. and OH, J. W. 1996. Sex discrimination by female *Procambarus clarkii* (Girard, 1852) (Decapoda, Cambaridae): Use of chemical and visual stimuli. *Crustaceana* 69:534–542.

DUNHAM, D. W., CIRUNA, K. A., and Harvey, H. H. 1997. Chemosensory role of antennules in the behavioral integration of feeding by the crayfish *Cambarus bartonii*. *J. Crustacean Biol.* 17:27–32.

DUNHAM, P. J. 1978. Sex pheromones in Crustacea. *Biol. Rev. Cambridge Philos. Soc.* 53:555–583.

EVANS, T. and MAIN, B. Y. 1993. Attraction between social crab spiders: Silk pheromones in *Diaea socialis*. *Behav. Ecol.* 4:99–105.

FERRARI, K. and TARGETT, N. 2000. Isolation of a chemical attractant in *Limulus polyphemus*. 29th Benthic Ecology Meeting, March 9–12, 2000, University North Carolina, Wilmington, Wilmington, North Carolina.

FREY, M. A., LONSDALE, D. J., and SNELL, T. W. 1998. The influence of contact chemical signals on mate recognition in a harpacticoid copepod. *Phil. Trans. R. Soc. London B* 353:745–751.

GAFFIN, D. D. and BROWNELL, P. H. 1992. Evidence of chemical signaling in the sand scorpion, *Paruroctonus mesaensis* (Scorpionida: Vaejovida). *Ethology* 91:59–69.

GLEESON, R. A. 1991. Intrinsic factors mediating pheromone communication in the blue crab, *Callinectes sapidus*, pp. 17–32, *in* R. T. Bauer and J. W. Martin (eds.). Crustacean Sexual Biology. Columbia University Press, New York.

HANSTRÖM, B. 1926. Das Nervensystem und die Sinnesorgane von *Limulus polyphemus*. *Lunds Univ. Årsskr*. 22:1–79.

HASSLER, C. 1999. Satellite male groups in horseshoe crabs (*Limulus polyphemus*): How and why males choose females. MS thesis. Department of Zoology, University of Florida, Gainesville, Florida.

HAYES, W. F. 1966. Chemoreceptor sensilla structure in *Limulus*. *J. Morphol.* 119:121–142.

HAYES, W. F. 1971. Fine structure of the chemoreceptor sensillum of *Limulus*. *J. Morphol.* 133:205–240.

HAYES, W. F. 1985. Chemoreceptor sensillum structure in *Limulus. J. Morphol.* 119:121–142.

HAYES, W. F. and BARBER, S. B. 1982. Peripheral synapses in *Limulus* chemoreceptors. *Comp. Biochem. Physiol.* 72A:287–293.

HAYES, W. F. and WATERMAN, T. H. 1969. Structural organization of *Limulus* flabellar chemoreceptors. *Am. Zool.* 9:1098.

HAZLETT, B. A. 1996. Reproductive behavior of the hermit crab *Clibanarius vittatus* (Bose, 1802). *Bull. Mar. Sci.* 58:668–674.

HAZLETT, B. A. 2000. Responses to single and multiple sources of chemical cues by New Zealand crustaceans. *Mar. Fresh. Behav. Physiol*. 34:1–20.

HERZOG, E. D., POWERS, M. K., and BARLOW, R. B. 1996. Limulus vision in the ocean day and night: Effects of image size and contrast. *Visual Neurosci.* 13:31–41.

KAPLAN, E. H. 1988. A Field Guide to Southeastern and Caribbean Seashores. Houghton Mifflin, Boston, Massecheusetts.

KARAVANICH, C. and ATEMA, J. 1998. Olfactory recognition of urine signals in dominance fights between male lobster, *Homarus americanus*. *Behaviour* 135:719–730.

KASTON, B. J. 1936. The sense involved in the courtship of some vagabond spiders. *Entomol. Am.* 16:97–167.

KRAPF, D. 1986. Contact chemoreception of prey in hunting scorpions (Arachnida: Scorpiones). *Zool. Antz.* 217:119–129.

KRIEGER, J. and BREER, H. 1999. Olfactory reception in invertebrates. *Science* 286:720–723.

LEVITAN, D. R. and PETERSEN, C. 1995. Sperm limitation in the sea. *Trends. Ecol. Evol.* 10:228–231.

LILEY, N. R. 1972. The effects of estrogens and other steroids on the sexual behaviour of the female guppy, *Poecilia reticulata*. *Gen. Comp. Endocrinol. Suppl.* 3:532–552.

LITTLE, E. E. 1983. Behavioral function of olfaction and taste in fish, pp. 351–376, *in* R. G. Northcutt and R. E. Davis (eds.). Fish Neurobiology, Vol. 1: Brain Stem and Sense Organs. University of Michigan Press, Ann Arbor, Michigan.

MACKIE, A. M. and GRANT, P. T. 1974. Interspecies and intraspecies chemoreception by marine invertebrates, pp. 105–141, *in* P. T. Grant and A. M. Mackie (eds.). Chemoreception in Marine Organisms. Academic Press, New York.

OH, J. W. and DUNHAM, D. W. 1991. Chemical detection of conspecifics in the crayfish *Procambarus clarkii*: Role of antennules. *J. Chem. Ecol.* 17:161–166.

PARTRIDGE, B. L., LILEY, N. R., and STACEY, N. E. 1976. The role of pheromones in the sexual behaviour of the goldfish. *Anim. Behav.* 24:291–299.

PASSAGLIA, C., DODGE, F., HERZOG, E., JACKSON, S., and BARLOW, R. 1997a. Deciphering a neural code for vision. *Proc. Natl. Acad. Sci. USA* 94:12649–12654.

PASSAGLIA, C. L., MCSWEENEY, M. E., STEWART, K. M., KIM, E., MOLE, E. J., POWERS, M. K., and BARLOW, R. B. 1997b. Visual performance of horseshoe crabs: Role of underwater lighting. *Biol. Bull.* 193:205–207.

PASSARO, S. and MARLER, P. 1999. Multi-modal communication. *Science* 283:1272–1273.

PATTEN, W. 1894. On the morphology and physiology of the brain and sense organs of *Limulus. Q. J. Microse. Sci.* 35:1–96.

PENN, D. and BROCKMANN, H. J. 1994. Nest site selection in the horseshoe crab, *Limulus polyphemus. Biol. Bull.* 187:373–384.

PENNINGTON, J. T. 1985. The ecology of fertilization of echinoid eggs: The consequences of sperm dilution, adult aggregation, and synchronous spawning. *Biol. Bull.* 169:417–430.

POLLARD, S. D., MACNAB, A. M., and JACKSON, R. R. 1987. Communication with chemicals: Pheromones and spiders, pp. 133–141, *in* W. Nentwig (ed.). Ecophysiology of Spiders. Springer, Berlin.

POMERAT, C. M. 1933. Mating in *Limulus polyphemus*. *Biol. Bull.* 64:243–252.

POWERS, M. K., BARLOW, R. B., JR., and KASS, L. 1991. Visual performance of horseshoe crabs day and night. *Visual Neurosci*. 7:179–190.

RUDLOE, A. E. 1980. The breeding behavior and patterns of movement of horseshoe crabs, *Limulus polyphemus*, in the vicinity of breeding beaches in Apalachee Bay, Florida. *Estuaries* 3:177–183.

SHOGER, R. L. and BISHOP, D. W. 1967. Sperm activation and fertilization in *Limulus polyphemus*. *Biol. Bull.* 133:485.

SHUSTER, C. N. 1982. A pictorial review of the natural history and ecology of the horseshoe crab *Limulus polyphemus*, with reference to other Limulidae, pp. 1–52, *in* J. Bonaventura, C. Bonaventura, and S. Tesh (eds.). Physiology and Biology of Horseshoe Crabs: Studies on Normal and Environmentally Stressed Animals. Alan R. Liss, New York.

STANHOPE, M. J., CONNELLY, M. M., and HARTWICK, B. 1992. Evolution of a crustacean chemical communication channel: Behavioral and ecological genetic evidence for a habitat-modified, race-specific pheromone. *J. Chem. Ecol.* 18:1871–1887.

TAVOLGA, W. N. 1956. Visual, chemical and sound stimuli as cues in the sex discriminatory behavior of the fish *Bathygobius soporator*. *Zoologica (NY)* 41:49–64.

TIETJEN, W. J. and ROVNER, J. S. 1982. Chemical communication in lycosids and other spiders, pp. 249–279, *in* P. N. Witt and J. S. Rovner (eds.). Spider Communication: Mechanisms and Ecological Significance. Princeton University Press, Princeton, New Jersey.

WASSERMAN, G. S. and CHENG, Z. 1996. Electroretinographic measures of vision in horseshoe crabs with uniform versus variegated carapaces. *Biol. Signals* 5:247–262.

WUDKEVICH, K., WISENDEN, B. D., CHIVERS, D. P., and SMITH, R. 1997. Reactions of *Gammarus lacustris* to chemical stimuli from natural predators and injured conspecifics. *J. Chem. Ecol.* 23:1163–1173.

WYSE, G. A. 1971. Receptor organization and functions in *Limulus* cheleae. *Z. Vergl. Physiol.* 73:249–273.

ZHOU, T. and REBACH, S. 1999. Chemosensory orientation of the rock crab *Cancer irroratus*. *J. Chem. Ecol.* 25:315–329.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

BOTH OVARIAN FLUID AND FEMALE URINE INCREASE SEX STEROID HORMONE LEVELS IN MATURE ATLANTIC SALMON (*Salmo salar*) MALE PARR

K. H. OLSÉN,[1,*] R. BJERSELIUS,[1] I. MAYER,[2] and H. KINDAHL[3]

[1]*Department of Environmental Toxicology, EBC*
Uppsala University
Norbyvägen 18A, SE-752 36 Uppsala, Sweden

[2]*Department of Zoology*
Stockholm University
SE-106 91 Stockholm, Sweden

[3]*Department of Obstetrics and Gynaecology*
Swedish University of Agricultural Sciences (SLU)
SE-750 07 Uppsala, Sweden

(Received December 27, 2000; accepted July 10, 2001)

Abstract—We compared the ability of urine and ovarian fluid from female Atlantic salmon (*Salmo salar*) to stimulate increase in plasma concentrations of sex steroid hormones in mature conspecific male parr (priming effect of the stimuli). We also tested the hypothesis that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) may act as a priming pheromone in the tested stimulants. Individual males of salmon parr were exposed to female urine, ovarian fluid, urine–ovarian fluid mix, or $PGF_{2\alpha}$. Plasma concentrations of the sex steroids of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) were higher in males exposed to urine, ovarian fluids, and $PGF_{2\alpha}$ compared to control males. $PGF_{2\alpha}$ and a mixture of urine and ovarian fluid also gave increased concentrations of 11-ketotestosterone (11-KT). Concentrations of $PGF_{2\alpha}$ were higher in ovarian fluids than in urine. A behavior test with mature male parr in a fluviarium showed neither attraction to nor avoidance of 0.1 nM $PGF_{2\alpha}$, but plasma levels of 17,20β-P were significantly higher in exposed males compared to controls.

Key Words—Atlantic salmon, salmonids, priming odors, urine, ovarian fluid, steroid hormones, prostaglandin $F_{2\alpha}$.

*To whom correspondence should be addressed. Present address: Södertörn University College, Department of Natural Sciences, Box 4101, SE-141 04 Huddinge, Sweden. E-mail: hakan.olsen@sh.se

0098-0331/01/1100-2337$19.50/0

INTRODUCTION

Previous studies in mature salmonid males have demonstrated both behavioral and endocrine responses to water conditioned by urine and/or ovarian fluid from mature females (e.g., Honda, 1982; Olsen and Liley, 1993; Scott et al., 1994; Waring et al., 1996; Vermeirssen et al., 1997; see also review by Liley, 1995). Scott et al. (1994) showed that plasma sex steroid concentrations in male rainbow trout (*Oncorhynchus mykiss*) were elevated following exposure to the urine of both ovulated and nonovulated conspecific females, but not to ovarian fluid. Waring et al. (1996) showed that sex steroid concentrations in male Atlantic salmon (*Salmo salar* Linné) parr were elevated by exposure to the urine of ovulated females, but they did not test ovarian fluid. Moore and Waring (1996) suggested that F-series prostaglandins such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were acting as priming pheromones. This substance has been reported to be inactive in rainbow trout (Kitamura et al., 1994; Scott and Vermeissen, 1995). Electrophysiological studies have shown that, in contrast to rainbow trout, the olfactory epithelium in salmon detects $PGF_{2\alpha}$ (Moore and Waring, 1995, 1996; Hara and Zhang, 1998).

It is known that prostaglandins are important for the process of ovulation in fish (Goetz et al., 1989), and ovulated eggs and ovarian fluid are present in the body cavity. If ovarian fluids contain high enough concentrations of $PGF_{2\alpha}$ and the prostaglandin has a priming effect, the ovarian fluids should be effective in increasing plasma levels of sex hormone steroids. In a recent study, we have observed that plasma sex steroid levels in male parr of both Atlantic salmon and brown trout (*Salmo trutta* L.) were elevated by exposing them to a mixture of urine and ovarian fluid derived from ovulated females of either species (Olsén et al., 2000). The volumes of strippable milt were also enhanced in Atlantic salmon parr. These results led us to the present study, which compares the responses of Atlantic male parr to urine and ovarian fluid. We wanted to test the hypothesis that the priming response to ovarian fluid should be at least as strong as to urine. We also analyzed the content of $PGF_{2\alpha}$ in the urine and ovarian fluid and detected much higher levels in the ovarian fluid. Furthermore, we tested whether $PGF_{2\alpha}$ had a behavioral effect on male Atlantic salmon parr, as some studies have shown behavioral effects in fish during exposure to F prostaglandins (Stacey et al., 1994; Sveinsson and Hara, 1995, 2000).

METHODS AND MATERIALS

Fish. Experiments were performed during 1996 and 1997 with Atlantic salmon obtained from the Swedish National Board of Fisheries hatchery at Älvkarleby (60°N). Male parr were raised from a wild brood stock, and the adult females were from the same wild stock caught in the River Dalälven. The parr were raised under standard hatchery conditions.

Collection of Female Stimulants. Urine and ovarian fluids were collected by catheters from anesthetized (2-phenoxy ethanol, 0.05%) ovulated Atlantic salmon females (2.780–5.960 g, $N = 8$) at the hatchery on the River Dalälven. Both urine and ovarian fluids were taken from each female. The urine and ovarian fluids were collected in 20-ml polythene tubes on ice and subsequently stored at -80°C. Care was taken to prevent contamination between the two fluid types. It was easy to fill the tubes with ovarian fluid, but the amounts of urine were often less than 10 ml. Prior to freezing, 0.5–1.5 ml of both fluids were decanted into microfuge tubes for analyses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-keto-dihydro-$PGF_{2\alpha}$ concentrations in individual fish. The remainder of the ovarian fluid and urine samples were pooled into an ovarian fluid mix and a urine mix, respectively, and then frozen in aliquots suitable for the experiments. We also sampled a mixture of urine and ovarian fluid in the same way as in Olsén et al. (2000). The mixture of urine and ovarian fluid was collected at the time (10.00–12.00 hr) when females were stripped of their eggs for artificial fertilization. The fluids used were collected during the process of stripping, up until the appearance of the first eggs in the ovipore. The concentrations of $PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ were also analyzed in urine and ovarian fluid that were collected in 1998 from another group of six ovulated Atlantic salmon females.

Experimental Procedure. Three separate experiments were conducted during 1996–1997 to investigate the priming effects of various substances on mature male parr. The first two were conducted at the Fisheries Research Station, Älvkarleby, while the last was conducted at Uppsala University.

Experiment 1. The priming effect of urine from ovulated females, ovarian fluid, or saline solution (control) was tested on mature male parr (22.9–52.7 g, $N = 23$) during the period November 3–5, 1996. Males were not stripped of milt before the experiment. In a previous study (Olsén et al., 2000), nonstripped salmon parr showed enhanced plasma levels of sex steroids and milt volumes after exposure to from ovulated females. We wanted to keep the effects of handling stress to a minimum.

Tests were done in direct connection to, and in the same way as, the experiments described by Olsen et al. (2000) and are briefly described here: Test areas (test volume: 60 cm long $\times$ 60 cm wide $\times$ 11 cm deep) were continuously supplied with aerated groundwater at a rate of 300–400 ml/min. Temperature of the water varied between 8 and 10°C. Mature male parr were individually placed in the test area ca. 18 hr before the first supply of 2 ml of stimulant at 10:00 hr. They were exposed a second time at 12:00 hr by adding another 2 ml. Two hours later, the first fish was sampled. Tanks were supplied with a continuous flow of ground water and had their own inlet and outlet. A submersible pump circulated the water in each tank and ensured that the stimulants were evenly mixed.

For each sampling, the male parr was first anesthetized (2-phenoxy ethanol, 0.05%), weighed, and the volume of milt determined by collecting in preweighed

glass tubes following gentle abdominal pressure (Olsén and Liley, 1993). Blood was subsequently collected by severing the caudal peduncle and collecting the blood in heparinized tubes. Fish were put to death after blood sampling. Following centrifugation, plasma was collected and stored at -80°C for sex steroid determination. Testes were weighed for the determination of gonadosomatic index [GSI, (gonad weight/body weight) $\times$ 100].

Experiment 2. During the period October 20–24, 1996, the priming effect of the following fluids was studied on mature male parr (45.9–120.2 g, $N = 26$): ovarian fluid/urine mixture (87.5 μl ethanol to 50 ml of mixture); $PGF_{2\alpha}$ (87.5 μl of $PGF_{2\alpha}$ stock solution, 1mg/ml of ethanol, added to 50 ml 0.9% NaCl); control solution (87.5 μl ethanol added to 50 ml 0.9% NaCl). The solution of prostaglandin was added to the test area to give an acute nominal concentration of 0.1 nM. Experiments were performed as in experiment 1, except that males were anesthetized (2-phenoxy ethanol, 2%) and stripped of milt before they were placed in the test area. As there was a lack of increase in milt volumes despite increased plasma concentrations of 17,20β-P in experiment 1, we stripped the fish to make it easier to observe an increase in milt volume. We showed in a previous study that a mixture of urine and ovarian fluid increased the volumes of strippable milt (Olsén et al., 2000).

Fluviarium Test. In this experiment, the behavior and endocrine responses to $PGF_{2\alpha}$ were studied. On November 5, 1996, male parr with running milt were transported to Uppsala University and placed in 400–liter rearing tanks continuously supplied with aerated tap water.

Individual fishes (33.4–81.0 g, $N = 20$) were tested in a fluviarium (Höglund, 1961; Bjerselius et al., 1995) during the period November 14 to December 9, 1996. Males were stripped of milt before they were placed in the test area of the fluviarium. One fish was tested at a time. Water, with or without $PGF_{2\alpha}$, was supplied to opposite halves of the fluviarium and mixed into the main stream of aerated tap water flowing to the test area (temperature 8.3–9.5°C). The test stimuli (ethanol or $PGF_{2\alpha}$ in ethanol added to tap water) were released from 12-liter stainless steel containers by two peristaltic pumps, at the rate of 30 ml/min through two separate magnetic valves. Two magnetic valves automatically switched the supply between the two halves of the test area at regular 90-min intervals. The nominal concentration of $PGF_{2\alpha}$ in the test area was 0.1 nM (stock solution: 7.4 mg in 10 ml 95% ethanol).

A video camera was placed over the test area, and the position of the fish was recorded by a videocomputer image analysis system in IR light ($>$750 nm) between 18:00 and 06:00 hr the next morning. No stimulus was added during the first four 90-min periods (18:00–24:00 hr), but were started at 24.00 hr and lasted until 06.00 hr. The water going through the magnetic valves shifted sides of the fluviarium every 90 min from the start to the end of the experiment. It was only during the last four 90-min periods (24:00–06:00 hr) that the pumps added water with ethanol or water containing $PGF_{2\alpha}$ and ethanol.

For each fluviarium test, we used data from the last 60 min in each of the last four 90-min periods of a test to calculate a reaction value (R_v) according to the equation: $R_v = [(N_L - N_0)/(N_L + N_0)] \times 100$, in which N_L and N_0 represent the number of observations in each side of the test area with water supplied with $PGF_{2\alpha}$ and ethanol or ethanol alone, respectively. The arithmetic means of reaction values from identical tests are designated MR_v. After each fluviarium test, blood samples were taken from fish as described above.

Hormone Measurements. Plasma levels of 11-KT, T (experiment 3 only) and 17,20β-P were measured by specific radioimmunoassay (RIA) according to the method of Mayer et al. (1990). The metabolite of prostaglandin $F_{2\alpha}$, 15-keto-dihydro-$PGF_{2\alpha}$, was analyzed in unextracted media by RIA (Kindahl et al., 1976; Granström and Kindahl, 1982). The antiserum cross-reacted 16% against 15-keto-$PGF_{2\alpha}$, 4% against 13,14-dihydro-$PGF_{2\alpha}$, 0.4% against $PGF_{2\alpha}$, and 1.7% with 15-keto-dihydro-PGE_2, a major metabolite of PGE_2. For other prostaglandins, the cross-reaction was <0.1%. Samples were diluted in a 0.25% bovine γ-globulin buffer. In most cases, 50 μl of media was diluted with 450 μl of the buffer. The practical detection limit of the assay was 300 pmol/liter. If necessary, samples were diluted to allow accurate readings on the standard curve. The interassay coefficient of variation was 14%. Samples were reanalyzed if the coefficient of variation exceeded 10% for duplicate determinations. The intraassay coefficient of variation was evaluated by analyses of 10 different pooled plasma samples in five separate radioimmunoassay tubes and was between 3.4 and 7.6% at different ranges of the standard curve.

The primary prostaglandins $F_{2\alpha}$ and E_2 were analyzed in the media by a modification of the method of Lindgren et al. (1974). Samples were diluted as for the analysis of 15-keto-dihydro-$PGF_{2\alpha}$, described above. The antiserum against $PGF_{2\alpha}$ cross-reacted 75% against $PGF_{1\alpha}$, 2% against $PGF_{2\beta}$, 1.5% against $PGF_{1\beta}$, and <0.1% for PGE_2, PGE_1, PGD_2, TXB_2, and 15-keto-dihydro-$PGF_{2\alpha}$. Samples were analyzed directly for concentrations of $PGF_{2\alpha}$. Then after reduction with sodium borohydride (Lindgren et al., 1974), the samples were reanalyzed for concentrations of $PGF_{2\alpha}$. Reduction with sodium borohydride reduces PGE_2 to a mixture of $PGF_{2\beta}$ and $PGF_{2\alpha}$. With samples containing known amounts of PGE_2, the ratio between the two isomers was found to be 1:1. Therefore, the following estimates, the increase in $PGF_{2\alpha}$ concentrations after reduction was multiplied by two to obtain the PGE_2 content. The practical limits of detection for PGE_2 and $PGF_{2\alpha}$ were 0.5 and 0.3 nmol/liter, respectively. The interassay coefficient of variation was 15%, and the intraassay coefficients of variation varied between 7.3 and 12.5% at different ranges of the standard curve.

Statistical Analyses. Plasma hormone levels and milt volumes were compared between treatments with Kruskal-Wallis nonparametric ANOVA, and in the case of significance, followed by Dunn's multiple-comparison test (Siegel and

Castellan, 1988; Motulsky, 1999). Calculations were performed with GraphPad Prism (Motulsky, 1999).

In the fluviarium tests, the Student's *t* test was used when *MRv*s were compared to the theoretically indifferent reaction, i.e., when $MRv = 0$. MRv is zero when the number of observations in each water quality is the same. The Mann-Whitney U test was used to compare hormone levels, milt volumes, and GSI of control and exposed fish.

RESULTS

Exposure to Female Odors in Tank. In experiment 1, Kruskal-Wallis (KW) analysis of variance showed a significant effect of treatment on plasma levels of 17,20β-P when control solution (0.9% NaCl), ovarian fluid, or urine was added to the tank (Figure 1). Dunn's multiple-comparison test revealed higher plasma 17,20β-P levels after exposure to both female stimuli compared to the control. No effects were observed in the plasma levels of 11-KT (KW = 0.71, $P = 0.70$) or T (KW = 2.18, $P = 0.34$). No differences in volumes of strippable milt (mean ±

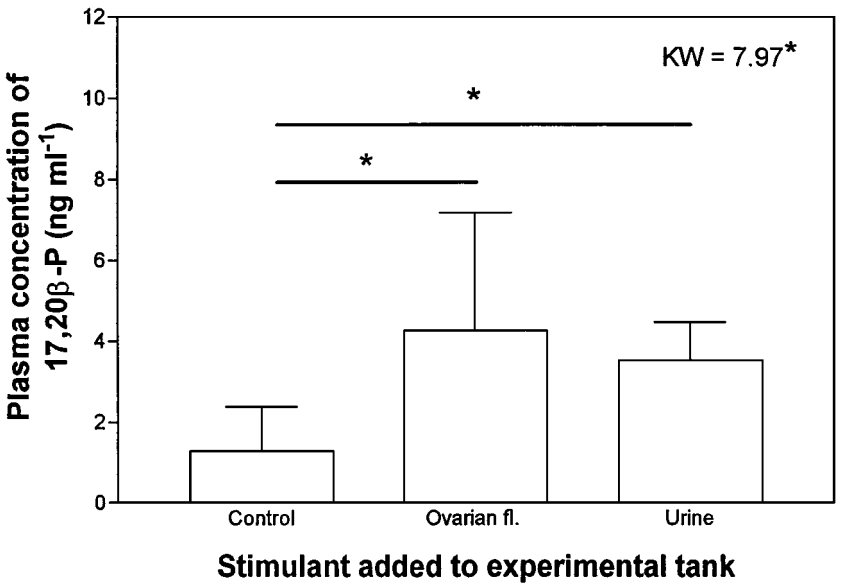


FIG. 1. Blood plasma concentrations of 17,20β-dihydroxy-4-pregnen-3-one in mature Atlantic salmon male parr after exposure to pure ovarian fluid, or urine from an ovulated conspecific female. The control solution contained 0.9% NaCl.* $P < 0.05$.

SD; control: 66.3 ± 18.7 mg/g fish, $N = 7$; urine: 57.9 ± 14.0, $N = 8$; ovarian fluid: 55.1 ± 13.3, $N = 8$; KW = 2.125, $P > 0.05$), and GSI [mean ± SD, (g/g fish) × 100; control: 11.9 ± 2.5, $N = 7$; urine: 10.8 ± 1.4, $N = 8$; ovarian fluid: 9.7 ± 1.7, $N = 8$; KW = 3.59, $P > 0.05$] were observed.

In experiment 2, Kruskal-Wallis analysis of variance showed an effect of treatment on plasma levels of 17,20β-P and 11-KT when control solution (ethanol added to 0.9% NaCl), ovarian fluid–urine mix (ethanol added to the mix), or $PGF_{2\alpha}$ (nominal conc. 0.1 nM in the tank) was added to the experimental tank (Figure 2A and 2B). Dunn's test showed a difference in 17,20β-P levels between the control and the $PGF_{2\alpha}$ -exposed fish. Plasma levels of 11-KT were increased both in the female-odor-exposed fish and fish exposed to $PGF_{2\alpha}$ (Figure 2B). There were no differences in volumes of strippable milt (mean ± SD, mg/g fish; control: 10.0 ± 6.7, $N = 9$; mix: 10.5 ± 4.6, $N = 9$; $PGF_{2\alpha}$: 11.0 ± 4.1, $N = 8$; KW = 0.13; $P > 0.05$) and GSI [mean ± SD, (g/g fish) × 100; control: 6.8 ± 1.5, $N = 9$; mix: 6.6 ± 1.7, $N = 9$; $PGF_{2\alpha}$: 6.5 ± 1.5, $N = 8$; KW = 0.26, $P > 0.05$].

Fluviarium Test. No behavioral responses were observed in parr exposed to 0.1 nM $PGF_{2\alpha}$ in the fluviarium (*MRv*s did not differ significantly from an indifferent response when $MRv = 0$) . The $PGF_{2\alpha}$ was added to one half of the test area (*MRv* ± SD; control: period 1–4: −8.5 ± 21.6, period 5–8: −4.9 ± 9.5; $PGF_{2\alpha}$: period 1–4: −3.7 ± 15.2, period 5–8 ($PGF_{2\alpha}$): 5.8 ± 13.3; *t* test gave $P > 0.05$ for all periods in both treatments). Plasma levels of 17,20β-P were, however, higher in the exposed fish compared to control (Mann-Whitney U test, $U = 11.0$, $P = 0.05$). No differences were observed in 11-KT (Mann-Whitney $U = 15.50$, $P = 0.15$) and T levels ($U = 15.50$, $P = 0.15$), respectively. No difference in volumes of strippable milt (mean ± SD, mg/g fish; control: 21.7 ± 18.2, $N = 7$; $PGF_{2\alpha}$: 25.8 ± 6.33, $N = 8$; $U = 24.0$, $P = 0.69$), and GSI (mean ± SD, (g/g fish) × 100; control: 7.4 ± 2.8, $N = 7$; $PGF_{2\alpha}$: 7.1 ± 0.9, $N = 8$; $U = 25$, $P = 0.78$) were observed.

Prostaglandin Concentrations. Concentrations of $PGF_{2\alpha}$ and its metabolite 15-keto-dihydro-$PGF_{2\alpha}$ were measured in urine and in ovarian fluid. There were high concentrations of $PGF_{2\alpha}$ in ovarian fluid (range: 200–796 nmol/liter, $N = 6$), and somewhat lower concentrations in the ovarian fluid–urine mixture (69–166 nmol/liters^{-1}, $N = 6$). Concentration of $PGF_{2\alpha}$ was low in urine (<0.3–1.1 nmol/liter, four samples <0.3, $N = 11$). The concentrations of 15-keto-dihydro-$PGF_{2\alpha}$ were lower than $PGF_{2\alpha}$ in ovarian fluid (5.4–29.4, $N = 7$, vs 200–796 nmol/liter), but in urine the reverse was found, with concentrations of the metabolite being somewhat higher than the hormone (0.5–4.6 vs <0.3–1.1 nmol/liter; $N = 11$ and $N = 7$, respectively). Levels of 15-keto-dihydro-$PGF_{2\alpha}$ were lower in urine (0.5–4.6 nmol/liter) compared to ovarian fluid (5.4–29.4 nmol/liter). Concentrations of 15-keto-dihydro-$PGF_{2\alpha}$ in the ovarian fluid–urine mix varied between 3.5 and 15.0 nmol/liter ($N = 7$). The concentration of PGE_2 varied in urine

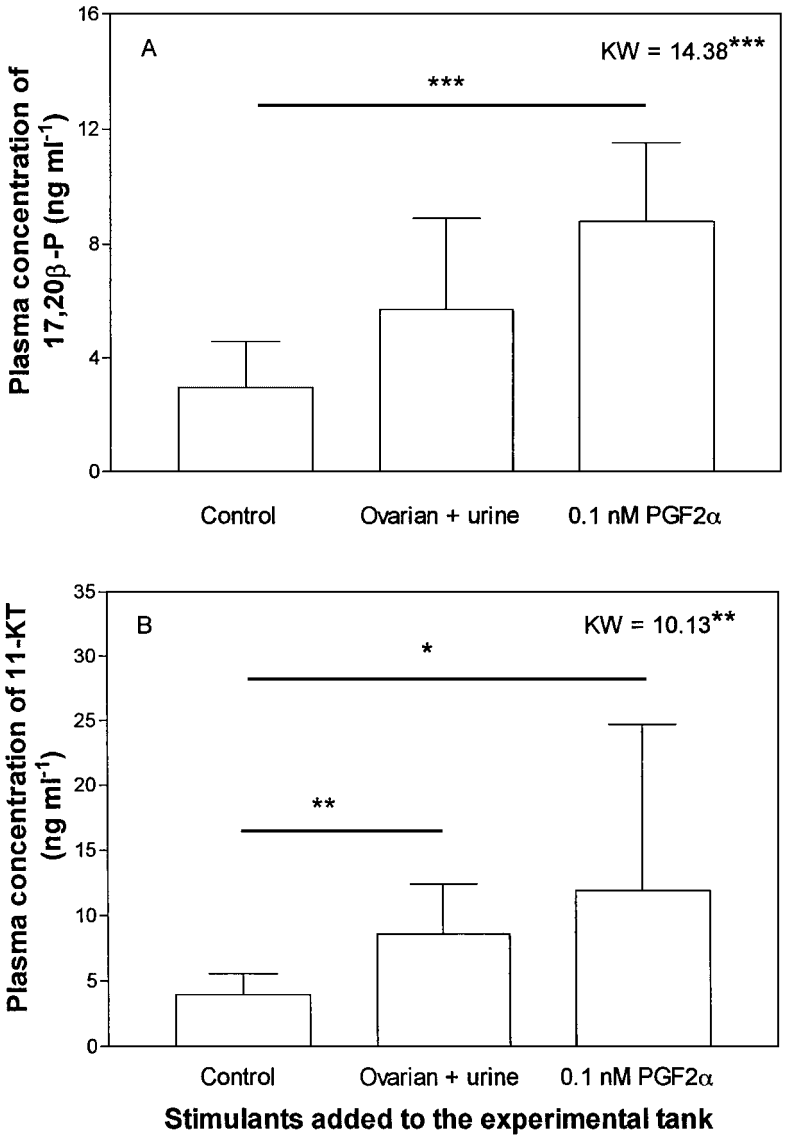


FIG. 2. Blood plasma concentrations of (A) 17,20β-dihydroxy-4-pregnen-3-one and (B) 11-ketotestosterone in mature Atlantic salmon male parr after exposure to conspecific female odor (mix solution of urine and ovarian fluid), 0.1 nM prostaglandin $F_{2\alpha}$ or control (ethanol added to 0.9% NaCl). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

between 1 and 6.8 nmol/liter ($N = 6$), but no PGE_2 was detected in ovarian fluid (detection threshold 0.5 nmol/liter).

DISCUSSION

The results of the present study show that both urine and ovarian fluid from ovulated Atlantic salmon females increase plasma levels of 17,20β-P in male parr. The mixture of urine and ovarian fluid and pure 0.1 nM $PGF_{2\alpha}$ also gave enhanced levels of 11-KT. In contrast to the previous study (Olsén et al., 2000) that was done with the same equipment and during the corresponding time of the spawning period (c.f., Moore and Waring, 1996), no difference in volumes of strippable milt was observed between control and exposed males. Previous studies with ovulated female odors have mostly reported enhanced plasma concentrations of the progestin 17,20β-P, which is involved in control of spermiation and sperm performance (e.g., Scott and Baynes, 1982; Ueda et al., 1983; Sakai et al., 1989; Miura et al., 1992), but the occurrence of increased milt volumes in these studies has varied (Scott et al., 1994; Moore and Waring, 1996; Waring et al., 1996; Vermeirssen et al., 1997; Olsén et al., 2000). This could reflect that stimulation of milt volumes is influenced by the season (Moore and Waring, 1996; Waring et al., 1996) and that there are individual differences among males (Vermeissen et al., 1997). The reason behind this lack of response in some males is not known. 17,20β-P is probably a good indicator of priming as it is directly connected to spermiation.

Analyses of the ovarian fluid and urine revealed that $PGF_{2\alpha}$ and its metabolite 15-keto-dihydro-$PGF_{2\alpha}$ are present in both fluids. The concentrations of $PGF_{2\alpha}$ were, however, much higher in ovarian fluid than in urine, while the metabolite was at about the same level. Previous studies have suggested $PGF_{2\alpha}$ as a priming pheromone in Atlantic salmon female urine (Moore and Waring, 1996). In some teleost species, prostaglandins can act as releaser pheromones (e.g., Sorensen et al., 1989; Sveinsson and Hara, 1995), and acute olfactory sensitivity has been demonstrated in different species (e.g., Cardwell et al., 1992; Bjerselius and Olsén, 1993; Kitamura et al., 1994; Essington and Sorensen, 1996; Sveinsson and Hara, 2000). In different species of Pacific salmon within the genus *Oncorhynchus*, priming and releasing sex pheromones are believed to be present, although the chemical identities of these substances are still unknown (Emanuel and Dodson, 1979; Honda, 1980, 1982; Olsén and Liley, 1993; Liley et al., 1993; Scott et al., 1994; Vermeirssen et al., 1997; Yambe et al., 1999). Enhanced plasma levels of sex steroids (but not milt volumes) were detected with mature rainbow trout female urine, but exposure to ovarian fluid failed to elicit an endocrine response (Scott et al., 1994), although the concentration of ovarian fluid in the experimental tanks was probably much higher than in the present study. To date, few sex pheromones

have been identified in fish, but the substances that have been identified are either steroids or prostaglandins (review by Stacey et al., 1994).

Prostaglandin concentrations in ovarian fluid were higher than in female urine. As prostaglandins are important for ovulation in fish, it is no surprise that they are present in ovarian fluids (e.g., Goetz et al., 1989; Peter and Yu, 1997). Both $PGF_{2\alpha}$ and PGE_2 have been detected in high concentrations in both blood plasma and ovarian fluids of brook trout, *Salvelinus fontinalis* (Mitchill), females during or immediately after ovulation (Cetta and Goetz, 1982). The question is whether the prostaglandins are released from the ovary and coelomic fluids into the water and detected by the males before spawning. Directly after spawning, the concentration of $PGF_{2\alpha}$ should be high in water close to the female and detected by males that will subsequently receive a priming effect. Males will, thus, have the advantage of increasing milt volumes until a spawning situation occurs a couple of hours later and probably with another female. There is also a possibility that small amounts of coelomic fluids are released into the water during nest digging when the abdominal pressure increases. Salmonid fish urinate in pulses (Curtis and Wood 1991), which makes it possible for the female to control the priming stimulation of the males. This should be an advantage for the courting males that will have high milt volumes ready for the spawning. A morphological study has, however, opened the possibility that ovarian fluids can also be released voluntarily. The study demonstrated the presence of a sphincterlike musculature that surrounds both the ureter and the oviduct of chum salmon (*Oncorhynchus keta*) (Uematsu and Hibiya, 1983). The authors suggested that the musculature is used to contract both the ureter and the reproductive duct, and, due to this arrangement, it may enclose the latter more tightly. This opens the possibility that both ovarian fluid and urine can be the source of priming chemicals.

Parr did not show any behavioral response to 0.1 nM $PGF_{2\alpha}$ in the fluviarium tests, but blood plasma levels of 17,20β-P were elevated. The same concentration of $PGF_{2\alpha}$ was used in the tank exposure tests that gave enhanced plasma levels of 17,20β-P and 11-KT. A recent study with male salmon parr supports the view that $PGF_{2\alpha}$ is not acting as an attractant (Olsén et al., unpublished results).

If F prostaglandins are the active substances, ovarian fluid should be at least as potent as urine as a priming agents. On the basis of the much greater content of $PGF_{2\alpha}$, it should be even more active than urine. The concentration of the hormone in the test area with urine added should have been 1000 times lower (0.1 pM) compared to the ovarian fluid exposure (experiment 1). One possibility is that in addition to $PGF_{2\alpha}$, other substances may be effective as priming pheromones in the urine. One candidate would be 15-keto-$PGF_{2\alpha}$, which is also detected by the olfactory senses of mature salmon male parr, but at much higher concentrations (Moore and Waring, 1996). In our studies, we do not know the concentrations of 15-keto-$PGF_{2\alpha}$. In addition to $PGF_{2\alpha}$, we measured its major metabolite 15-keto-dihydro-$PGF_{2\alpha}$ (the difference between the two metabolites is

saturation of the 13,14 double bound in 15-keto-dihydro-$PGF_{2\alpha}$). No data are available concerning the olfactory sensitivity of Atlantic salmon and other salmonids to 15-keto-dihydro-$PGF_{2\alpha}$, which means that its importance as a chemical signal and priming pheromone is not known. If it had an effect as a priming substance, ovarian fluid should have been more potent. We found PGE_2 in the urine of adult salmon females at about the same level as $PGF_{2\alpha}$, but in contrast to $PGF_{2\alpha}$, nothing could be detected in ovarian fluid. This suggests that the effect of PGE_2 is of little or no significance.

In summary, the results indicate that both female urine and ovarian fluid contain substances that enhance plasma sex steroid levels in Atlantic salmon male parr. They confirm that $PGF_{2\alpha}$ is one active priming substance. In addition, another unknown substance may be of importance for the potency of female urine.

Acknowledgments—This study was supported by grants from Carl Tryggers Foundation (to K.H.O.) and the Swedish Research Council for Agriculture and Forestry (SJFR; to K.H.O., I.M., and H.K.). Dr. R. W. Schulz (University of Utrecht) is thanked for supplying the steroid antisera. We thank Dr. T. Järvi and Dr. E. Petersson for placing the facilities at the Stream Water Ecology Lab., National Board of Fisheries, at our disposal.

REFERENCES

BJERSELIUS, R. and OLSÉN, K. H. 1993. A study of the olfactory sensitivity of crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) to 17α,20β-dihydroxy-4-pregnen-3-one and prostaglandin $F_{2\alpha}$. *Chem. Senses* 18:427–436.

BJERSELIUS, R., OLSÉN, K. H., and ZHENG, W. 1995. Behavioural and endocrinological responses of mature male goldfish to the sex pheromone 17α,20β-dihydroxy-4-pregnen-3-one in the water. *J. Exp. Biol.* 198:747–754.

CARDWELL, J. R., DULKA, J. G., and STACEY, N. E. 1992. Acute olfactory sensitivity to prostaglandins but not to gonadal steroids in two sympatric species of *Catostomus* (Pisces: Cypriniformes). *Can. J. Zool.* 70:1897–1903.

CETTA, F. and GOETZ, F. W. M. 1982. Ovarian and plasma prostaglandin E and F levels in brook trout (*Salvelinus fontinalis*) during pituitary-induced ovulation. *Biol. Reprod.* 27:1216–1221.

CURTIS, B. J. and WOOD, C. M. 1991. The function of the urinary bladder in vivo in the freshwater rainbow trout. *J. Exp. Biol.* 155:567–583.

EMANUEL, M. E. and DODSON J. J. 1979. Modification of the rheotropic behavior of male rainbow trout (*Salmo gairdneri*) by ovarian fluid. *J. Fish. Res. Board Can.* 36:63–68.

ESSINGTON, T. E. and SORENSEN, P. W. 1996. Overlapping sensitivities of brook charr and brown trout to putative hormonal pheromones. *J. Fish Biol.* 48:1027–1029.

GOETZ, F. W., DUMAN, P., RANJAN, M., and HERMAN, C. A. 1989. Prostaglandin F and E synthesis by specific tissue components of the brook trout (*Salvelinus fontinalis*) ovary. *J. Exp. Zool.* 250:196–205.

GRANSTRÖM, E. and KINDAHL, H. 1982. Radioimmunoassay of the major plasma metabolite of $PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$. *Methods Enzymol.* 86:320–339.

HARA, T. J. and ZHANG, C. 1998. Topographic bulbar projections and dual neural pathways of the primary olfactory neurons in salmonid fishes. *Neuroscience* 82:301–313.

HÖGLUND, L. B. 1961. The reaction of fish in concentration gradients. *Rep. Inst. Freshwater Res., Drottningholm, Sweden* 43:1–147.

HONDA, H. 1980. Female sex pheromone of rainbow trout, *Salmo gairdneri*, involved in courtship behavior. *Bull. Jpn. Soc. Sci. Fish.* 46:1109–1112.

HONDA, H. 1982. On the female sex pheromones and courtship behaviour in the salmonids, *Oncorhynchus masou* and *O. rhodurus. Bull. Jpn. Soc. Sci. Fish.* 48:47–49.

KINDAHL, H., EDQVIST, L.-E., GRANSTRÖM, E., and BANE, A. 1976. The release of prostaglandin $F_{2\alpha}$ as reflected by 15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$ in the peripheral circulation during normal luteolysis in heifers. *Prostaglandins* 11:871–878.

KITAMURA, S., OGATA, H., and TAKASHIMA, F. 1994. Olfactory responses of several species of teleost to F-prostaglandins. *Comp. Biochem. Physiol.* 107A:463–467.

LILEY, N. R. 1995. Reproductive pheromones in salmonids, pp. 1–8, *in* V. M. Canário and D. M. Power (Compilers). Fish Pheromones—Origins and Modes of Action. Proceedings of a Workshop. Centre of Marine Sciences, University of Algarve, Faro, Portugal.

LILEY, N. R., OLSÉN, K. H., FOOTE, C. J., and VAN DER KRAAK, G. J. 1993. Endocrine changes associated with spawning behavior in male kokanee salmon (*Oncorhynchus nerka*) and the effects of anosmia. *Horm. Behav.* 27:470–487.

LINDGREN, J. Å., KINDAHL, H., and HAMMARSTRÖM, S. 1974. Radioimmunoassay of prostaglandins E_2 and $F_{2\alpha}$ in cell culture media utilizing antisera against prostaglandins $F_{2\beta}$ and $F_{2\alpha}$. *FEBS Lett.* 48:22–25.

MAYER, I., BERGLUND, I., RYDEVIK, M., BORG, B., and SCHULZ, R. 1990. Plasma levels of five androgens and 17α-hydroxy-20β-dihydroprogesterone in immature and mature male Baltic salmon (*Salmo salar*), and the effects of castration/androgen-replacement in mature parr. *Can. J. Zool.* 68:263–267.

MIURA, T., YAMAUCHI, K., TAKAHASHI, H., and NAGAHAMA, Y. (1992). The rule of hormones in the acquisition of sperm motility in salmonid fish. *J. Exp. Zool.* 107:95–103.

MOORE, A. and WARING, C. P. 1995. Seasonal changes in olfactory sensitivity in mature male Atlantic salmon (*Salmo salar* L.) parr to prostaglandins, p. 273, *in* F. W. Goetz and P. Thomas (eds.). Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. 1995.

MOORE, A. and WARING, C. P. 1996. Electrophysiological and endocrinological evidence that F-series prostaglandins function as priming pheromones in mature male Atlantic salmon (*Salmo salar*) parr. *J. Exp. Biol*. 199:2307–2316.

MOTULSKY, H. 1999. Analysing data with GraphPad Prism. GraphPad Software Inc., San Diego, California.

OLSÉN, K. H. and LILEY, N. R. 1993. The significance of olfaction and social cues in milt availability, sexual hormone status and spawning behavior of male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol*. 89:107–118.

OLSÉN, K. H., BJERSELIUS, R., PETERSSON, E., JÄRVI, T., MAYER, I., and HEDENSKOG, M. 2000. Lack of species-specific primer effects of odours from female Atlantic salmon, *Salmo salar*, and brown trout, *Salmo trutta. Oikos* 88:213–220.

PETER, R. E. and YU, K. L. 1997. Neuroendocrine regulation of ovulation in fishes: Basic and applied aspects. *Rev. Fish Biol. Fish* 7:173–197.

SAKAI, N., UEDA, H., SUZUKI, N., and NAGAHAMA, Y. 1989. Involvement of sperm in the production of 17α,20βdihydroxy-4-pregnen-3-one in the testis of the spermiating rainbow trout, *Salmo gairdneri*. *Biomed. Res.* 10:131–138.

SCOTT, A. P. and BAYNES, S. M. 1982. Plasma levels of sex steroids in relation to ovulation and spermiation in rainbow trout (*Salmo gairdneri*), pp. 103–106, *in* C. J. J. Richter and H. J. T. Goos (eds.). Proceedings of the International Symposium of Reproductive Physiology of Fish. Pudoc, Wageningen, The Netherlands.

SCOTT, A. P. and VERMEIRSSEN, E. L. M. 1995. Research on reproductive priming pheromones in fish, pp. 15–23, *in* V. M. Canário and D. M. Power (Compilers). Fish Pheromones—Origins and Modes of Action. Proceedings of a Workshop. Centre of Marine Sciences, University of Algarve, Faro, Portugal.

SCOTT, A. P., LILEY, N. R., and VERMEIRSSEN, E. L. M. 1994. Urine of reproductive mature female rainbow trout, *Oncorhychus mykiss* (Walbaum), contains a priming pheromone which enhaces plasma levels of sex steroids and gonadotrophin II in males. *J. Fish Biol.* 44:131–147.

SIEGEL, S. and CASTELLAN, N. J. 1988. Nonparametric Statistics for Behavioral Sciences. McGraw-Hill, New York.

SORENSEN, P. W., STACEY, N. E., and CHAMBERLAIN, K. J. 1989. Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. *Horm. Behav.* 23:317–332.

STACEY, N. E., CARDWELL, J. R., LILEY, N. R., SCOTT, A. P., and SORENSEN, P. W. 1994. Hormones as sex pheromones in fish, pp. 438–448, *in* K. G. Davey, R. E. Peter, and S. S. Tobe (eds.). Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, Canada.

SVEINSSON, T. and HARA, T. J. 1995. Mature males of Arctic charr, *Salvelinus alpinus*, release F-type prostaglandins to attract conspecific females and stimulate their spawning behaviour. *Environ. Biol. Fish* 42:253–266.

SVEINSSON, T. and HARA, T. J. 2000. Olfactory sensitivity and specificity of Arctic charr, *Salvelinus alpinus*, to a putative male pheromone, prostaglandin $F_{2\alpha}$. *Physiol. Behav.* 69:301–307.

UEDA, H., YOUNG, G., CRIM, L. W., KAMBEGAWA, A., and NAGAHAMA, Y. 1983. 17α,20β-dihydroxi-4-pregnen-3-one: Plasma levels during sexual maturation and in vitro production by the testis of amago salmon *Oncorhynchus rhodurus* and rainbow trout *Salmo gairdneri*. *Gen. Comp. Endocrinol.* 51:106–112.

UEMATSU, K. and HIBIYA, T. 1983. Sphincter-like musculature surrounding the urino-genital duct of some teleosts. *Jpn. J. Ichthyol.* 30:72–80.

VERMEIRSSEN, E. L. M., SCOTT, A. P., and LILEY, N. R. 1997. Female rainbow trout urine contains a pheromone which causes a rapid rise in plasma 17,20β-dihydroxy-4-pregnen-3-one levels and milt amounts in males. *J. Fish Biol.* 50:107–119.

WARING, C. P., MOORE, A., and SCOTT, A. P. 1996. Milt and endocrine responses of mature male Atlantic salmon (*Salmo salar* L.) parr to water-borne testosterone, 17,20-beta-dihydroxy-4-pregnen-3-one 20-sulfate, and the urines from adult female and male salmon. *Gen. Comp. Endocrinol.* 103:142–149.

YAMBE, H., SHINDO, M., and YAMAZAKI, F. 1999. A releaser pheromone that attracts males in the urine of mature female masu salmon. *J. Fish Biol.* 55:158–171.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

A SIMPLE METHOD TO EXTRACT ESSENTIAL OILS FROM TISSUE SAMPLES BY USING MICROWAVE RADIATION

NÉLIDA E. GÓMEZ[1,*] and LUDGER WITTE[2]

[1]*Smithsonian Tropical Research Institute*
PO Box 2072, Balboa, Panama

[2]*Institut für Pharmazeutische Biologie*
Technische Universität Braunschweig
Mendelssohnstrasse 1, D-38106 Braunschweig, Germany

(Received January 23, 2001; accepted July 18, 2001)

Abstract—A microwave protocol to extract lipophilic substances from tissue was modified to extract essential oils (EOs) from plant tissue and insect feculae. The material, in a solvent transparent to microwave radiation, is exposed for a short time to steam in a microwave oven. EO extracts are analyzed directly by GC or GC-MS when plant material is fresh and terpenes contained in glandular structures on leaf surfaces are readily released into the solvent. For dried material or insect feculae, mechanical means are utilized first to break up tissue; however, the complete procedure is carried out inside the same vial to reduce losses. Statistical analysis shows that the reproducibility of the modified method is high. Several samples can be run within an hour with this method.

Key Words—Microwave, essential oil, *Cordia curassavica*, Chrysomelidae, Boraginaceae, lower terpenes, extraction method, chamomile.

INTRODUCTION

Terpenoids have been studied for different reasons. Leaf terpenoids have been evaluated for chemosystematic and geographic variability (Salgueiro et al., 1997; Fahlen et al., 1997; Souto et al., 1997), biosynthetic studies (Gershenzon, 1994; Clark et al., 1997), herbivore–host-plant relationships (Steinbauer et al., 1998; Gómez et al., 1999), bioassays (Lis et al., 1998), proof of authenticity (Casabianca et al., 1998), and aroma chemistry (Pallado et al., 1997).

A number of methods for obtaining essential oils (EOs) from plant foliage have been published (Ruberto et al., 1999; Simandi et al., 1999; Lis et al., 1998;

*To whom correspondence should be addressed. E-mail: gomezn@tivoli.si.edu

0098-0331/01/1100-2351$19.50/0

Anitescu et al., 1997; Laenger et al., 1997; Muzika et al., 1990). However, many of these techniques require gram amounts of samples and long extraction times. In contrast, methods to extract EOs from insect wastes appear to have received little attention.

A common problem encountered by chemical ecologists during their assessments is the small amount of sample material available for chemical analysis, especially when that material is insect physiological wastes and discharges, e.g., feculae and regurgitates. Similar situations are encountered with plant material, for instance, when herbivory causes defoliation, especially of young leaves (Gómez, 1997; Coley and Kursar, 1996), or conversely, when considerable quantities of plant tissue are needed for studies of geographical distributions of EOs.

We developed a method based on the work of Pare (1994, 1995) and modified by Clark et al. (1997) to quantitatively extract EOs from plant tissue and insect feculae. Here we present data on the reproducibility of that method by quantifying EOs in plant material and insect feculae with known and unknown terpenoid levels and EOs. We also compare our results with those obtained from steam distillation.

METHODS AND MATERIALS

Plant Material. We tested five samples (40 mg) from *Matricaria chamomilla* L. from the company Caolo (30.06.95, Ch. B. 53618395), with 0.63% EO to assess the efficacy of the modified method (see below). Five (40-mg) samples from *Mentha piperita* L. and *Salvia triloba* L., with unknown terpenoid levels, were also examined. Foliage of *Cordia curassavica* (Jacq.) Roem. & Schult. (field plants from Central Panama) also were examined. Branches of *C. curassavica* were collected in the field and transported to the laboratory in plastic bags. Young leaves (newly expanded at nodes 1 and 2) were excised, pooled, dried at room temperature, and then coarsely ground before weighing and extracting. Vouchers of *C. curassavica* were deposited in the Herbarium of the Smithsonian Tropical Research Institute.

Insect Feculae. Larval feculae came from *Eurypedus nigrosignata* Boh. (Coleoptera: Chrysomelidae), a beetle that feeds on *Cordia curassavica* in Central Panama. Feculae were collected from third instars that fed on plants with differing compositional profiles of EOs (Gómez et al., 1999). Larvae of *E. nigrosignata* produce elaborate dorsal structures, by using feculae and cast skins, that can be detached easily from their bodies with forceps. The size and weight of dorsal structures vary with larval age, and their chemical composition varies depending on plant terpenoid content (Gómez et al., 1999). Three groups of feculae (40 mg) were examined. Feculae for batch 1 ($N = 5$) and batch 2 ($N = 4$) came from larvae feeding on *C. curassavica* shrubs carrying the α-pinene profile, and

batch 3 ($N = 5$) came from larvae raised on *C. curassavica* cultivated in a green house (Gómez, 1997) and carrying the β-terpinene profile. Replicates in each batch contained several dorsal structures. Only quantifiable monoterpenoids and caryophyllene were considered for total concentration.

Steam Distillation. Distillation was performed in a steam-distillation apparatus as described in the German Pharmacopoeia (DAB10, 1991). Samples (1 g, $N = 2$) of dried material from *C. curassavica* (see above) were placed in a flask, 200 ml distilled water was added, and the distillation carried out for 5 hr. The distillate was collected in 0.75-ml *n*-hexane. *n*-Octadecane was added to the samples to arrive at a final concentration of 20 μg/mg. Only major terpenoids were used for comparison among methods.

Microwave Method. A method to extract lipophilic substances from fresh material as developed by Pare (1994, 1995) and modified by Clark et al. (1997) was adjusted further to extract EOs from small amounts of fresh and dried samples. The material was extracted with 0.75 ml *n*-hexane (p.a.) containing 50 μg/ml of *n*-octadecane in a vial (2.0-ml, conical, polypropylene Twist Cap vials, with screw caps with an O-ring seal). Two glass beads (4 mm diam.) were added to the sample vial, and shaken in a Vortex at medium speed for approximately 120 sec (or until tissue was crushed). A polypropylene lid with five to six radially symmetrical holes served as a vial holder (Clark et al., 1997). The lid was placed on a 250-ml glass beaker containing 50 ml water at room temperature. Sample vials were then heated in a microwave oven at full power (800 W) for 60 sec. After the microwave exposure, the vials with lids were cooled in a polypropylene jar of cold water and centrifuged at 14,000 rpm for 3 min. The clear supernatant was transferred and kept in 2.0-ml glass vials (screw cap with Teflon lining) at -18°C until GC and GC-MS were carried out (see below). When fresh plant material was used, a clear extract was obtained without the need for maceration or centrifugation. The total EO concentration, and the concentration of the major terpenoids was determined for each sample (see below). Only major terpenoids were considered for comparison of methods.

GC and GC-MS. Separation, quantification, and identification of terpenoids were carried out as described in Gómez et al. (1999).

Scanning Electron Microscopy (SEM). The surface of young *C. curassavica* leaves was viewed directly before and after microwave extraction, by using backscattered electrons at low vacuum, and they were photographed in a Jeol model 5300LV scanning electron microscope.

Statistical Analysis. The reproducibility of the microwave method was tested by comparing coefficients of variation (CV) (Muzika et al., 1990; SYSTAT, 1992), and the intraclass correlation coefficient (ICC) within groups (Sokal and Rohlf, 1995; SYSTAT, 1992). Yields obtained by the microwave method and the distillation method were compared by linear regression (LR) analysis, and the adjusted

squared multiple R was utilized to compensate for unequal sample sizes. In one case, a LR was made by considering the mean values of the distillation method as the independent variable. If the microwave method extracts a higher quantity of terpenoids than the distillation method, then the slope of the LR equation should be larger than unity. A second LR was made by considering the mean values of the microwave method as the independent variable. Correlation coefficients were used to compare the fit and the variance explained by the method.

RESULTS

Plant Material. The EO concentration of commercial *M. chamomilla* samples determined by the microwave method was $0.65 \pm 0.05\%$ (Table 1), the same as the reported one of 0.63%. Terpenoids detected in the yellowish extract of chamomile were β-farnesene, α-bisabolol oxide B, α-bisabolone oxide, α-bisabolol, bisabolol oxide A, and spiroether. Plant material (40 mg) of *M. piperita*, *S. triloba*, and *C. curassavica* (wild plants) with unknown levels of EO also were examined (Table 1). The CV varied from 5% to 15% for the total concentration, and from 3% to 16% for the amount of the major terpenoid.

The ICC was used to evaluate the reproducibility of the microwave method and showed 9% variation for the total concentration within the groups, and 11% for the main component.

Comparison of Extraction Methods. The first LR demonstrates that the microwave method was more efficient in obtaining higher concentrations of components (slope $= 1.729$; $p < 0.001$). The microwave method produced less variable yields ($R^2 = 0.900$) than the distillation method ($R^2 = 0.787$).

SEM. After exposure to hot hexane, glandular trichomes on the surface of fresh leaves collapsed (Figure 1A, B).

Insect Material. Three different batches of insect feculae were utilized to evaluate this method. CVs varied from 14% to 21% for the total concentration of EOs and from 17% to 20% for the main component, depending on fecula origin

TABLE 1. EO CONCENTRATION OF DIFFERENT PLANT SPECIES SAMPLES WITH MICROWAVE METHOD[a]

Plant species	Total concentration	%CV	Major terpenoid concentration	%CV
Matricaria chamomilla	6.59 ± 0.43	7	3.66 ± 0.11^a	3
Mentha piperita	5.30 ± 0.29	5	2.14 ± 0.19^b	8
Salvia triloba	13.50 ± 2.20	15	6.89 ± 1.25^c	16
Cordia curassavica	12.66 ± 0.90	7	3.74 ± 0.54^d	16

[a] $N = 5$; concentration (mean $\pm$ SD) in mg/g, dry weight; %CV, coefficient of variation in percentage; a, bisabololoxide A; b, menthol; c, 1,8-cineol; d, β-terpinene.

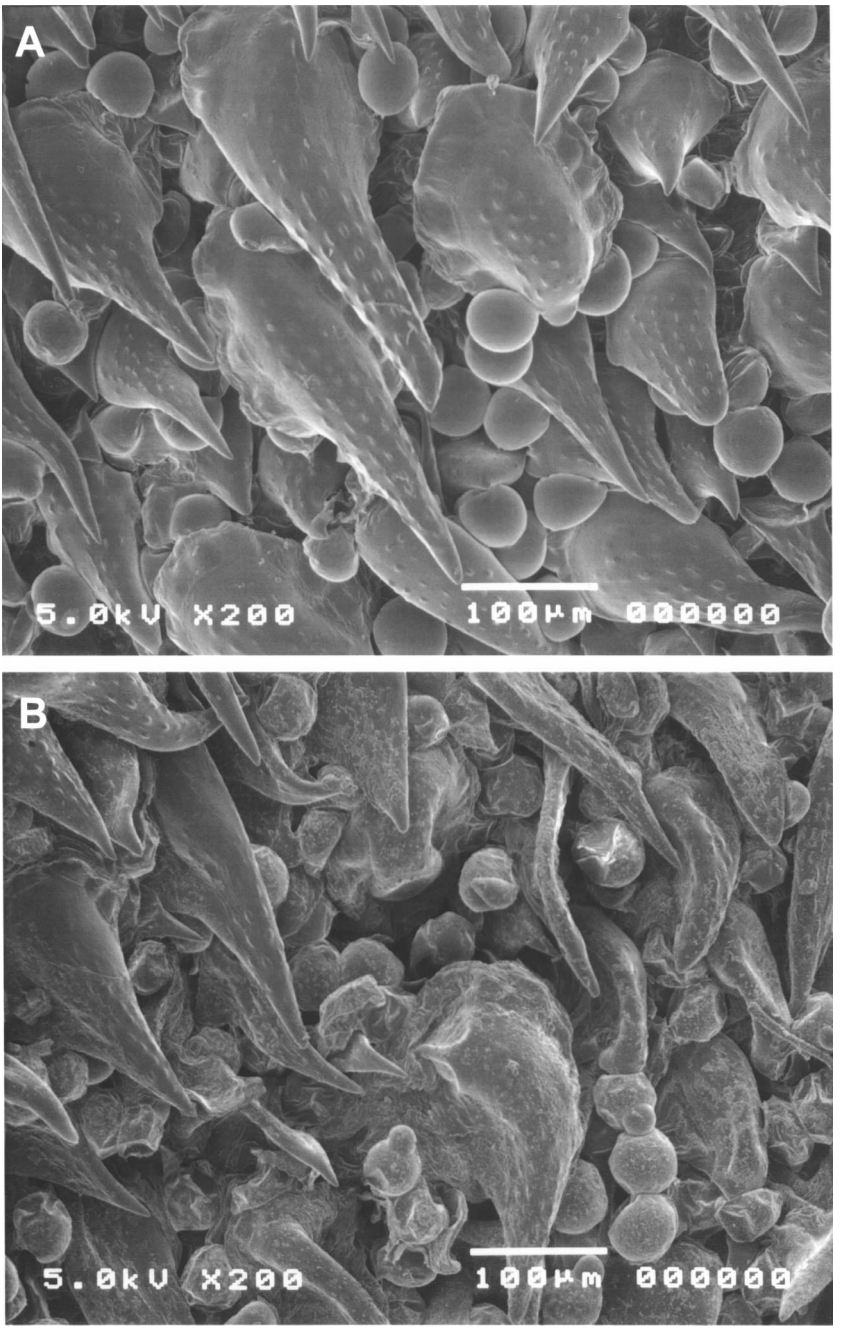


FIG. 1. Adaxial surface of *C. curassavica* showing glandular round trichomes (A) before and (B) after exposure to hot hexane.

TABLE 2. EO CONCENTRATION FOR THREE FECULA BATCHES COLLECTED FROM THIRD INSTARS OF *Eurypedus nigrosignata*[a]

Batch	Avg. weight (mg) (±SD)	Avg. number of dorsal structure per replicate (±SD)	Total concentration (mean ±SD)	%CV	Major terpenoid concentration (mean ± SD)	%CV
Wild larvae ($N = 5$)[b]	40.9 ± 1.2	6.6 ± 0.5	2.88 ± 0.61	21	1.12 ± 0.22	20
Wild larvae ($N = 4$)	39.9 ± 2.6	7.3 ± 0.8	4.99 ± 0.92	18	2.57 ± 0.46	18
Greenhouse larvae ($N = 5$)[c]	37.8 ± 3.2	7.0 ± 1.4	2.67 ± 0.39	14	1.58 ± 0.26	17

[a] Concentration in μg/mg, dry weight; %CV, coefficient of variation in percentage.
[b] α-Pinene profile.
[c] β-Terpinene profile.

(Table 2). Feculae from animals feeding on field plants registered higher CVs than those from animals raised on greenhouse plants.

The ICC was calculated also to evaluate the microwave method for insect samples. The ICC percentage variation within groups was 23% for the total concentration, and 17% for the major component.

DISCUSSION

For fresh plant material containing EOs in glands, the hot solvent was efficient at collapsing glandular structures and extracting terpenes (Clark et al., 1997) without need of maceration. Indeed, treatment of fresh plant material with glass beads in these trials did not cause tissue destruction. Destruction of plant tissue other than that containing EOs may lead to undesirable plant contaminants in the extract. By extracting fresh plant material as described above, the clear extract obtained can be analyzed directly by GC.

In the case of insect material, we found that the use of glass beads to macerate both fresh and dried insect feculae before microwave exposure, in order to destroy the matrix holding EOs, facilitates their release into the solvent. Maceration with glass beads was a good alternative to pulverization with a mill or maceration with a mortar and pestle, because these procedures lead to significant sample loss, particularly when dealing with small samples containing volatile substances. This step usually produced fine particles that were separated by centrifugation. Losses of volatiles can be reduced further by carrying the procedure to completion in the same extraction vial and by avoiding rotary evaporation to eliminate the solvent, because it may lead to the losses of volatile compounds.

The coefficient of variation (CV) has been used as an index of reproducibility of five techniques for extracting volatile compounds from needles of two conifer

species (Muzika et al., 1990). The CV was developed to compare the relative amounts of variation in populations having different means (Sokal and Rohlf, 1995, p. 58). CV analysis indicates that the modified microwave method is reproducible both for total terpenoid concentration and for individual terpenoids. The intraclass correlation coefficient (ICC) measures the similarity of individuals within a group, relative to the degree of difference found among the groups (Sokal and Rohlf, 1995, p. 213). A low value for ICC indicates that there is little or no variance within groups. The ICC showed low variation within groups for both plant and insect materials, supporting the reproducibility of the modified method.

Higher EO yields were attained with the microwave method compared to yields obtained by steam distillation. In addition, this method yields less variable results. Several authors have reported that steam distillation produces lower extraction yields (Simandi et al., 1999; Tuan and Ilangantileke, 1997; Stashenko et al., 1997), contributes to thermolysis of sensitive terpenes (Ruberto et al., 1999; Ammann et al., 1999; Eikani et al., 1999), and takes longer than other techniques (Muzika et al., 1990). With the modified method, we can not rule out the possibility that some thermal degradation may occur. However, we were not able to detect this in our samples. On the other hand, comparison of various methods that have been used to extract EOs from conifer needles has shown that circular steam distillation of 10-g samples for 8 hr yielded higher quantities (Muzika et al., 1990) than did solvent extraction, liquid carbon dioxide extraction, or rapid steam distillation.

The tremendous natural variation in leaf terpenoids (Langenheim, 1994; Gershenzon, 1994) may also influence the composition of physiological discharges from phytophagous insects, e.g., many insects prefer to eat young leaves (Coley and Kursar, 1996). We suggest collecting and extracting newly expanded leaves (assuming that they are small enough to fit inside a vial) and/or removing a predetermined leaf area from more mature leaves. Analysis of fresh material results in higher yields of secondary metabolites (Gómez et al., 1999; Coley and Kursar, 1996) and prevents the loss of volatiles due to drying (Ross and ElSohly, 1996; Yen and McGaw, 1996).

When analyzing insect material with the microwave method, one should consider that variation due to the nature of the sample rather than the method itself may occur. Our particular plant–insect system is under the strong chemical influence of a particular host plant as well as the larval age (Gómez et al., 1999).

Specific Considerations. Because hexane is transparent to microwave radiation, water steam is the heating source. A solvent not transparent to microwave radiation would itself boil, making water steam unnecessary. However, solvent polarity may be important, depending on the chromatographic analysis chosen. Additionally, an explosion-proof oven should be used, in case extraction vials have leaks or if open containers are used. It is advisable to use high-quality, seal-proven extraction vials to assure that solvent fumes (and volatile substances) are not released into the oven chamber. The watt output of the microwave oven

may vary depending on brand, country of manufacture, and local electrical characteristics, so that the exposure time may need adjustment. We found that dried leaflets longer than half of the vial height were difficult to grind with two glass beads. In such a case, smaller beads especially designed for tissue analysis may be needed. Alternatively, the leaf can be broken manually.

Chemical ecologists usually are confronted with a limited sample of insect waste material for analysis. Factors such as insect age and insect preferences for specific plant organs at specific developmental stages are important considerations during the analysis of EOs.

In summary, the microwave method is reproducible, easy to run, allows simultaneous analysis of many samples, and offers an excellent alternative to methods that utilize gram amounts of material, i.e., steam distillation and solvent extraction. Researchers engaged in evaluating the role of EOs in plant–insect interactions, terpenoid differences in individual plants, and biogeographical studies of EOs, may profit greatly from using this modified method.

Acknowledgments—We thank Dr. H.-J. Hammerschmidt of the perfume company Dragoco, Germany, for kindly identifying many sesquiterpenes, T. Hartmann (TU-BS) and P. Solís (University of Panama) for comments on the manuscript, A. Aiello (STRI) for improving the language, and R. Cordero for helping with the statistics. This work was financially supported by a fellowship (to N.E.G.) from the German Academic Exchange Service (DAAD), and by Research Opportunity Funds from the Smithsonian Tropical Research Institute (STRI).

REFERENCES

AMMANN, A., HINZ, D.-C., ADDLEMAN, R.-S., WAI, C.-M., and WENCLAWIAK, B. 1999. Superheated water extraction, steam distillation and SFE of peppermint oil. *J. Anal. Chem.* 364:650–653.

ANITESCU, G., DONEANU, C., and RADULESCU, V. 1997. Isolation of coriander oil: Comparison between steam distillation and supercritical CO_2 extraction. *Flavour Fragrance J.* 12:173–176.

CASABIANCA, H., GRAFF, J. B., FAUGIER, V., FLEIG, F., and GRENIER, C. 1998. Enantionmeric distribution studies of linalool and linalyl acetate. A powerful tool for authenticity control of essential oils. *J. High Resolut. Chromatogr.* 21:107–112.

CLARK, J. L., HAMILTON, J. G. C., CHAPMAN, J. V., RHODES, M. J. C., and HALLAHAN, D. L. 1997. Analysis of monoterpenoids in glandular trichomes of the catmint *Nepeta racemosa*. *Plant J.* 11:1387–1393.

COLEY, P. D. and KURSAR, T. A. 1996. Anti-herbivore defenses of young tropical leaves: Physiological constraints and ecological trade-offs, pp. 305–336, *in* S. S. Mulkey, R. L. Chazdon, and A. P. Smith (eds.). Tropical Forest Plant Ecophysiology. Chapman & Hall, New York.

DAB10 (DEUTSCHES ARZNEIBUCH). 1991. Gehaltsbetimmung des ätherisches Oelen, Grundlfg., v.4.5.8., amtliche Ausgabe. Deutscher Apotheker Verlag, Stuttgart.

EIKANI, M. H., GOODARZNIA, I., and MIRZA, M. 1999. Supercritical carbon dioxide extraction of cumin seeds. *Flavour Frangance J.* 14:29–31.

FAHLEN, A., WELANDER, M., and WENNERSTEN, R. 1997. Effects of light-temperature regimes on plant growth and essential oil yield of selected aromatic plants. *J. Sci. Food Agric.* 73:111–119.

GERSHENZON, J. 1994. The cost of plant chemical defense against herbivory: A biochemical perspective, pp. 105–173, *in* Elizabeth A. Bernays (ed.). Insect–Plant Interactions, Vol. V. CRC Press, Boca Raton, Florida.

GÓMEZ, N. E. 1997. The fecal shields of larvae of tortoise beetles (Cassidinae: Chrysomelidae): A role in chemical defense using plant-derived secondary metabolites. Dissertation. Naturwissenschafts-fakultät, Technische Universität Braunschweig, Braunschweig. 124 pp.

GÓMEZ, N. E., WITTE, L., and HARTMANN, T. 1999. Chemical defense in larval tortoise beetles: Essential oil composition of fecal shields of *Eurypedus nigrosignata* and foliage of its host plant, *Cordia curassavica. J. Chem Ecol.* 25:1007–1027.

LAENGER, R., MECHTLER, C., and JURENITSCH, J. 1997. Composition of the essential oils of commercial samples of *Salvia officinalis* L. and *S. fruticosa* Miller: A comparison of oils obtained by extraction and steam distillation. *Phytochem. Anal.* 7:289–293.

LANGENHEIM, J. H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol.* 20:1223–1280.

LIS, B. M., BUCHBAUER, G., RIBISCH, K., and WENGER, M. T. 1998. Comparative antibacterial effects of novel *Pelargonium* essential oil and solvent extracts. *Lett. Appl. Microbiol.* 27:135–141.

MUZIKA, R.-M., CAMPBELL, C. L., HANOVER, J. W., and SMITH, A. L. 1990. Comparison of techniques for extracting volatile compounds from conifer needles. *J. Chem. Ecol.* 16:2713–2722.

PALLADO, P., TASSINATO, G., D'ALPAOS, M., and TRALDI, P. 1997. Gas chromatography/mass spectrometry in aroma chemistry: A comparison of essential oils and flavors extracted by classical and supercritical techniques. *Rapid Commun. Mass Spectrom.* 11:1335–1341.

PARE, J. R. J. 1994. Microwave extraction of volatile oils. U.S. Patent No. 5,338,557.

PARE, J. R. J. 1995. Microwave-assisted extraction from materials containing organic matter. U.S. Patent No. 5,458,897.

ROSS, S. A. and ELSOHLY, M. A. 1996. The volatile oils composition of fresh and air-dried buds of *Cannabis sativa*. *J. Nat. Prod.* 59:49–51.

RUBERTO, G., BIONDI, D., and RENDA, A. 1999. The composition of the volatile oil of *Ferulago nodosa* obtained by steam distillation and supercritical carbon dioxide extraction. *Phytochemi. Anal.* 10:241–246.

SALGUEIRO, L. R., VILA, R., TOMÀS, X., CAÑIGUERAL, S., PROENÇA DA CUNHA, A., and ADZET, T. 1997. Composition and variability of the essential oils of *Thymus* species from section *Mastichina* from Portugal. *Biochem. Syst. Ecol.* 25:659–672.

SIMANDI, B., DEAK, A., RONYAI, E., YANXIANG, G., VERESS, T., LEMBERKOVICS, E., THEN, M., SASS-KISS, A., and VAMOS-FALUSI, Z. 1999. Supercritical carbon dioxide extraction and fractionation of fennel oil. *J. Agric. Food Chem.* 47:1635–1640.

SOKAL, R. R. and ROHLF, F. J. 1995. Biometry, 3rd ed. W. H. FREEMAN, New York, 887 pp.

SOUTO BACHILLER, F., DE JESUS ECHEVERRIA, M., CARDENAS GONZALEZ, O. E., ACUNA RODRIGUEZ, M. F., MELENDEZ, P. A., and ROMERO RAMSEY, L. 1997. Terpenoid composition of *Lippia dulcis. Phytochemistry* 44:1077–1086.

STASHENKO, E. E., PUERTAS, M. A., and COMBARIZA M. Y. 1996. Volatile secondary metabolites from *Spilanthes americana* obtained by simultaneous steam distillation–solvent extraction and supercritical fluid extraction. *J. Chromatogr.* 752:223–232.

STEINBAUER, M. J., CLARKE, A. R., and MADDEN, J. L. 1998. Oviposition preference of a *Eucalyptus* herbivore and the importance of leaf age on interspecific host choice. *Ecol. Entomol.* 23:201–206.

SYSTAT. 1992. SYSTAT for Windows: Statistics, Version 5 ed. SYSTAT, Inc., Evanston, Illinois, 750 pp.

TUAN, D. Q. and ILANGANTILEKE, S. G. 1997. Liquid CO_2 extraction of essential oil from star anise fruits (*Illicium verum H.*). *J. Food Eng.* 31:47–57.

YEN, V. C. R. S. and MCGAW, D. 1996. Yield and chemical composition of essential oils of Grenadian nutmegs. *Trop. Agric.* 73:301–304.

Journal of Chemical Ecology, Vol. 27, No. 11, November 2001 (© 2001)

CALL FOR PAPERS

SPECIAL ISSUE ON AQUATIC CHEMICAL ECOLOGY

There will be a special issue of the *Journal of Chemical Ecology* devoted entirely to papers dealing with "Aquatic Chemical Ecology". Publication is planned for late summer/early fall 2002. The issue will consist of invited mini-reviews and contributed papers. Those wishing to submit a paper for consideration for this special issue should do so by February 1, 2001.

Please send the original and two copies of the manuscript (including illustrations) and the names and addresses of three potential reviewers to:

John T. Romeo
Journal of Chemical Ecology
Department of Biology
University of South Florida
Tampa, Florida 33620-5200

0098-0331/01/1100-2361$19.50/0

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

APPLICATION OF HYPHENATED CHROMATOGRAPHY–MASS SPECTROMETRY TECHNIQUES TO PLANT ALLELOPATHY RESEARCH

TERRY HAIG[1,2]

[1]*School of Science & Technology*

[2]*Farrer Centre for Conservation Farming*
Charles Sturt University
Wagga Wagga, New South Wales 2678, Australia
E-mail: thaig@csu.edu.au

(Received December 1, 2000; accepted July 28, 2001)

Abstract—Plant allelopathy offers hope as an additional means of weed control in modern agriculture. Its mechanisms and molecular basis are not yet well understood. Research on the chemical basis for allelopathy has often been hindered by the complexity of plant and soil matrices, making it difficult to track active compounds. Recent improvements in the cost and capabilities of bench-top chromatography–mass spectrometry instruments make these tools more powerful and more widely available to assist with molecular studies conducted in today's expanding field. Such instrumental techniques are herein recommended as economically efficient means of advancing the rigor of allelopathy research and assisting the development of a better understanding of the chemical basis for the allelopathy phenomenon.

Key Words—Allelopathy, phytotoxicity, sustainable agriculture, weed management, allelochemicals, chromatography, mass spectrometry, hyphenation, GC-MS, LC-MS, MS-MS.

INTRODUCTION

Within the broader field of chemical ecology, the expanding science of plant allelopathy (biochemical interactions between plants) possesses potential to contribute to the development of sustainable agriculture, especially in the area of weed management, as currently, weed science possesses few alternatives to tillage and to artificial herbicides as means for economically and environmentally sound weed control.

0098-0331/01/1200-2363/0

However, for an adequate understanding of allelopathic effects and mechanisms, a knowledge of specific allelochemicals is required (among other things); yet it is only in comparatively recent times that this sort of information has become readily available. This lag in chemical information is due first to the fact that the compounds from a given plant, microorganism, or soil source usually occur within complex metabolic mixtures and in tiny quantities, thus creating a formidable problem in analysis. Second, the most powerful tool for dealing with such complex organic mixtures, gas chromatography coupled with mass spectrometry, (GC-MS), has only been readily available in commercial economic form since about 1975, while the useful (but presently less powerful) companion tool liquid chromatography-mass spectrometry (LC-MS), has been available for even less time. It is the physical on-line coupling, software-controlled, separate systems unitization, and computerized output data handling (collectively known as "hyphenation") between the physical mixture separation process of chromatography and the structure identifying (and quantifying) ability of mass spectrometry that allows such analytical power to bypass the need for the elaborate and expensive isolation of individual compounds. The key idea behind hyphenation is the significant gain in analyte-signal to background-noise ratio. While with hyphenation the signal decreases with each additional hyphenated analysis stage, the background chemical noise decreases even faster, owing to the higher selectivity of the coupled system. Although the individual compounds within complex mixtures in GC- or LC-MS work may not be actually isolated, the identifying data so obtained can enable the researcher to carry out bioassays and chemical assays with authentic compounds from other sources available in larger quantity.

The major purposes of this review are to assist with the advancement and rigor of plant allelopathy research, in accordance with journal editors' calls (Romeo, 2000) by promoting the use of hyphenated chromatography–mass spectrometry techniques to provide confirming and defining chemical data and also to provide sufficient examples of these techniques to allow the evaluation of their efficacy for the solution of new allelopathic problems that may involve the identification and/or quantification of numerous organic components from complex mixtures. In this respect, the review is meant to be indicative and not exhaustive.

For those newly moving into allelopathy studies, its more recent historical development can be conveniently traced through such works as Rice (1984, 1995), Thompson (1985), Putnam (1985), Putnam and Tang (1986), Waller (1987), Rizvi and Rizvi (1992), Narwal and Tauro (1994), Inderjit et al. (1995, 1999), Olofsdotter (1998), Macias et al. (1999), Narwal (1999a,b), Cutler and Cutler (1999), and Blum et al. (1999), although the phenomenon of allelopathy has a much longer history than this. Chromatography–mass spectrometry has been given little prominence in these works until the late 1990s, and although its analytical power has been widely recognized for some time, the cost of instruments until now has been a hindrance to widespread use, particularly in developing countries. Thus, as late as 1986,

Putnam and Tang (1986) commented that "chemistry has been the Achilles heel of allelopathy." Today, however, most researchers would agree with Weidenhamer's (1996) comment that the Putnam-Tang assertion is no longer true. More specifically, with respect to LC-MS and GC-MS, the 1990s have seen the development of small, relatively inexpensive, bench-top versions of these instruments, with sophisticated software on dedicated personal computers, which require much less operator expertise, and display great reliability and robustness—most notably in the more difficult interface between LC and MS.

For those readers not necessarily intimate with general high-resolution (HR) chromatographic separations, there are numerous descriptive works available to give a sound introduction to each type. For HRLC, examples include: Meyer (1988) and Gooding and Regnier (1990); while for LC-MS, such works as Brown (1990), Lin and Voyksner (1995), Kleintop et al. (1995), and Niessen (1999) serve well. For HRGC, examples include Jennings et al. (1997), Grob (1998), and Heath and Dueben (1998); while for GC-MS the works of Karasek and Clement (1988) and McMaster and McMaster (1998) are helpful. Closer to the theme of this review are the edited publications of Millar and Haynes (1998) (providing a current summary of a range of useful analytical methods in chemical ecology, including mass spectrometry), Linskens and Jackson (1986) (use of GC-MS in plant analysis), Newton and Walton (1996) (plant GC-MS), Alborn and Stenhagen (1987) (LC-MS in allelopathy), and Waller et al. (1999) (allelopathic compounds).

While both hyphenated techniques of LC-MS and GC-MS combine the resolving power of chromatography with the detection specificity of mass spectrometry and possess about the same sensitivity in analysis, together with similar fingerprint-identifying power for unknown compounds (where pure reference compound spectra can be run to build small user-created libraries, or where EI particle beam LC-MS is available), the LC-MS technique has a great advantage over GC-MS in being able to handle effectively the separation of labile, heat-sensitive, involatile, and high-molecular-weight samples, and can, therefore, cover an estimated 80% of all known compounds. The remaining 20% of lighter, more stable, and volatile compounds are best handled by capillary GC-MS (HRGC-MS), which has a much higher chromatographic resolving power than LC-MS because of the long and narrow GC separation columns that help to overcome a large part of the tendency for analyte coelution that occurs with complex mixture analysis. Another significant advantage of GC-MS [whose volatile analyte range may be further extended by chemical derivatization, Halket (1993)] is the availability, for the sake of identification, of quite large (approx. 320,000 Wiley/NIST) commercial libraries of computer-searchable standard electron-impact mass spectra accumulated over many years of research by using magnetic sector and transmission quadrupole spectrometers (classical mass spectra). Such library spectra have been run and collected under physical conditions similar to that occur in the mass spectral detector used in most hyphenated GC-MS instruments, and can, therefore, be directly

compared with analytical spectra by a software computer algorithm for compound matching. For a library-to-analyte computer match number in excess of 800 (1000 is a perfect match), a tentative identification is accepted and will become definitive if the pure reference compound can be found (perhaps by synthesis or from commercial sources) and determined to have the same chromatographic retention time under the same instrumental conditions. For most LC-MS, there is at present no large spectral library equivalent to the GC-MS fingerprint technique (unless an EI particle beam instrument is available) because optimal LC-MS instrument operating conditions and methods of ionization are so variable.

ALLELOPATHY STUDIES ASSISTED BY CHROMATOGRAPHY AND MASS SPECTROMETRY

Studies Using Nonhyphenated Techniques

The allelopathy literature has, of course, many fine examples of chemical identifications (and a few quantifications) carried out by various means that use combinations of separate chromatographic and spectroscopic analyses performed upon the isolated individual compounds. Such papers as Chou and Waller (1980), Phillips and Hedin (1990), Yamane et al. (1992b), Inoue et al. (1992), Li et al. (1993), Waller et al. (1993a,b), Ohwaki et al. (1993), Korhammer and Haslinger (1994), Nakahisa et al. (1994), Macias et al. (1996, 1997), Bah and Pereda-Miranda (1996), Pereda-Miranda (1997), Gumnicka and Oleszek (1998), and Huang et al. (1999) are typical of these types of studies. Provided the investigator is prepared to meet the higher costs involved in time, manpower, instrumentation, and consumables for the isolation and purification of individual compounds from a complex matrix, then the advantages of other powerful analytical tools such as FTIR, PMR, CMR, and MS-MS, can also be brought to bear upon structural elucidation and chemical identifications. In this review, however, the concern is with cheaper, faster, hyphenated chromatography–mass spectrometry methods that rely more upon fast computer spectral library matching for identifications. Hyphenation of GC with FTIR plus MS, and recently between LC and NMR, offers additional analytic power within the range of hyphenated techniques, but again, present instrument cost is a considerable barrier to their widespread use.

Allelochemical Identifications Using Hyphenated Chromatography–Mass Spectrometry

Table 1 lists a comparison of research papers in summary form published between 1979 and 2000 that establish the identity of plant allelochemicals by using chromatography–mass spectrometry. As might be expected for the more mature GC-MS techniques, the number of examples of their use is considerably

TABLE 1. ALLELOCHEMICAL IDENTIFICATIONS USING CHROMATOGRAPHY–MASS SPECTROMETRY

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To develop a procedure for the separation, detection, and identification of hydroxamates and lactams in maize extracts (*Zea mays* L.)	Bioassay not required	GC-MS GC: Varian Aerograph 2700; packed column: glass, 1.8 m × 2 mm ID; stat. phase 3% OV-1; MS: Du Pont 21-491B; EI at 70 eV: source 200°C; inlet 250°C	Six different 1,4-benzoxazin-3-ones (three × hydroxamic acids, three × lactams) and 6-methoxybenzo-xazolinone	TMS derivatives using BSTFA at 70°C for 20 min	Woodward et al. (1979); packed GC column—sensitivity to 100 ng
To examine the weed thorn-apple (*Datura stramonium*) for its allelopathic potential against linseed (*Linum usitatissimum* L.) and to search the seed and leaves aqueous leachate for allelopathic alkaloids	% germination and radicle elongation of linseed (*Linum usitatissimum* L.)	GC-MS Finnigan Quadrupole 3200 system with Incos 2300 Data System; column glass, 1.5 m × 2 mm ID; stat. phase 3% OV-17 and temp. prog.; carrier gas methane at 20 ml/min; ionization: methane CI with ion source pressure 0.8 torr	Alkaloids; hyoscyamine and scopolamine	pentafluoro-propionate derivatives using PFPA	Lovett et al. (1981) methane carrier gas used as CI gas also; seed leachate contained more alkaloid than leaf

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To isolate, identify and measure the biological activities of allelochemicals from water nutgrass (*Cyperus serotinus* Rottb.)	Radicle length and 2nd leaf sheath length of rice seedlings (*Oryza sativa* L. cv. Honenwase) and lettuce (*Lactuca sativa* L. cv. New York) hypocotyl and root length	GC-MS LKB 9000 system; column: glass, 3 m; stat. phase 10% PEG-20 M and a 2% OV-17 column; molec. separator 240°C; source 270°C; EI at 20 eV; ionising current 60 μA	Sesquiterpenoids; β-selinene; 2,6-farnesol; methyl farnesate; *trans*-2, *trans*-6-farnesyl acetate	Steam-distilled volatiles	Komai et al. (1981); packed column GC
To determine if aqueous extracts of wheat straw were inhibitory towards the weed morning glory (*Ipomoea lacunosa* L.) and to identify any inhibitory component	% germination and root length measured on morning glory and on ragweed (*Ambrosia artemisiifolia*)	GC-MS LKB 2091 system; column: 6 ft $\times$ 1/8 in. ID ultrabond at 230°C; helium carrier 8 ml/min; source 210°C; EI at 70 eV; mass scan cycle 2.0 sec	Ferulic acid	No derivatization (some adsorption and thermal decomposition of analyte)	Liebl and Worsham (1983); ferulic acid readily decarboxylated by bacteria to 2-methoxy-4-ethenylphenol, itself phytotoxic

To identify components of the volatiles emitted from harvested Palmer amaranth (*Amaranthus palmeri* S. WATS.) root, stem, and seedhead, and to test their capacity to inhibit germination of onion, carrot, tomato, and Palmer amaranth seeds	Vapor phase seed germination inhibition on onion, carrot, and tomato	GC-MS Finnigan MAT 4000 system; column: 10 ft × 1/8 in. ID nickel tube; stat. phase 8% poly-*m*-phenoxylene; temp. prog. from initial 30°C; MS: scan time 2.0 sec.; mass range 33–450 m/z	20 compounds, mainly simple methyl ketones, alcohols, aldehydes, and two furans	Volatiles stripped off a Tenax trap by thermal desorption at 200°C in a He flow at 20 ml/min into GC inlet	Connick et al. (1987); packed column GC
To identify the major active components in manila-grass [*Zoysia matrella* (L.) Merr.] shoot extracts	% germination and seedling growth of livid amaranth [*Amaranthus lividus* (L.) Merr.]	GC-MS H-P 5980/ MS5970 system; GC column: FSOT, stat. phase DB-1; 15 m × 0.25 mm ID; He carrier gas; temp. prog. from 260°C to 310°C	Two phenolic acids (4-hydroxybenzoic and *p*-coumaric acids), plus nine fatty acids	Diazomethane for methyl esters, and MSTFA for TMS derivatives	Laosinwattana et al. (1999); 4-hydroxybenzoic and *p*-coumaric acids were highly inhibitory to livid amaranth at 62.5 ppm

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To use a circulating root exudate trapping system (CRETS) to collect exuded compounds from Bigalta limpograss (*Hemarthria altissima*) on XAD-4 resin and identify the compounds	Radicle length of lettuce seedlings	GC-MS GC: Varian Aerograph 1400; column 30 m SCOT glass capil.; stat. phase OV-17; injection 220°C, temp. prog. from 150°C; MS: Finnigan 3000 with EI at 69.5 eV	12 compounds, mostly phenolic acids (substituted benzoic and cinnamic acids)	Methyl esters and ethers formed from diazomethane and deuterated diazomethane	Tang and Young (1982); the first successful report of the isolation and identification of phytotoxic compounds from the environment of undisturbed living plant roots
To isolate, identify and determine the biological activities of the non-phenolic phytotoxins found in prostate knotweed (*Polygonum aviculare*) residues and surrounding soil	% germination and seedling growth (radicle, epicotyl, and whole seedling) of Bermuda-grass (*Cynodon dactylon*)	GC-MS H-P 5985 system; column: 25 m × 0.2 mm ID FSOT; stat. phase methyl silicone/ Carbowax-20M; He carrier at 20 psi and 0.6 ml/min; split ratio 50:1; temp. prog. from initial 150°C	Nine compounds identified as various saturated and unsaturated fatty acids	Methyl ester derivatives formed with diazomethane	Alsaadawi et al. (1983); gives the first report implicating long-chain fatty acids in the allelopathic effects of higher plants

To determine if the ability of rye (*Secale cereale* L.) to suppress the growth of weeds Lambsquarters (*Chenopodium album* L.) and Common ragweed (*Ambrosia artemisiifolia*) is caused by phytotoxic compounds, and to identify such possible compounds	% germination and root and hypocotyl lengths of *Chenopodium album* L. seedlings after treatment with extracts of rye	GC-MS H-P 5985B system; column: 30 m × 0.25 mm ID; stat. phase DB-5; FSOT; He carrier at 1.0 ml/min; injection at 250°C of 1-4 μl; temp. prog. from 50°C to 280°C; MS: EI 70 eV, also methane positive CI	3-phenyllactic and 3-hydroxybutanoic acids	TMS derivatives formed using BSTFA	Shilling et al. (1986); TLC then HPLC then GC-MS separation sequence; positive methane CI spectra included std. adduct ions at (M + 1), (M + 29), (M + 41) to assist MW determinations
To investigate the rhizosphere surrounding marigold (*Tagetes patula* L.) roots for the presence of phytotoxins known to be present within the roots	Bioassay not required	GC-MS Finnigan 1020 system; column: 30 m FSOT; stat. phase SE-54; MS scan 40–400 m/z	Four thiophenes and two benzofurans	Volatiles stripped off XAD-4 resin trap	Tang et al. (1987); CRETS methodology

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To describe new developments in micro LC-MS techniques for the analysis of labile allelochemicals from plant materials	Bioassay not required	LC-MS LC: micro-columns of 20–50 cm × 0.22 mm ID packed with 3-5 μm particles; flow rates 1-5 μl/min; injection 0.5 μl; MS: EI positive ions magnetic sector; detection limits ng-μg.	Separations and identifications achieved with cardenolides, chlorogenic acid, and phenolic acids	LC-MS—no derivatization necessary	Alborn and Stenhagen (1987); caffeic, *p*-coumaric, and sinapic acids separated in 10 min on a 18 cm × 0.22 mm column using 3 μm Spherisorb ODS particles in MeOH/water/ HOAc isocratic mobile phase
To investigate the chemical properties of *Calmintha ashei*, a scrub plant with inhibitory effects on Florida sandhill grasses	Seed germination inhibition of *Schizachyrium scoparium*	GC-MS column: 30 m bonded FSOT capillary; injection 250°C; temp. prog. 45°–250°C; MS: 70 eV EI	Evodone, menthofuran, 2,3-dihydroevodone	Steam-distilled volatiles	Tanrisever et al. (1988); results suggest that ursolic acid in aqueous leaf leachate of *C. ashei* may act as micellar host for aqueous transportation of other bioactive compounds

To identify the volatile allelochemicals emitted from *Amaranthus* spp. by adsorption on Tenax and Carbotrap	Seed germination of carrot, tomato, and onion	GC-MS Finnigan MAT 4000 quadrupole system; and a Perkin-Elmer Sigma 300 GC with ion trap detector; GC/ITD column: 50 m × 0.31 mm ID × 0.52 μm with H-P ultra-2 stat. phase; He carrier at 12 psi; MS scan 1.0 sec for 33–250 m/z	34 compounds identified, mostly simple alcohols, aldehydes, esters, ketones, three alkanes, three terpenes, two furans, and dimethylsulfide	Headspace emitted volatiles desorbed off Tenax and Carbotrap tubes over 3 min at 200°C in 10 ml/min He gas flow. Cryoscopic focussing at −30°C	Connick et al. (1989); 31 compounds used in vapor phase bioassay at conc. 34.4 μmol/liter; all compounds showed some level of inhibition
To investigate the leaching of allelochemicals from the leaves of the allelopathic mint *Conradina canescens*	Seed germination and radicle lengths of *Schizachyrium scoparium, Andropogon gyrans,* and *Leptochloa dubia*	GC-MS H-P 5985 system; column: 30 m bonded phase FSOT; injection 250°C; temp. prog. 60°C–210°C; MS: EI at 70 eV	26 terpenoids	Steam-distilled leaf volatiles and bioactive LC column fraction	Williamson et al. (1989)
To develop an improved LC-MS system of micro eluant flow into an EI-MS source to enable identifying mass spectra of thermo-labile allelochemicals	Bioassay not required	LC-MS LC column: 20–50 cm × 0.2 mm ID with 3–5 μm packing; flow rate <2 μl/min with solvent gradient; MS: 70 eV EI; source 140°C	Dhurrin (a cyanogenic glucoside) from sorgum leaf extract	No derivatization necessary	Stenhagen and Alborn (1989)

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To identify compounds which may be responsible for autotoxicity in alfalfa	% germination of alfalfa or velvetleaf seeds	GC-MS (no details given)	Medicarpin; sativan; 4-methoxymedicarpin; 5-methoxysativan	(No details given)	Dornbos et al. (1990); medicarpin is an isoflavanoid phytoalexin
To determine the profiles of the volatiles emitted by residues of winter cover crop legumes berseem clover (*Trifolium alexandrinum* L.), hairy vetch [*Vicia hirsuta* (L.) S. F. Gray], and Crimson clover (*Trifolium incarnatum* L.)	% germination of wetted seeds of onion, carrot, and tomato from volatile test compounds in their vapor state at 34.4 μmol/2.5 liters and other concentrations	GC-MS GC: Sigma 300; column: 50 m $\times$ 0.31 mm ID $\times$ 0.52 μm; stat. phase H-P ultra-2; MS: Finnigan MAT 700 ITD	31 compounds; hydrocarbons, alcohols, aldehydes, ketones, esters, furans, and monoterpenes. (*E*)-2-hexenal was the most inhibitory compound tested	Tenax-trapped volatiles thermally desorbed in an external closed inlet system interfaced with GC	Bradow and Connick (1990); the even-numbered methyl ketones from anaerobic plant residues were among the most inhibitory group of volatiles
To investigate and compare the allelochemicals extracted from soils in which no-tillage and conventional tillage wheat have been grown	Lengths of wheat seedling root and shoot	GC-MS LKB-2091 system; column: 30 m $\times$ 0.32 mm ID; stat. phase DW-5; injection 275°C; He carrier 2-3 ml/min; temp. prog. 65°C–300°C; MS: 70 eV EI; source 265°C; separator 275°C	Soil extracts identified as fatty acids	Diazomethane derivatization to methyl esters	Cast et al. (1990); greater autotoxicity shown in no-tillage soil over that of conventional tillage

To investigate the components of Devil's claw (*Proboscidea louisianica*) oil as potential allelochemical agents on cotton and wheat plants	Radicle length of cotton and wheat seedlings	GC-MS GC: Varian 3700; column: 50 m × 0.32 mm ID FSOT; stat. phase OV-1; temp. prog. 50°C–225°C; He carrier 0.5 ml/min; run time 140 min; MS: Kratos MS-50 with DS-55 data system	150–200 compounds found, mostly hydrocarbons and terpenoids; *p*-vinylphenol; piperitenone; vanillin; ionol; traxolide; 2-methyl-1, 4-naphthoquinone; 1,3,5-tri-t-butyl benzene; alpha-bisabolol; hexa-decanoic acid; delta-cadinene; anthracenedione	Steam-distilled volatiles from roots	Riffle et al. (1990); the monoterpenoid piperitenone was the most inhibitory compound tested
To identify a biologically active constituent from corn pollen (*Zea mays* L., Gramineae)	% germination and radicle length of *Amaranthus leucocarpus* Wats, and of *Echinochloa crusgalli* L. Beauv	GC-MS H-P 5985B system; column 30 m × 0.32 mm ID FSOT bonded RSL-400; stat. phase OV-210, QF-1, or DB-210; He carrier; temp. prog. 100°C–240°C; MS: 70 eV EI	Phenylacetic acid	Diazomethane derivatization to methyl ester	Anaya et al. (1992)

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To identify the active allelopathins present in *Rorippa indica* Hiern roots (Cruciferae)	Lettuce seed germination assay, and for some, a lettuce hypocotyl and radicle growth assay	GC-MS JEOL DX-300 system; column: 25 m; stat. phase OV-1; MS: 70 eV EI; some FDMS done on a JEOL JMS-01SG-2	Hirsutin, arabin, camelinin, and three new methylsulfonylalkyl isothiocyanates	Extracted volatiles directly injected	Yamane et al. (1992a); CRETS used with XAD-4 resin trap; 9-methylsulfonylnonyl isothiocyanate showed remarkable inhibition at just 26 ppm
To identify the leaf volatiles of *Ceratiola ericoides* in conjunction with allelopathic studies on this shrub	Bioassay not required	GC-MS H-P 5792 GC with a H-P 5970 MSD; column: 60 m $\times$ 0.25 mm ID $\times$ 0.25 μm; stat. phase Supelcowax 10; carrier gas 15 psi and 0.74 ml/min; temp. prog. 30°C–175°C; MS:70 eV EI	94 compounds identified, including hydrocarbons, aromatics, esters, alcohols, aldehydes, ketones, monoterpenes, sesquiterpenes	Volatiles collected on Tenax TA trap by a He gas purge (dynamic headspace)	Jordan et al. (1992); thermal desorption off Tenax followed by cryogenic focussing in liquid nitrogen before chromatography
To investigate the chemical basis for cucumber plant allelopathy on weeds	Lettuce seedling radicle length	GC-MS Hitachi M80-B system; GC column: 2 m; stat. phase OV-17; MS: 70 eV EI	*p*-Thiocyanatophenol (at about 0.7 mg/g root exudate)	Volatiles stripped off XAD-4 resin trap	Yu and Matsui (1993); CRETS trapping on XAD-4 resin; the first time such a compound has been isolated from a plant root

To identify volatile compounds from soil and litter associated with the shrub *Ceratiola ericoides* which shows field evidence for allelopathy	Bioassay not required	GC-MS H-P 5792 GC with a H-P 5970 MSD; column: 60 m × 0.25 mm ID × 0.25 μm; stat. phase Supelcowax 10; carrier gas 15 psi and 0.74 ml/min; temp. prog. 30°C–175°C; MS: 70 eV EI	66 compounds identified, including hydrocarbons, aromatics, monoterpenes, sesquiterpenes, alcohols, aldehydes, ketones; the most abundant in litter were 1-octene, 3-octanol, 1-pentanol, and in soil were 3-methyl-1-butanol, 3-octanol, and 3-octanone	Dynamic headspace sampling with He gas purge and trap on Tenax	Jordan et al. (1993)
To identify the components present in an ethereal extract of wheat (*Triticum aestivum*) which shows a positive bioassay for allelopathic activity	Bioassay not required	LC-MS and GC-MS LC: Gilson; column 250 mm × 4 mm ID with 5 μm Chrosorb RP Select B; acetonitrile eluent; MS: VG Trio-1000 with particle beam interface and 70 eV EI GC: Carlo Erba Vega 5300; MS: Shimadzu QP-1000; source 250°C	37 compounds; 21 esters of linear and branched saturated and unsaturated fatty acids; eight ketosterols; seven 3-hydroxysterols, and syringic aldehyde	For GC-MS, TMS derivatives formed using HMDS/TMCS; for double bond localization by MS, dimethyl disulfide derivatives made of the unsaturated esters	Chaves das Neves and Gaspar (1995); *Z* and *E* unsaturated isomers differentiated by diastereomeric adduct formation with dimethyl disulfide

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To compare the essential oil composition of *Hesperozygis ringens* (Benth) Epling and *H. rhododon* Epling and verify their allelopathic potential	Lettuce seed germination	GC-MS a H-P 5970 system; column: 25 m × 0.23 mm ID × 0.25 μm; stat. phase DB-1; He carrier 0.9 ml/min; MS: 70 eV EI	24 compounds, mostly terpenoids; main component of *H. ringens* was pulegone, and of *H. rhododon* was menthone	Steam-distilled volatiles	von Poser et al. (1996); both *cis* and *trans*-pulegone oxides were synthesised for authentic reference compounds
To identify Aponin-3 from the green alga *Nannochloris oculata* as an allelopathic inhibitor of the red-tide dinoflagellate phytoplankton *Gymnodinium breve*	Cell counts of *Gymnodinium breve* cultures	GC-MS a H-P 5890(II) with H-P MSD 5972 system (no other data)	Methyl palmitate and methyl stearate	Extracted volatiles directly injected	Perez et al. (1997); optimum concentration of allelochemicals was about 3 ppm for bioactivity
To investigate the allelochemicals in Beijing wheat #437 rhizosphere fungi and bacteria	Length of radicle in wheat and maize seedlings	GC-MS GC: H-P 5890; column: 30 m × 0.25 mm ID FSOT; stat. phase DB-5; injection 240°C; He carrier 1.0 ml/min; temp. prog. 50°C–275°C; MS: TR 102000 with 70 eV EI; source 150°C; mass scan 30–600 m/z	Approx. 40 compounds; hydrocarbons, acids, alcohols, esters, aldehydes, ketones, nitriles, amines, and some heterocycles but only by library matching	Extracted volatiles directly injected	Ma et al. (1997)

To evaluate the allelopathic potential of several members of the *Brassicaceae* family (crucifers) to suppress weed emergence and growth when green tissues are incorporated into soil; and to determine the major volatiles released by chopped tissues	Inhibition of seed germination and growth of *Sesbania exaltata* and *Triticum aestivum* on exposure to soil green manures. Seed germination of soybean, corn, wheat, rapeseed, dandelion, alfalfa, and cucumber on exposure to *Brassica* volatiles	GC-MS GC: a H-P 6890; column: 30 m × 0.25 mm ID × 0.25 μm; stat. phase HP-5MS; MS: H-P 5973 MSD	Five volatiles found in greatest concentration were allyl isothiocyanate, 3-butenyl isothiocyanate, benzyl isothiocyanate, *cis*-hexen-1-ol, *trans*-2-hexenal	SPME collection of headspace volatiles with direct thermal desorption into GC inlet	Vaughn and Boydston (1997); allyl isothiocyanate completely inhibited the germination of all seven bioassay test species at a level less than 1 ppm
To identify the compounds responsible for the allelopathic effect of rice seedlings upon the aquatic weed ducksalad [*Heteranthera limosa* (SW.) Willd]	Not required in laboratory—preestablished by field studies against ducksalad	GC-MS a Varian Saturn 2 system with septum programable inlet and 8100 auto-sampler; column: 30 m × 0.25 mm ID × 0.25 μm; stat. phase DB-5MS; He carrier 40 cm/sec; temp. prog. 80°C–290°C; MS: 70 eV EI; mass scan 50–600 m/z	41 compounds identified including hydroxy and methoxy substituted benzoic and cinnamic acids, phenylpropanoic and long chain aliphatic acids, some benzaldehydes, phenylalkanols, resorcinol, and 5-hydroxy-2-indolecarboxylic acid	TMS derivatives formed using MSTFA & TMCS	Mattice et al. (1998); simple diethyl ether extraction was found to be superior for recovery of the moderately polar rice allelochemicals, than was SPE, charcoal, styrene-DVB, or XAD-4 resin trapping

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To evaluate the essential oils of 32 aromatic plants for allelopathic properties and to identify constituents in the most active ones	Wheat seed radicle and coleoptile lengths, and seed germination of wheat, black mustard [*Brassica nigra* (L.) Koch], Palmer amarant (*Amaranthus palmeri* S. Watson)	GC-MS (no details given)	From the three most significant aromatic plants *Origanum syriacum, Micromeria fruticosa*, and *syriacum, Cymbopogon citratus*, were isolated 15 terpenoids, of which geranial and neral proved the most active	Steam-distilled volatiles	Dudai et al. (1999); seed germination was inhibited at 20–80 ppm of essential oils
To identify the major active allelopathins in Manila-grass [*Zoysia matrella* (L.) Merr.] shoot extracts	% germination and root and shoot length of livid amaranth [*Amaranthus lividus* (L.) Merr.] seeds	GC-MS a H-P system; GC: 5980; column: 15 m × 0.25 mm ID FSOT; stat. phase DB-1; He carrier; injector 260°C; temp. prog. 100°C–310°C; MS: 5980 MSD; 70 eV EI	Nine fatty acids and *p*-hydroxybenzoic and *p*-coumaric acids	Diazomethane derivatization to methyl esters/ethers, and TMS derivatives from MSTFA	Laosinwattana et al. (1999); preliminary fractionations achieved by solvent partitioning, by LC, and by HPLC
To investigate the culture media of the macrophyte *Pistia stratiotes* and its capacity to allelopathically inhibit algal growth	Growth-water aliquots from *Pistia stratiotes* were tested on agar plates against alga *Selenastrum capricornutum*	GC-MS (no data were given)	Active compounds include 9-hydroxylinolenic acid; 9-hydroxy and 13-hydroxy linoleic acids; α-ascarone; stigmasta-4, 22-diene-3, 6-dione; sinapyl and coniferyl alcohols	An ethyl acetate extract treated with TMCS, and with diazomethane	Greca et al. (1999); α-ascarone was most active compound showing 40% inhibition of algal growth at 10 ppm

To identify the constituents of the volatile oil from *Ageratum conyzoides* and to determine their allelopathic potential upon cultivated crops	Fresh weight, root length and shoot length of seedlings of cucumber, wheat, radish, ryegrass, tomato, and mungbean	GC-MS a Finnigan TSQ 70B-4000 system; GC column: 50 m × 0.32 mm ID FSOT; stat. phase HP-1; He carrier 12 psi; temp. prog. 60°C–280°C; MS: 50 eV EI; source 280°C; scan 35–450 m/z	11 compounds, largely terpenoids	Steam-distilled volatiles	Kong et al. (1999); the most active components were precocene I and beta-caryophyllene
To isolate and identify the water-soluble allelochemicals from extracts of *Scirpus acutus* Muhlenb. rhizomes and *Eleocharis smallii* L. shoots, both aquatic plants associated with wild rice	Lettuce seedling germination and the lengths of radicle and primary shoots	GC-MS a VG Auto Spec high resolution system; GC injection port 280°C; column temp. prog. 50°C–350°C; (no other details)	In excess of 40 compounds from *S. acutus* rhizome including lactic, fumaric, 2-phenyllactic, 3- and 4-hydroxybenzoic, protocatechuic, ferulic, and dehydroabietic acids; *E. smallii* gave 4-methoxyphenol, benzofuran, benzeneacetic acid and some acetophenones	TMS derivatives formed using BSTFA	Quayyum et al. (1999)

TABLE 1. CONTINUED

Purpose of study	Bioassay used	LC-MS or GC-MS conditions	Allelochemicals identified	If GC-MS, then volatiles or derivatization	Reference and comments
To develop a method based upon LC/MS/MS capable of identifying benzoxazinoids as both glycones and aglycones within 5- to 15-day-old maize seedlings	Bioassay not required	LC-MS LC: Spectra System AS3000; column: 25 cm × 2.1 mm ID; 5 μm particles of C-18 Adsorbosphere; injection 5 μl; flow rate 200 μl/min using linear binary gradient. MS: Finnigan MAT TSQ 7000 triple quad with APCI interface	Seven benzoxazinoid glucosides and two aglycones; 2-β-D-glucopyranosyloxy-4,7,8-trimethoxy-1, 4-benzoxazin-3-one (HDM2BOA-glucoside) observed for first time	No derivatization required; negative ion mass spectral mode used	Cambier et al. (1999); MS/MS fragmentation analysis used to identify particular glucoside products

higher than that of the presently developing LC-MS techniques, of which only some five qualitative analysis examples in allelopathy could be found. Data extracted from these papers include: purpose of the study; bioassay used; chromatography–MS conditions; types of compounds identified and if GC-MS, whether volatiles or derivatization; as well as literature reference and comment.

Allelochemical Quantifications Using Hyphenated Chromatography–Mass Spectrometry

Although quantitative studies on plant allelopathic substances yield more useful information per compound than qualitative studies, the literature, as yet, contains few quantitative investigations. This is despite the fact that because of the great selectivity of the MS detector to perform extracted ion, selected ion (SIM), and product ion (MS-MS) analyses, analysis problems of chromatographic coelution in complex mixtures can mostly be avoided. It appears that the greater time, effort, and expense associated with the more demanding quantitative requirements, together with the nonavailability of pure reference compounds for calibration purposes, has often prevented allelochemical analyses from proceeding beyond the qualitative level. Nevertheless, a number of quantitative studies have been published, thereby stimulating deeper levels of scientific enquiry into the molecular mechanisms associated with plant allelopathy.

One such study performed in the early period of GC-MS is that by Lovett and Potts (1987) who investigated the levels of the alkaloids scopolamine and hyoscyamine in thorn apple (*Datura stramonium*) seeds. They wished to test the hypothesis that the early seedling growth of crop plants such as barley and wheat was affected by alkaloid interference with the gibberellin-stimulated food reserve metabolism of germinating seeds. Their GC-MS equipment consisted of a Pye 204 GC using a 25-m $\times$ 0.33-mm-ID FSOT HP-1 column operated isothermally at 220°C interfaced at 220°C to a VG MM70-70F mass spectrometer and VG 2235 Data System, which performed their qualitative work. Quantitative analysis was then carried out by GC-FID with the same column on a HP 5880A instrument. Allelochemical samples were obtained by aqueous leaching of thorn apple seeds over seven days, with both alkaloids being quantitatively determined each day. The aqueous seed leachates were made alkaline and extracted with ether–chloroform. After evaporation of the solvent, the residue was taken up in ethyl acetate and derivatized with pentafluoropropionic anhydride (PFP). The alkaloid scopolamine varied in leached amounts from 0.577 mg/g seed on day1 to 0.14 mg/g seed on day 7, with a total of 2.507 mg/g seed over seven days. Hyoscyamine varied from 0.377 mg/g seed on day 2 to 0.098 mg/g seed on day 7, with a total of 1.825 mg/g over seven days. The authors believed that these seed alkaloids could be leached for up to two weeks into the soil, thereby maintaining a continual supply for allelopathic effects upon the surrounds.

More recent studies by researchers at the University of Bonn (Schulz et al., 1994; Friebe et al., 1995), have been carried out on the allelopathins of quackgrass, a widespread Northern Hemisphere weed. This group's 1994 objective was to identify and quantify constituents in the exudate from quackgrass (*Agropyron repens* L.) rhizome-borne roots. Water flowing at 1 liter/hr through a root exudate recirculation system (RERS) over a period of 10 days was acidified and extracted with ethyl acetate. The organic solvent was removed by evaporation and the residue taken up in a little methanol, followed by transfer to microreaction vials, further solvent evaporation, and then silylation of the residue with BSTFA to volatile TMS derivatives. GC-MS analysis was performed on an Automass 100 (Delsi Nermag, Unicam) instrument using a HP Ultra-2 5% phenylmethylpolysilane, 25-m $\times$ 0.2-mm ID column, 2-μl injections, and a temperature program from 100°C to 280°C in stages. The injector port and detector temperature were both 280°C, while the He carrier gas head pressure was 1 bar. Mass spectra were collected at 70 eV using EI. A cress seedling bioassay based upon biomass and shoot length was used to monitor the phytotoxic effects of quackgrass from variously aged plants, up to 92 days. Exuded compounds were identified by comparison of their mass spectra with those in standard reference data, thereby establishing the presence of 3-hydroxybutyric, vanillic, ferulic, 4-hydroxycinnamic, protocatechuic, and syringic acids, plus vanillin and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). For vanillic, ferulic, and syringic acids, the concentrations in the aqueous recirculated exudate ranged from 0.1 to 0.2 μM, with other phenolics only in traces. The most active aqueous exudate constituent, DIBOA, was present in 0.01–0.03 μM concentrations from older plants (54 days), while for younger plants it was present at 0.24 μM (0.52 μmol/kg fresh weight quackgrass). The authors carried out a systematic bioassay study using up to six different defined concentrations of the two allelochemicals DIBOA and ferulic acid against eight different test plant species and made the important finding that plant response was species related and dose dependent.

In a second study by the same Bonn group, Friebe et al. (1995) set out to identify and quantify the allelopathic constituents of the roots and shoots of quackgrass seedlings. Aqueous shoot extracts of 10-day-old quackgrass plants were acidified and extracted with ethyl acetate. Rather more importantly, root exudates were obtained from 10-day-old quackgrass seedlings that had been transferred to freshwater alone for a further three days of growth. The water from these plants was extracted with ethyl acetate, and after removal of the organic solvent, the residue was silylated with BSTFA to form TMS derivatives. The GC-MS work was carried out on the same instrument and conditions as for the 1994 study above. Compounds were identified by their retention times and mass spectra as compared with published and authentic reference samples. The significant compounds identified were vanillic, ferulic, and 3-hydroxybutyric acids, together with the benzoxazinoids DIMBOA, DIBOA, HBOA, and DIM_2BOA. Reference

compound calibrations and chromatographic peak areas led to quantitative values in quackgrass root exudates of 0.4 μM for DIMBOA and 0.2 μM for DIBOA. In terms of the allelopathic phenomenon, knowledge of the constituents and the composition of plant root exudate are vital to understanding the plant's allelopathic potential. The authors suggest that the allelopathic effectiveness of quackgrass is due to the result of synergistic interactions among the different allelochemicals exuded.

Another study of the same period is by Yamamoto (1995), who investigated the chemical basis for allelopathy in the pasture plant *Anthoxanum odoratum* towards the native grass *Zoysia japonica*. Yamamoto employed a Petri dish bioassay based upon the hypocotyl length of germinated lettuce seedlings. These seedlings had been treated with chemical and/or solvent fractionated and thin-layer-chromatographed *A. odoratum* extracts, in order to direct his search for allelochemicals. One neutral fraction at R_f 0.6–0.7 on TLC was identified by GC-MS as coumarin, which had the greatest inhibitory activity. The instrument used for identification was a Shimadzu GCMS-QP1000S using an FSOT CBP1-M25-0.25 25-m $\times$ 0.25-mm-ID column with He as carrier gas at 40 ml/min, injector 250°C, and oven temperature programed from 50°C to 250°C at 5°C/min. Quantitative work was done with a Shimadzu GC-15A chromatograph. Coumarin content within the roots, stubble, leaf, stem, and dead litter of *A. odoratum* was measured for various times of the year and was found to vary significantly. Thus, June contents yielded 461 $\pm$ 62 μg/g dry sample for roots and 26,577 $\pm$ 4,531 μg/g for leaf, while November contents were 47 $\pm$ 13 μg/g for roots and 2,773 $\pm$ 956 μg/g dry sample for leaf.

Biofumigation (Angus et al., 1994) was the possible effect being explored in the thorough study conducted by Brown and Morra (1995) on both the volatiles and water-soluble allelochemicals of *Brassica napus* L. These researchers used a GC-MS identification and quantification system from Hewlett-Packard (HP 5890II GC with HP 5972 MSD) operating with a 30-m $\times$ 0.25-mm ID $\times$ 0.25-μm HP-5MS column, injector at 260°C, interface at 320°C, He carrier at 1.14 ml/min, splitless injection, and oven temperature program from 130°C to 320°C at 15°C/min. The mass detector scanned from *m/z* 25 to 470 using 70 eV EI. Glucosinolates (sulfur-containing organic anions with a β-D-thioglucose moiety and variously structured side groups) were identified as the hydrolyzable precursors to a range of allelopathic volatiles from *Brassica napus* seed meal. The volatiles consisted of a range of nitriles and isothiocyanates. Bioassay was conducted by allowing only the volatile compounds released from hydrolyzed glucosinolates to impact lettuce seed germination through vapor-phase contact alone, inside a closed 1.45-liter germination chamber. Glucosinolates were quantified from 0.2 g plant samples, which were first desulfated on Sephadex columns, and the dried products then silylated with BSTFA–TMCS–methylimidazole before GC-FID analysis on a HP 5890II 15-m $\times$ 0.32-mm-ID $\times$ 1.0-μm DB-1 column. The *Brassica*

volatiles were trapped by dynamic headspace sweep onto 100 mg of Super Q porous polymer contained in 16-cm × 0.4-mm-ID glass tubes, which were then stripped with dichloromethane before GC-MS injection. Six nitriles and nine isothiocyanates were identified. Of the volatiles analyzed from a 16-h collection period of headspace sweeping, quantitative results ranged from 0.2 nmol/g meal for phenylethylcyanide and 5-methylhexylisothiocyanate, up to 260.2 nmol/g meal for 3-butenylisothiocyanate. The authors believed their experimental evidence suggested that the volatile glucosinolate degradation products are the causative agents responsible for the observed lettuce seedling inhibition.

Heavy losses in cereal and pasture production southern Australia through the allelochemical effects of residues from the weed silvergrass (*Vulpia myuros*) prompted An et al. (1996) to investigate the chemical constituents of aqueous extracts of *V. myuros* residues. A bioassay based upon radicle and coleoptile elongation of germinated wheat seeds directed solvent fractionation of aqueous *Vulpia* extracts to an active ether fraction that was evaporated to dryness and silylated with PowerSil-prep mixture to give TMS derivatives. These derivatives were injected onto a GC-MS system consisting of a HP 5890II GC fitted with an FSOT DB-5 30-m × 0.32-mm-ID × 1.0-μm column with He carrier at 38 cm/s, injector port 290°C, 3 μl split injection (1:100), oven temperature programed in stages from 80°C to 280°C, and a VG Trio-2 quadrupole MS using 70 eV EI. The GC-MS interface was kept at 295°C, the MS ion source at 250°C, and the mass scan covered m/z 60–460 in 0.8 sec. Twenty-one phytotoxins consisting mainly of shikimic acid metabolites, such as vanillic, ferulic, and 4-hydroxybenzoic acids, were identified and quantified using an internal standard technique and multipoint calibration curves. The quantities of the 21 individual compounds ranged from 1.36 to 81.0 μg/g dry plant residue, and in total accounted for 0.0502% per dry weight of *Vulpia* residue. The authors noted that those allelochemicals present in large amounts possessed low biological activities, while those present in small amounts possessed strong inhibition. Their bioassay studies on artificial solutions of the pure individual allelochemicals and also of mixtures composed of 20 of these compounds demonstrated that a modest level of synergism was exerted by the multicompound mixtures. The synergism of allelochemical mixtures is a dimension of the allelopathic phenomenon that is deserving of further research, as little is presently known of the mechanisms by which allelopathic substances bring about their inhibitory affects.

Developing an analytical method to quantify the allelopathin batatasin-III (3,3′-dihydroxy-5-methoxydihydrostilbene) in the leaves of the dwarf shrub *Empetrum hermaphroditum* was the task undertaken by Wallstedt et al. (1997). They extracted leaf material with either ethyl acetate or water, cleaned up the samples by SPE (Isolute or C-18), and eluted the batatasin-III off the SPE columns with EtOAc–HOAc or EtOAc–MeOH–HOAc. The respective fractions containing the

target compound were each lyophilized and redissolved in 1000 μl of organic solvent, ready for subsample volumes of 20 μl (EtOAc) or 50 μl (water extracts) to be lyophilized before treatment with BSTFA–pyridine for silylation. A standard calibration curve (using 1,6-dihydroxynaphthalene as internal standard) was prepared by plotting area ratios of analyte to internal standard across 14 different concentrations of batatasin-III from 5 to 300 ng/μl. The derivatized analyte was identified by GC-MS using a VG Trio-3 tandem quadrupole MS scanning m/z 50–450, using EI at 70 eV, and ion source at 250°C. Silylated batatasin-III gave characteristic ions at m/z 388 (M^+), 209, and 179. The GC was a HP 5890 containing a DB-5 30-m $\times$ 0.25-mm-ID $\times$ 0.25-μm column with He carrier at 1.0 ml/min, injection volume 1 μl, and initial oven temperature rising from 150°C to 290°C at 7°C/min. Quantifications were performed under the same chromatographic conditions using GC-FID, with detection levels of analyte down to 1 ng. The contents of batatasin-III in *E. hermaphroditum* varied with plant age, and ranged from 0.027 to 0.062-mg/g leaf dry weight across three years of growth.

The power to deal with the analytical complexity of allelochemicals immersed in a matrix of aqueous wheat shoots extract by using GC-MS-MS on a Varian 3400 CX/Saturn 2000 ion-trap spectrometer system was demonstrated by Wu et al. (1999), who focused their attention upon the quantification of DIMBOA and five phenolic acids of recognized phytotoxicity obtained from 17-day-old wheat seedlings. Their tandem (in time) mass spectrometric detector used the chromatographic helium carrier gas (flow rate 34 cm/sec) inside the ion trap as the collision gas for collision-induced dissociation (CID) of selected, single, characteristic, analyte, precursor ions arising from the normal electron impact spectra obtained at the first MS stage of molecular ion fragmentations. The selection again of single, characteristic, quantitation ions from these second-stage product-ion spectra produced at characteristic retention times allowed for great analytical selectivity and significant improvement in the analyte signal-to-noise ratios. Chromatographic conditions consisted of a DB-5 MSITD 30-m $\times$ 0.25-mm-ID $\times$ 0.25-μm column with temperature program from 80°C to 280°C in stages, and run time 29.9 min, divided into nine separate MS-MS acquisition time segments. Injector temperature was 280°C with a splitless 1-μl injection of silylated derivatives. The transfer line was held at 250°C and the ion trap detector at 200°C, while the mass scan covered m/z 50–450 in 1 sec (3 microscans). Nonresonant CID was used for MS-MS with an excitation time of 20 msec. Compound identifications were achieved by comparison of both normal fragment ion and product-ion spectra for the TMS derivatives used against user libraries of the TMS derivatives of authentic reference compounds, as well as by comparison of retention times. Using this approach, both the *cis* and *trans* geometrical isomers of coumaric and ferulic acids could easily be recognized and quantified. Characteristic product-ion chromatographic peak area ratios, with 4-chlorobenzoic acid as internal standard,

were used as the analytical parameter. Results for allelochemicals present in wheat shoots ranged from 2.9 ± 0.1 mg/kg for *cis*-4-coumaric acid to 110 ± 4.8 mg/kg dry shoot mass for DIMBOA.

Gardiner et al. (1999) used GC-MS to confront the problem of measuring glucosinolate breakdown products in field soil following incorporation of winter rapeseed (*Brassica napus*) green manure. Soil samples at 0.0- to 7.5-cm and at 7.5- to 15.0-cm depths were taken from field soils over three weeks in plots that had had *Brassica napus* plants plowed under. Their analytical equipment consisted of a HP 5890II+ GC using a DB-5 30-m × 0.25-mm-ID × 0.25-μm column, He carrier gas, oven temperature program from 40°C to 285°C at 15°C/min, and a HP 5972 quadrupole mass selective detector. Nine phytotoxic glucosinolate degradation products were identified in dichloromethane soil extracts, including five isothiocyanates, three nitriles, and one oxazolidinethione (goitrin). Maximum concentrations were observed 30 h after plowdown, but for all compounds, soil concentrations were quite low, seldom exceeding 1 nmol/g dry weight soil. The plant parts of two *Brassica napus* cultivars (Dwarf Essex and Humus) were also analyzed for precursor glucosinolate contents. This analysis, based upon a 0.2-g sample of plant tissue, took place after desulfation on a Sephadex column treated with imidazole and formic acid, followed by silylation. The GC-MS conditions for this part of the analytical work were similar to those of the breakdown products but used a HP-5MS 30-m × 0.25-mm-ID × 0.25-μm column running He carrier at 1.14 ml/min, with splitless injection at 260°C and interface 320°C. The oven temperature program covered 130°C–320°C at 15°C/min, while mass scanning ranged over m/z 25–470 on the MSD 5972. Glucosinolates were identified by comparison with previously reported mass spectra, and their quantification was assisted by the use of previously published response coefficients and benzyl glucosinolate as an internal standard. The glucosinolate content of tops and roots from cultivar Dwarf Essex at the time of incorporation into the soil ranged from 0.01 μmol/g tops for 4-methoxyindole-3-methylglucosinolate to 12.86 μmol/g roots for 2-phenylethylglucosinolate, causing the authors to comment that the roots of *B. napus* were a more important source of toxic fumigants arising from glucosinolate degradation in the soil than were the shoots.

LC-MS-MS was the analysis technique chosen by Lee et al. (1999) in their development of a quantitative method for the estimation of momilactones A and B in rice straw across a range of different growth stages. Dried rice straw samples were extracted with aqueous methanol, the solutions evaporated to dryness, and the residue taken up in ethyl acetate, washed with $NaHCO_3$ solution, and then concentrated by evaporation. The resulting residue was dissolved in methanol, and the solution was passed through a Sep-Pak Light C-18 cartridge. Five-microliter injections of the eluate were made on the LC-MS-MS system , which was composed of a HP 1100 LC using an Inertsil ODS-2 150-mm × 4.6-mm- ID × 5-μm particle column, 80% aqueous CH_3CN containing 0.1% formic acid at a flow rate of

0.6 ml/min. The detector was a Sciex API-300 (Perkin-Elmer) with atmospheric pressure chemical ionization (APCI) inlet that used the positive ion mode and nitrogen as collision gas for CID. Momilactone A gave a precursor ion $(M+1)^+$ at m/z 315 with corresponding product ion at m/z 271. For momilactone B, a $(M+Na)^+$ ion was observed at m/z 353, and a useful precursor ion at m/z 331 that afforded two product ions at m/z 313 (-18, H_2O) and m/z 269 ($313 - 44$). For MS-MS quantifications, the m/z 315/271 pair was used for momilactone A and the m/z 331/269 pair for momilactone B under metastable reaction monitoring (MRM) conditions. Calibration curves for both compounds were obtained by using two reference standard concentrations for each at 0.5 and 2.5 ng on the same instrument. Detection limits of the method were established at 0.5–1.0 ng. On the basis of 180 days of growth and a water-only extraction of rice straw, the concentrations of momilactone A were determined as 1.01 μg/g dry weight straw and those of momilactone B as 0.81 μg/g dry weight straw. Using an 80% methanol extraction of straw of 180-day-old rice, momilactone A rose to 3.80 μg/g and momilactone B to 2.01 μg/g dry weight straw.

DISCUSSION

The foregoing material constitutes a cross-section of the types of LC/GC-MS-aided plant allelopathy studies described in the literature and illustrates the capacity of the mass spectrometric detector to cope selectively with complex plant mixtures. The older (photodiode array) (PDA) and UV-V detectors for HPLC, and the older FID-type detectors for HRGC, all with their limited selectivity and capacity to deal with chromatographic coelution in complex matrices, are gradually being replaced by highly selective, chemical noise-reducing mass spectrometric detectors. The high level of integration found in the modern, inexpensive, bench-top, hyphenated, chromatography–mass spectrometric systems with their fast, sophisticated, software-driven control from dedicated system computers, makes it possible for technicians of only modest training to achieve remarkable results.

One important point not to overlook, when involved in pioneering work on new allelopathy investigations, concerns the need for a means of recognizing those individual compounds within a complex plant-related sample that actually carry the biological activity. If the sample were a simple mix of several components, then the task of tracking compound-related bioactivity and identifying each separate substance would be comparatively easy (although not trivial), but in the more typical complex sample cases, with so many different compounds present, the investigator is faced with the difficult problem of knowing which substances to focus upon. The usual means of addressing this problem is to perform bioassay-directed fractionations (Rimando et al., 2001), whereby the original mixture is gradually

separated in stages (using such means as differential chemical solubility, sequential solvent partitioning, preparative column chromatography, TLC, HPLC, etc.) into fractions that demonstrate no activity or are determined to be active under the bioassay. The process of fractionation and bioassay is repeated until pure active compounds are isolated. It is at the last fractionation stage that chromatography–mass spectrometry has its most valuable input because of the usually small amount of active fraction remaining and the probable need for still further resolution and identification of compounds. Even with the availability of large mass spectral libraries, it will still often be necessary to isolate sufficient quantities of the pure active components in order to perform structure analysis with spectrometric techniques such as HRMS, PMR, CMR, FTIR, and UV-V. Once qualitative analysis is complete, the now identified allelochemicals can be quantified if a sample of the pure reference compound is available.

As mentioned earlier, hyphenation between chromatographic and mass spectrometric components is not the only type available. Such systems as GC-FTIR-MS (Auger and Ferary, 1994; Ferary et al., 1996), and LC-NMR also offer high analytic power, but like the magnetic sector, high mass-resolution spectrometer systems, such instruments are costly and, therefore, are not likely to be in widespread use. Indeed, the high analyte selectivity afforded by the magnetic sector HRMS (and its various hybrid configurations) brought about by its capacity to separate original ions on the basis of their slight differences in precise mass, is nowadays being replaced by the inexpensive tabletop MS-MS system that uses an ion-trap detector. The prior MS-MS systems (in space) that use a series of beam-quadrupole mass filters (QqQ, or triple quads) are again much more expensive and are losing ground to the simpler and more economical ion-trap spectrometers that conduct time-based tandem MS analyses. The latter do not exhibit the spectral skewing caused by variable analyte concentration across the duration of a chromatographic peak, which often affects the output of a transmission quadrupole. The more expensive triple quad instrument retains a superior (approx. 10-fold) sensitivity to the ion-trap detector in comparable product ion monitoring modes and a superiority in the ability to perform precursor ion scans and neutral loss scans (useful in metabolite searches). However, the cheaper ion-trap excels the triple quad sensitivity (approx. 100-fold) once a full mass scan in product-ion mode (valuable for fingerprinting) is required. In addition, an ion-trap detector can deliver both EI and CI (chemical ionization) spectra in a single chromatographic run. This is achievable automatically through software switching alone, thus easily generating both molecular weight and structural information without hardware reconfiguration of the instrument. With respect to the polarity of ion analysis, however, if one desires to be able to analyze in both positive and negative ion modes like the switchable transmission quadrupole, then the instrument design must include external source ionization, as the simpler internal ion source delivers only positive ions.

The popular tandem MS mode for chromatographic detection has been especially advantageous to LC-MS studies. This is not just because the additional capability of MS-MS can often eliminate the need for extra sample clean-up steps, and provide for more rapid analytical method development and limits of detection, but also because it brings to bear more structural analysis power supplementing the usual molecular weight data on unknown compounds. The ion-trap's ability to perform multistage (in time) MS-MS (MS^2), $MS^3, \ldots,$ MS^n spectra, has opened up a wide range of structure determining experiments upon the initial ions formed by electrospray ionization (ESI), APCI, or EI in today's LC-MS systems, which significantly outstrip the capacity of the earlier, bench-top, single transmission quadrupole. There is a whole new range of opportunities for the use of these powerful techniques in application to plant allelopathy studies where molecular information is required. Apart from the rarer particle beam EI systems, LC-MS is now mostly run by using APCI or ESI methods to cover most compound masses and polarities. Such instruments may possess quaternary solvent gradient delivery, HPLC columns from <1 mm ID up to 4.6 mm ID, with mobile phase flow rates from 50 μl/min to 2 ml/min, and run in either positive or negative ion mode, or even, for some instruments, in both polarities during the same run time. Both LC-MS and GC-MS instruments frequently come with dual detectors built in. LC systems often possess a PDA that can operate simultaneously with MS and that is still useful for determining chromatographic peak homogeneity, generating UV-V chromatograms, and comparing results against the earlier literature. For the GC-MS types, simultaneous detection may also take place using FID or NPD. For bench-top LC- and GC-MS systems, depending upon the type of MS, the mass scan range can presently cover up to 3000 m/z, with scan rates around 6000 Da/sec, and with sensitivities down to the femtogram range. Within GC-MS instruments, electronic pneumatic control (EPC) is now standard and provides constant carrier gas flow rates, automatically compensating for the vacuum environment of the MS detector. This maximizes retention time and spectrum reproducibilities between chromatographic runs. Under EPC, the MS sensitivity and column resolution are nearly unaffected by oven temperature programing. At least one manufacturer offers a special retention time locking capability that helps to ensure reproducibility of retention times for the same compounds under the same instrument conditions, between different laboratories.

In conclusion, the use of the modern, economical, bench-top, chromatography–mass spectrometry system is commended to those researchers interested in the chemical basis for plant allelopathy. The use of these systems is expected to increase rapidly in the near future, especially among the developing countries. Such relatively cheap instruments offer considerable analytic power and are ideally suited to investigate the lighter mass range of secondary plant metabolites typically responsible for plant allelopathy.

REFERENCES

ALBORN, H. and STENHAGEN, G. 1987. Micro liquid chromatography-mass spectrometry combination: Application to allelochemical compounds, chapter 29, *in* G. R. Waller (ed.). Allelochemicals: Role in Agriculture and Forestry. American Chemical Society Symposium Series 330, ACS, Washington, D.C.

ALSAADAWI, I. S., RICE, E. L., and KARNS, T. K. B. 1983. Allelopathic effects of *Polygonum aviculare* L. III. Isolation, characterization, and biological activities of phytotoxins other than phenols. *J. Chem. Ecol*. 9:761–774.

AN, M., PRATLEY, J. and HAIG, T. 1996. Applications of GC/MS in allelopathy research: A case study. *Rapid Commun. Mass Spectrum* 10:104–105.

ANAYA, A. L., HERNANDEZ-BAUTISTA, B. E., JIMENEZ-ESTRADA, M., and VELASCO-IBARRA, L. 1992. Phenylacetic acid as a phytotoxic compound of corn pollen. *J. Chem. Ecol*. 18:897–905.

ANGUS, J. F., GARDNER, P. A., KIRKEGAARD, J. A., and DESMARCHELIER, J. M. 1994. Biofumigation: Isothiocyanates released from *Brassica* roots inhibit growth of the take-all fungus. *Plant Soil* 162:107–112.

AUGER, J. and FERARY, S. 1994. First results in trace identification of allelochemicals and pheromones by combining gas chromatography–mass spectrometry and direct deposition gas-chromatography–Fourier transform infrared spectrometry. *J. Chromatogr*. *A* 683:87–94.

BAH, M. and PEREDA-MIRANDA, R. 1996. Bioactive natural products from traditionally used Mexican plants. Part IV. Detailed FAB-mass spectrometry and high resolution NMR investigations of tricolorins A-E, individual oligosaccharides from the resins of *Ipomoea tricolor* (Convolvulaceae). *Tetrahedron* 52:13063–13080.

BLUM, U., SHAFER, S. R., and LEHMAN, M. E. 1999. Evidence for inhibitory allelopathic interactions involving phenolic acids in field soils: Concepts vs. an experimental model. *Crit. Rev. Plant Sci*. 18:673–679.

BRADOW, J. M. and CONNICK W. J., JR., 1990. Volatile seed germination inhibitors from plant residues. *J. Chem. Ecol*. 16:645–666.

BROWN, M. A. (ed.). 1990. Liquid Chromatography–Mass Spectrometry: Applications in Agricultural, Pharmaceutical, and Environmental Chemistry. American Chemical Society Symposium Series 420, ACS, Washington, D.C.

BROWN, P. D. and MORRA, M. J. 1995. Glucosinolate-containing plant tissues as bioherbicides. *J. Agric. Food Chem*. 43:3070–3074.

CAMBIER, V., HANCE, T., and DE HOFFMANN, E. 1999. Non-injured Maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal*. 10:119–126.

CAST, K. G., MCPHERSON, J. K., POLLARD, A. J., KRENZER JR., E. G., and WALLER, G. R. 1990. Allelochemicals in soil from no-tillage versus conventional-tillage wheat (*Triticum aestivum*) fields. *J. Chem. Ecol*. 16:2277–2289.

CHAVES das NEVES, H. J. and GASPAR, E. M. M. S. 1995. HRGC-MS and HPLC-MS identification of new ketosterols in an extract of wheat straw. *J. High Resolut. Chromatogr*. 18:299–303.

CHOU, C.-H., and WALLER, G. R. 1980. Possible allelopathic constituents of *Coffea arabica*. *J. Chem. Ecol*. 6:643–654.

CONNICK, W. J., JR., BRADOW, J. M., LEGENDRE, M. G., VAIL, S. L., and MENGES, R. M. 1987. Identification of volatile allelochemicals from *Amaranthus palmeri* S. WATS. *J. Chem. Ecol*. 13:463–472.

CONNICK W. J., JR., BRADOW, J. M., and LEGENDRE, M. E. 1989. Identification and bioactivity of volatile allelochemicals from Amaranth residues. *J. Agric. Food Chem*. 37:792–796.

CUTLER, H. G. and CUTLER, S. J. (eds.). 1999. Biologically Active Natural Products: Agrochemicals. CRC Press, Boca Raton, Florida.

DORNBOS, D. L., JR., SPENCER, G. F., and MILLER, R. W. 1990. Medicarpin delays alfalfa seed germination and seedling growth. *Crop Sci.* 30:162–166.

DUDAI, N., POLJAKOFF-MAYBER, A., MAYER, A. M., PUTIEVSKY, E., and LERNER, H. R. 1999. Essential oils as allelochemicals and their potential use as bioherbicides. *J. Chem. Ecol*. 25:1079–1089.

FERARY, S., AUGER, J., and TOUCHE, A. 1996. Trace identification of plant substances by combining gas chromatography–mass spectrometry and direct deposition gas chromatography-Fourier transform infrared spectrometry. *Talanta* 43:349–357.

FRIEBE, A., SCHULZ, M., KUCK, P., and SCHNABL, H. 1995. Phytotoxins from shoot extracts and root exudates of *Agropyron repens* seedlings. *Phytochemistry* 38:1157–1159.

GARDINER, J. B., MORRA, M. J., EBERLEIN, C. V., BROWN, P. D., and BOREK, V. 1999. Allelochemicals released in soil following incorporation of rapeseed (*Brassica napus*) green manures. *J. Agric. Food Chem*. 47:3837–3842.

GOODING, K. M. and REGNIER, F. E. (eds.). 1990. High-Performance Liquid Chromatography of Biological Macromolecules, Methods and Applications, Marcel Dekker, New York.

GRECA, M. D., FIORENTINO, A., MONACO, P., PREVITERA, L., PINTO, G., and POLLIO, A. 1999. Release of potential allelochemical from aquatic plants, chapter 24, *in* Recent Advances in Allelopathy, Vol. 1. A Science for the Future, International Allelopathy Society, University of Cadiz, Cadiz, Spain.

GROB, R. L. 1998. Modern Practice of Gas Chromatography, 3rd ed. Wiley-Interscience, New York.

GUMNICKA, O. and OLESZEK, W. 1998. Triterpene saponins from the aerial parts of *Dianthus caryophyllus* var. remontant Hort. *Acta Soc. Bot. Pol*. 67:65–68.

HALKET, J. M. 1993. Derivatives for gas chromatography-mass spectrometry, Chapter 14, *in* K. Blau and J. Halket (eds.). Handbook of Derivatives for Chromatography, 2nd ed. Wiley and Sons, New York.

HEATH, R. R. and DUEBEN, B. D. 1998. Analytical and preparative gas chromatography, chapter 3, *in* J. G. Millar and K. F. Haynes (eds.). Methods of Chemical Ecology, Vol. 1, Chapman and Hall, London.

HUANG, S., WANG, W., MA, K., ZHOU, H., XU, Y.-Z., WU, H., SUN, W.-H., YANG, S.-Y., HUANG, H., HUANG, Z.-A., WU, H., and YU, S.-W. 1999. Allelochemicals from root exudates and extracts of water hyacinth *Eichhornia crassipes*, chapter 18, *in* F. A. Macias, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler. Recent Advances in Allelopathy, Vol. 1: A Science for the Future (eds.). International Allelopathy Society. University of Cadiz, Cadiz, Spain.

INDERJIT, S., DAKSHINI, K. M. M., and EINHELLIG, F. A. (eds.). 1995. Allelopathy: Organisms, Processes, and Applications. American Chemical Society Symposium Series 582, ACS, Washington, D.C.

INDERJIT, S., DAKSHINI, K. M. M., and FOY, C. L. (eds.). 1999. Principles and Practices in Plant Ecology: Allelochemical Interactions. CRC Press, Boca Raton, Florida.

INOUE, M., NISHIMURA, H., LI, H.-H., and MIZUTANI, J. 1992. Allelochemicals from *Polygonum sachalinense* FR. SCHM. (Polygonaceae). *J. Chem. Ecol*. 18:1833–1840.

JENNINGS, W., MITTLEFEHLDT, E., and STREMPLE, P. 1997. Analytical Gas Chromatography, 2nd ed. Academic Press, New York.

JORDAN, E., HSIEH, T., and FISCHER, N. H. 1992. Volatile compounds from leaves of *Ceratiola ericoides* by dynamic headspace sampling. *Phytochemistry* 31:1203–1208.

JORDAN, E., HSIEH, T., and FISCHER, N. H. 1993. Volatiles from litter and soil associated with *Ceratiola ericoides*. *Phytochemistry* 33:299–302.

KARASEK, F. W. and CLEMENT, R. E. 1988. Basic Gas Chromatography–Mass Spectrometry: Principles and Techniques. Elsevier, New York.

KLEINTOP, B. L., EADES, D. M., JONES, J. A., and YOST, R. A. 1995. Liquid chromatography–mass spectrometry, chapter 5, *in* R. E. March and J. F. J. Todd (eds.). Practical Aspects of Ion Trap Mass Spectrometry, Vol. 3, CRC Press, Boca Raton, Florida.

KOMAI, K., SUGIWAKA, Y., and SATO, S. 1981. Plant-growth retardant of extracts obtained from water nutgrass (*Cyperus serotinus* Rottb.). *Mem. Fac. Agric. Kinki Univ*. 14:57–65.

KONG, C., HU, F., XU, T., and LU, Y. 1999. Allelopathic potential and chemical constituents of volatile oil from *Ageratum conyzoides*. *J. Chem. Ecol*. 25:2347–2356.

KORHAMMER, S. and HASLINGER, E. 1994. Isolation of a biologically active substance from rhizomes of quackgrass [*Elymus repens* (L.) Gould]. *J. Agric. Food Chem*. 42:2048–2050.

LAOSINWATTANA, C., YONEYAMA, K., TAKEUCHI, Y., OGASAWARA, M., and KONNAI, M. 1999. Purification of allelopathic compounds from manilagrass [*Zoysia matrella* (L.) Merr.] plants. *J. Jpn. Soc. Turfgrass Sci*. 28:27–36.

LEE, C. W., YONEYAMA, K., TAKEUCHI, Y., KONNAI, M., TAMOGAMI, S., and KODAMA, O. 1999. Momilactones A and B in rice straw harvested at different growth stages. *Biosci. Biotechnol. Biochem*. 63:1318–1320.

LI, H. H., LAJIDE, L., NISHIMURA, H., HASEGAWA, K., and MIZUTANI, J. 1993. Allelochemicals in the soil beneath *Quercus mongolica* Fisch var. grosseserrata Rehd. Wils. *Zasso Kenkyu* 38:282–293.

LIEBL, R. A. and WORSHAM, D. 1983. Inhibition of pitted Morning glory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J. Chem. Ecol*. 9:1027–1043.

LIN, H.-Y. and VOYKSNER, R. D. 1995. Electrospray-ion trap mass spectrometry: Applications to trace analysis, chapter 14, *in* R. E. March and J. F. J. Todd (eds.). Practical Aspects of Ion Trap Mass Spectrometry, Vol. 3, CRC Press, Boca Raton, Florida.

LINSKENS, H. F. and JACKSON, J. F. (eds.). 1986. Modern Methods of Plant Analysis: GC-MS. Vol. 3. Springer-Verlag, Berlin, Germany.

LOVETT, J. V. and POTTS, W. C. 1987. Primary effects of allelochemicals of *Datura stramonium* L. *Plant Soil* 98:137–144.

LOVETT, J. V., LEVITT, J., DUFFIELD, A. M., and SMITH, N. G. 1981. Allelopathic potential of *Datura stramonium* L. (thorn-apple). *Weed Res.* 21:165–170.

MA, R., LIU, X., YUAN, G., and SUN, S. 1997. Allelochemicals and allelopathy from microorganisms in wheat rhizosphere. *J. Environ. Sci. (China)* 9:108–112.

MACIAS, F. A., SIMONET, A. M., ESTEBAN, M. D., and GALINDO, J. C. G. 1996. Triterpenoids from *Melilotus messanensis*; soyasapogenol G, the first natural carbonate derivative. *Phytochemistry* 41:1573–1577.

MACIAS, F. A., SIMONET, A. M., and GALINDO, J. C. G. 1997. Natural products as allelochemicals 5. Bioactive steroids and triterpenes from *Melilotus messanensis* and their allelopathic potential. *J. Chem. Ecol*. 23:1781–1803.

MACIAS, F. A., GALINDO, J. C. G., MOLINILLO, J. M. G., and CUTLER, H. G. (eds.). 1999. Recent Advances in Allelopathy, Vol. 1: A Science for the Future. International Allelopathy Society. University of Cadiz, Cadiz, Spain.

MATTICE, R., LAVY, T., SKULMAN, B., and DILDAY, R. 1998. Searching for allelochemicals in rice that control ducksalad, chapter 8, *in* M. Olofsdotter (ed.). Allelopathy in Rice. International Rice Research Institute, Los Banos, Philippines.

MCMASTER, M. and MCMASTER, C. 1998. GC-MS: A Practical Users Guide. Wiley-Interscience, New York.

MEYER, V. R. 1988. Practical High-Performance Liquid Chromatography. Wiley Sons, New York.

MILLAR, J. G. and HAYNES, K. F. (eds.). 1998. Methods in Chemical Ecology, Vol. 1, Chemical Methods. Chapman and Hall, London, England.

NAKAHISA, K., TSUZUKI, E., TERAO, H., and KOSEMURA, S. 1994. Study on the allelopathy of alfalfa (*Medicago sativa* L.). II. Isolation and identification of allelopathic substances in alfalfa. *Nippon Sakumotsu Gakkai Kiji* 63:278–284.

NARWAL, S. S. (ed.). 1999a. Allelopathy Update, Vol. 1: International Status. Science Publishers, Enfield, New Hampshire.

NARWAL, S. S. (ed.). 1999b. Allelopathy Update, Vol. 2: Basic and Applied Aspects. Science Publishers, Enfield, New Hampshire.

NARWAL, S. S. and TAURO, P. (eds.). 1994. Abstracts of the International Symposium: Allelopathy in sustainable agriculture, forestry & environment. India Agricultural Research Institute, New Delhi, India.

NEWTON, R. P. and WALTON, T. J. 1996. Applications of Modern Mass Spectrometry in Plant Science Research. Clarendon Press, Oxford, United Kingdom.

NIESSEN, W. M. A. 1999. Liquid Chromatography–Mass Spectrometry, 2nd ed. Marcel Dekker, New York.

OHWAKI, Y., OGINO, J., and SHIBANO, K. 1993. 3-Hydroxy-5-methoxystilbene-2-carboxylic acid, a phytotoxic compound isolated from methanolic extracts of pigeonpea (*Cajanus cajan* Millsp.) *Soil Sci. Plant Nutr*. 39:55–61.

OLOFSDOTTER, M. (ed.). 1998. Allelopathy in Rice. International Rice Research Institute, Los Banos, Philippines.

PEREDA-MIRANDA, R. 1997. Biodynamic oligosaccharides from Convolvulaceae: Isolation, structural elucidation and biological activities. *Rev. Latinoam. Quim*. 25:97–102.

PEREZ, E., SAWYERS, W. G., and MARTIN, D. F. 1997. Identification of allelopathic substances produced by *Nannochloris oculata* that affect a red-tide organism, *Gymnodinium breve*. *Biomed. Lett*. 56:7–14.

PHILLIPS, V. A. and HEDIN, P. A. 1990. Spectral techniques for structural analysis of the cotton terpenoid aldehydes gossypol and gossypolone. *J. Agric. Food Chem*. 38:525–528.

PUTNAM, A. R. 1985. Weed allelopathy, chapter 5, *in* S. O. Duke (ed.). Weed Physiology, Vol. 1, Reproduction and Ecophysiology. CRC Press, Boca Raton, Florida.

PUTNAM, A. R. and TANG, C.-S. (eds.). 1986. The Science of Allelopathy. John Wiley & Sons, New York.

QUAYYUM, H. A., MALLIK, A. U., ORR, D. E., and LEE, P. F. 1999. Allelopathic potential of aquatic plants associated with wild rice: II. Isolation and identification of allelochemicals. *J. Chem. Ecol*. 25:221–228.

RICE, E. 1984. Allelopathy, 2nd ed. Academic Press, London, England.

RICE, E. L. 1995. Biological Control of Weeds and Plant Diseases: Advances in Applied Allelopathy. University of Oklahoma Press, Norman, Oklahoma.

RIFFLE, M. S., WALLER, G. R., MURRAY, D. S., and SGARAMELLO, R. P. 1990. Devil's claw (*Proboscidea louisianica*), essential oil and its components. *J. Chem. Ecol*. 16:1927–1940.

RIMANDO, A. M., OLOFSDOTTER, M. O., DAYAN, F. E., and DUKE, S. O. 2001. Searching for rice allelochemicals: An example of bioassay-guided isolation. *Agron. J*. 93:16–20.

RIZVI, S. J. H. and RIZVI, V. (eds.). 1992. Allelopathy: Basic and Applied Aspects. Chapman & Hall, London, England.

ROMEO, J. T. 2000. Raising the beam: Going beyond phytotoxicity. *J. Chem. Ecol*. 26:2011–2014.

SCHULZ, M., FRIEBE, A., KUCK, P., SEIPEL, M., and SCHNABL, H. 1994. Allelopathic effects of living quackgrass (*Agropyron repens* L.). Identification of inhibitory allelochemicals exuded from rhizome borne roots. *Angew. Bot*. 68:195–200.

SHILLING, D. G., JONES, L. A., WORSHAM, A. D., PARKER, C. E., and WILSON, R. F. 1986. Isolation and identification of some phytotoxic compounds from aqueous extracts of rye (*Secale cereale* L.). *J. Agric. Food Chem*. 34:633–638.

STENHAGEN, G. and ALBORN, H. 1989. Developments of micro liquid chromatography-mass spectrometry with gradient elution. Improvements to obtain less thermal decomposition of labile compounds. *J. Chromatogr*. 474:285–300.

TANG, C.-H. and YOUNG, C.-C. 1982. Collection and identification of allelopathic compounds from the undisturbed root system of Bigalta limpograss (*Hemarthria altissima*). *Plant Physiol*. 69:155–160.

TANG, C. S., WAT, C. K., and TOWERS, G. H. N. 1987. Thiophenes and benzofurans in the undisturbed rhizosphere of *Tagetes patula* L. *Plant Soil* 98:93–97.

TANRISEVER, N., FISCHER, N. H., and WILLIAMSON, G. B. 1988. Menthofurans from *Calamintha ashei*: Effects on *Schizachyrium scoparium* and *Lactuca sativa*. *Phytochemistry* 27:2523–2526.

THOMPSON, A. C. (ed.). 1985. The Chemistry of Allelopathy: Biochemical Interactions among Plants. American Chemical Society Symposium Series 268, ACS, Washington, D.C.

VAUGHN, S. and BOYDSTON, R. A. 1997. Volatile allelochemicals released by crucifer green manures. *J. Chem. Ecol*. 23:2107–2116.

von POSER, G. L., MENUT, C., TOFFOLI, M. E., VERIN, P., SOBRAL, M., BESSIERE, J.-M., LAMATY, G., and HENRIQUES, A. T. 1996. Essential oil composition and allelopathic effect of the Brazilian Lamiaceae *Hesperozygis ringens* (Benth.) Epling and *Hesperozygis rhododon* Epling. *J. Agric. Food Chem*. 44:1829–1832.

WALLER, G. R. (ed.). 1987. Allelochemicals: Role in Agriculture and Forestry. American Chemical Society Symposium Series 330, ACS, Washington, D.C.

WALLER, G. R., JURZYSTA, M., and THORNE, R. L. Z. 1993a. Allelopathic activity of root saponins from alfalfa (*Medicago sativa* L.) on weeds and wheat. *Bot. Bull. Acad. Sin*. 34:1–11.

WALLER, G. R., WEST, P. R., CHENG, C. S., LING, Y. C., and CHOU, C. H. 1993b. The occurrence of soyasaponin I in *Vigna radiata* L. (mungbean) sprouts as determined by fast atom bombardment, liquid secondary ion mass spectrometry, and linked scanning at constant B/E MS/MS. *Bot. Bull. Acad. Sin*. 34:323–334.

WALLER, G. R., FENG, M.-C., and FUJI, Y. 1999. Biochemical analysis of allelopathic compounds: Plants, micro-organisms, and soil secondary metabolites, *in* S. Inderjit, K. M. M. Dakshini, and C. L. Foy (eds.). Principles and Practices of Plant Ecology. CRC Press, Boca Raton, Florida.

WALLSTEDT, A., NILSSON, M.-C., ODHAM, G., and ZACKRISSON, O. 1997. A method to quantify the allelopathic compound batatasin-III in extracts from *Empetrum hermaphroditum* using gas chromatography: Applied on extracts from leaves of different ages. *J. Chem. Ecol*. 23:2345–2355.

WEIDENHAMER, J. D. 1996. Distinguishing resource competition and chemical interference: Overcoming the methodological impasse. *Agron. J*. 88:866–875.

WILLIAMSON, G. B., FISCHER, N. H., RICHARDSON, D. R., and DE LA PENA, A. 1989. Chemical inhibition of fire-prone grasses by fire-sensitive shrub, *Conradina canescens*. *J. Chem. Ecol*. 15:1567–1577.

WOODWARD, M. D., CORCUERA, L. J., SCHNOES, H. K., HELGESON, J. P., and UPPER, C. D. 1979. Identification of 1,4-benzoxazin-3-ones in maize extracts by gas–liquid chromatography and mass spectrometry. *Plant Physiol*. 63:9–13.

WU, H., HAIG, T., PRATLEY, J., LEMERLE, D., and AN, M. 1999. Simultaneous determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in wheat (*Triticum aestivum* L.) by gas chromatography–tandem mass spectrometry. *J. Chromatogr. A* 864:315–321.

YAMAMOTO, Y. 1995. Allelopathic potential of *Anthoxanthum odoratum* for invading *Zoysia* grassland in Japan. *J. Chem. Ecol*. 21:1365–1373.

YAMANE, A., FUJIKURA, J., OGAWA, H., and MIZUTANI, J. 1992a. Isothiocyanates as allelopathic compounds from *Rorippa indica* Hiern. (Cruciferae) roots. *J. Chem. Ecol*. 18:1941–1954.

YAMANE, A., NISHIMURA, H., and MIZUTANI, J. 1992b. Allelopathy of yellow fieldcress (*Rorippa sylvestris*): Identification and characterization of phytotoxic constituents. *J. Chem. Ecol*. 18:683–691.

YU, J. Q., and MATSUI, Y. 1993. *p*-Thiocyanatophenol as a novel allelochemical in exudates from the root of cucumber. *Chem. Expr*. 8:577–580.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

MALE-SPECIFIC SESQUITERPENES FROM *Phyllotreta* AND *Aphthona* FLEA BEETLES[1]

ROBERT J. BARTELT,* ALLARD A. COSSÉ, BRUCE W. ZILKOWSKI, DAVID WEISLEDER, and FRANK A. MOMANY

USDA Agricultural Research Service
National Center for Agricultural Utilization Research
1815 North University Street
Peoria, Illinois 61604

(Received March 10, 2001; accepted August 6, 2001)

Abstract—It was previously reported that males of the crucifer flea beetle, *Phyllotreta cruciferae*, feeding on host foliage are attractive to both males and females in the field. Based on this evidence for an aggregation pheromone, volatiles were collected from male and female *P. cruciferae* feeding on cabbage (*Brassica oleracea*) and analyzed. For comparison, volatiles were also collected from males and females of three other flea beetle species, *Aphthona flava, A. czwalinae*, and *A. cyparissiae*, all feeding on their host, leafy spurge foliage (*Euphorbia esula*). Six male-specific compounds were isolated from *P. cruciferae*, and the same compounds plus two additional ones were isolated from males of *Aphthona flava, A. czwalinae*, and *A. cyparissiae*. The blends of compounds were relatively consistent within species, but there were characteristic differences between species. Compound structures were studied by mass spectrometry, NMR spectroscopy, UV spectroscopy, polarimetry, chiral and achiral gas chromatography, molecular modeling, and microchemical tests. Three of the compounds were identified as (+)-*ar*-himachalene; (+)-*trans*-α-himachalene; (+)-γ-cadinene. Two others were new enantiomers of himachalene hydrocarbons that were previously identified from the fir trees, *Abies alba* and *Abies nordmanniana*. Finally, there were two himachalene alcohols and one norsesquiterpene ketone that is a himachalene analog. Only (+)-*ar*-himachalene and (+)-γ-cadinene are previously known natural products. Electrophysiological activity was demonstrated for five of the compounds. The chemical and electrophysiological patterns are consistent with, but do not prove, a pheromonal function.

* To whom correspondence should be addressed. E-mail: BartelRJ@NCAUR.USDA.gov

[1] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

0098-0331/01/1200-2397/0 © 2001 Plenum Publishing Corporation

Key Words—Sesquiterpene, himachalene, cadinene, flea beetle, *Aphthona*, *Phyllotreta*, Chrysomelidae, Alticinae.

INTRODUCTION

Flea beetles constitute the largest subfamily (Alticinae) of the leaf beetle family, Chrysomelidae (Arnett, 1993). The beetles are known for their jumping ability and have greatly enlarged hind femora. Adults feed on host plant foliage, and the larvae typically feed on the roots of the same plant (Borror and DeLong, 1964). The group includes both agricultural pests and beneficial species. One of the four species used in this study, the crucifer flea beetle, *Phyllotreta cruciferae* Goeze, is a significant pest of canola and rapeseed in the northern prairie areas of the United States and Canada, and it also attacks cabbage, horseradish, and other crucifers in many regions (Lamb, 1989). The other three species, *Aphthona flava* Guillebeau, *A. czwalinae* (Weise), and *A. cyparissiae* (Koch), are effective biocontrol agents of the introduced rangeland weed, leafy spurge (*Euphorbia esula* L.) (Gassmann et al., 1996). These *Aphthona* species are native to Europe but have been established in the United States and Canada.

Little is known about pheromones and mating systems of flea beetles. The results of Zhang and McEvoy (1994) suggested that a female-produced sex pheromone exists in the ragwort flea beetle, *Longitarsus jacobaeae* (Waterhouse), and that only males respond to it. The chemical cue persists for hours in areas formerly occupied by females, but its chemical nature is not known. It was not clear from the report whether the pheromone is volatile enough to be attractive at a distance. Conclusions for *P. cruciferae* were quite different. Peng and Weiss (1992) demonstrated that unsexed *P. cruciferae* feeding on canola plants were clearly more attractive to both males and females than were damaged plants, beetles alone, or other controls, both in the field and in the laboratory. Then, Peng et al. (1999) compared male *P. cruciferae* to females in the field bioassay and concluded that only the males are demonstrably attractive, again to both sexes. Thus, in this species there was evidence for a male-produced aggregation pheromone.

The first objective of the present study was to discover possible pheromone component(s) in *P. cruciferae* by chemically comparing males and females. These comparisons employed collections of volatiles from beetles feeding on host plant material, to reflect the conditions of the previous, successful bioassays as closely as possible. Male-specific compounds were revealed, but they were clearly complicated in structure and the amounts collected were small. Chemical identification required key spectral techniques such as NMR, but a modest-scale program to collect volatiles from *P. cruciferae* could not provide enough material for this. During a preliminary examination of other flea beetle species, male-specific compounds were also found from *A. flava*, and coincidentally, by GC retention

times and mass spectra, these included all of the male-specific compounds from *P. cruciferae*. Although the *A. flava* compound ratios were different from *P. cruciferae*, the total amounts were considerably larger. Thus, by concentrating efforts on *A. flava*, identifying the male-specific chemicals of both species became feasible. *A. czwalinae* and *A. cyparissiae* were included in the study to further explore differences in chemical patterns among species, and these also shared the same chemical structures.

Eight male-specific compounds were chromatographically isolated, spectroscopically analyzed, and identified. Microchemical conversions among the compounds were vital to identification. Chemical standards derived from selected botanical sources aided in the characterization of the ring systems and stereochemistry. Preliminary analysis by GC-EAD indicated that identified compounds could be sensed well by beetle antennae, which would be expected for pheromone components. However, final conclusions about function must await field testing with synthetic compounds.

METHODS AND MATERIALS

Source and Care of Beetles. *P. cruciferae* for this work were field collected at various locations: canola fields in North Dakota, USA (from C. Peng), canola fields in Saskatchewan, Canada (from J. Soroka), and cabbage plots in Peoria, Illinois, USA. In the laboratory, the beetles were kept in cages and fed on seedlings of canola or cabbage, or later, on the core tissue of mature heads of cabbage. *Aphthona* species were field collected in eastern Montana and western North Dakota (from A. Caesar) or from western Montana (from R. Nowierski) from spurge-infested rangeland where *Aphthona* introductions were established. Beetles were kept in cages containing shoots of leafy spurge with cut ends in bottles of water. Spurge cuttings were from plants maintained in our greenhouse. *Aphthona* species identifications were based on LeSage and Paquin (1996) and McDaniel et al. (1992). *A. cyparissiae* is difficult to separate from another species in the United States, *A. nigriscutis* (Foidras), by external morphology. Males of *A. cyparissiae* were explicitly linked to one of the species-specific GC profiles of volatiles (presented below in the Results section) by genitalic dissection (McDaniel et al., 1992) of all individuals of a group that had produced the typical pattern.

Collection of Volatiles. Beetles were sexed by inspection of the ventral side of the abdominal tip under the microscope (LeSage and Paquin, 1996); insects were immobilized by gently guiding each one into the tapered end of a small glass tube and keeping it there with a narrow wooden rod. Beetles (usually 10–12 males or females) were introduced into volatiles collectors, which consisted of vertical glass tubes (5 cm diam. $\times$ 20 cm long) containing host plant material (chunks of mature cabbage head for *P. cruciferae* or spurge shoots with ends in water vials

for *Aphthona* species). The tube ends were closed with corks, each of which held a volatiles trap. Traps were made from 5-mm-diam. glass tubing and contained a 5 to 8-mm plug of Super-Q porous polymer (Allech Associates, Deerfield, Illinois). The Super-Q was held in place by fine stainless steel screen (which was fused into the trap wall) and glass wool. A gentle vacuum was drawn on the upper trap so that air flowed through the tubes at about 100 ml/min. The lower trap served to clean the incoming air, and the upper trap collected the emitted volatiles. The volatiles collectors were kept in an incubator at 27°C with a relative humidity of about 50%. Light was provided by three 40-W fluorescent tubes set about 0.5 m above the apparatus. The daily light cycle was 14L:10D.

The usual procedure was to service the collectors every two or three days. This included rinsing of volatiles from the filters into vials using 300 μl hexane, replacement of host plant material as needed, and analysis of each collection by GC (1 μl of the undiluted collection). There were some modifications of the above scheme: Initially, collections were processed daily, but longer periods did not lead to noticeable changes or degradation of collected chemicals, and the longer periods were adopted for convenience. Late in the study, 8–10 collections from *A. flava* were routinely combined for GC monitoring, after the consistency of GC patterns for this species had been established. Finally, occasional collections were made from host material without beetles, for comparison, but the major need was to use the equipment to accumulate the male-specific compounds.

For *P. cruciferae*, about 350 beetle-days of volatiles were accumulated for males, and about 120 for females. (One beetle-day is the amount of volatiles emitted by one feeding beetle in one day.) For the *Aphthona* species, totals for male *A. cyparissiae, A. flava*, and *A. czwalinae* were about 300, 7200, and 750 beetle-days, respectively. Totals for females were 35, 44, and 70 beetle-days, respectively. The total numbers of GC analyses made to monitor the collections were 38 for *P. cruciferae*, 15 for *A. cyparissiae*, 74 for *A. flava*, and 32 for *A. czwallinae*. Eleven GC analyses were made for uninfested cabbage, and seven were made for uninfested spurge. Samples from all groups were scrutinized by GC-MS, especially those for which GC comparisons indicated the presence of sex-specific peaks. Quantitation of GC peaks was by the external standard method, relative to heptadecane.

Instrumentation. Gas chromatography (GC) was conducted on a Hewlett Packard 5890 Series II instrument equipped with a split/splitless inlet, flame ionization detector, and autosampler and interfaced to an HP ChemStation data system. Columns were DB-5MS (30 m $\times$ 0.25 mm ID with 0.25 μm film, J&W Scientific, Folsom, California) or DB-1 (15 m $\times$ 0.25 mm ID with 0.10 μm film, J&W Scientific). A typical oven program began at 50°C for 1 min, increased at 10°C/min to 250°C, and remained at 250°C for 5 min. Inlet temperature was usually 200°C, and the detector temperature was 250°C. The carrier gas was helium, with a column head pressure of 140 kPa. The GC inlet temperature was normally cooler

than the maximum oven temperature because two of the analyzed compounds were somewhat heat-labile (see Results section below). The 200°C temperature provided acceptable compound stability while retaining injection efficiency.

High-performance liquid chromatography (HPLC) was done using a Waters 6000A pump and Waters R401 differential refractometer detector. Columns included a 25-cm × 4.6-mm-ID silica column (Adsorbosphere Silica 5μ, Alltech), treated with silver nitrate (Heath and Sonnet, 1980) for separation of unsaturated hydrocarbons, and a 25-cm × 4.6-mm-ID silica column (Econosphere Silica 5μ, Alltech) for separations by polarity. Elution solvents are given with the results for specific compounds. The flow rate was 1 ml/min.

Coupled GC–mass spectrometry (GC-MS) was conducted on a Hewlett Packard 5973 instrument, interfaced to an HP 6890 GC and HP ChemStation data system. The spectra were acquired in EI mode, with an ionization energy of 70 eV. The Wiley MS library with 275,821 spectra (Wiley, 1995) was installed on the data system. For achiral analyses, an EC-1 capillary column (30 m × 0.25 mm ID with 0.25 μm film, Alltech) or the columns described above were used, and the oven program was as for GC. The carrier gas was helium, with an inlet head pressure of 41 kPa. The temperatures for the inlet and transfer line were usually 200°C.

For chiral analysis, a Cyclodex-B column (J&W Scientific, 30 m × 0.25 mm ID × 0.25 μm film) was installed in the GC-MS system. To separate the enantiomers of compound **C**, the oven temperature was initially 50°C for 1 min, increased at 30°C/min to 140°C, and held at 140°C for 20 min; the oven was heated to 230°C between samples. Other programs were tried for other compounds. (All of the lettered compounds mentioned in the methods section are defined in the Results section.)

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance 400 instrument. Proton spectra were acquired at 400 MHz and carbon spectra, at 100 MHz. Many samples were small (e.g., less than 100 μg) and were run in a Bruker Microprobe Tube (product 520-1A, Wilmad, Buena, New Jersey). The solvent was either benzene-d_6 or $CDCl_3$. Some samples were stable only in benzene, but comparing to literature spectra required acquisition in chloroform. Reported shifts are relative to TMS. Proton spectra were acquired for all samples. Carbon, DEPT, proton COSY, proton decoupling, HMQC, and nuclear Overhauser difference experiments were also run for some samples, depending on amount of available material and specific research needs. To prepare samples for NMR (always after purification by HPLC), the solvent was carefully removed by evaporation under a stream of nitrogen, followed by introduction of the NMR solvent and transfer to the NMR tube using a micropipette. If the NMR spectrum revealed the presence of nondeuterated solvent, the evaporation process was repeated.

Ultraviolet (UV) spectra were acquired on a Perkin Elmer Lambda 4B UV/Vis spectrophotometer relative to solvent blank; cuvettes had a 1-cm light path. Optical rotations were measured with a Perkin Elmer 341 Polarimeter, using a microcell

1-dm long and of 1 ml volume. Analyses of both types were run in hexane. Sample concentrations were measured by GC. An uncertainty of 15% in optical rotations and UV extinction coefficients was not unlikely.

Column Chromatography. Open columns of silica in Pasteur pipets (about 6 cm $\times$ 0.5 cm ID) were used to fractionate volatile collections and other samples by polarity. The series of elution solvents included hexane, increasing percentages of ethyl ether in hexane (5, 10, 25), and pure ether (about 5 ml, or three column volumes per solvent). Larger columns were used for some samples, depending on the amount of material to be separated.

Botanical Sources of Chemicals for Comparative Analysis. Anise seed (*Pimpinella anisum* L.) was used as a source of the sesquiterpene, α-himachalene (Burkhardt et al., 1986). Seed was purchased from a local grocery store, crushed, and extracted with hexane. The hexane extract was applied to an open column of silica and eluted with hexane to separate the sesquiterpenes from anisaldehyde and other polar constituents. α-Himachalene was purified from the other hydrocarbons by HPLC on the silver nitrate column (elution solvent 1% 1-hexene in hexane, retention volume 4.4–4.8 ml). It was recognized by mass spectral comparison to the Wiley library.

Fir tree foliage was used as the source of two rare himachalene sesquiterpenes (Khan et al., 1989), which a literature search suggested were identical to two of the beetle-derived compounds (see Results). Four small living specimens of European silver fir (*Abies alba* Miller) and one of Nordmann fir [*Abies nordmanniana* (Steven) Spach] were obtained from a nursery company (Forest Farm, Williams, Oregon). Subsequently, about 10 kg of freshly cut foliage of *A. nordmanniana* was obtained from a Christmas tree plantation (Harold Miller Landscapes, Jefferson, Oregon). The fir needles were finely chopped and extracted with hexane in small vials for initial samples and in percolators for larger batches. Purification of sesquiterpenes generally followed the scheme for *Aphthona* compounds.

"Citronella oil" from *Cymbopogon* spp., obtained from (Nucara, Coralville, Iowa) was used as the source of natural (+)-γ-cadinene (Vig et al., 1970). The oil was diluted with hexane and applied to an open column of silica. The fraction eluted with hexane was then handled further as described for the *Aphthona* hydrocarbon compounds.

Microchemical Reactions. Catalytic hydrogenation followed by GC-MS was used to determine the numbers of double bonds in the male-specific hydrocarbons and also in the comparison of ring systems between three insect-derived compounds (**A**, **B**, and **C**) and a selected plant-derived standard (α-himachalene). Hydrogen was gently bubbled from a fine needle for about 5 min at room temperature into 100 μl of methylene chloride in a conical vial, which contained 10–100 ng of sample and a trace ($<$1 mg) of platinum oxide (Adam's catalyst). Samples were analyzed by GC-MS. Hydrogenation has often been applied in sesquiterpene analysis (e.g., Andersen et al., 1977).

A microscale Wittig reaction was used to prove the structure of the beetle-derived ketone (compound **H**) by converting it into a hydrocarbon that had been previously characterized. A 0.4 M solution of methylenetriphenylphosphorane in tetrahydrofuran was prepared (Sonnet, 1974), and 20 μl of this reagent was transferred by syringe to a solution of compound **H** in a conical vial (about 1 μg in 10 μl hexane). The sample was mixed by vortexing, then allowed to stand at room temperature for 15 min. The solution was diluted with about 50 μl hexane and 20 μl water, and the layers were separated. The organic layer was passed through a small silica column with hexane, concentrated under a stream of N_2, and then analyzed by GC-MS.

Determination of the absolute configuration of compound **A** involved converting it to a product (*ar*-himachalene) for which the stereochemistry had been established. Aromatization of compound **A** (5 mg) from Nordmann fir was conducted with chloranil (2,3,5,6-tetrachlorohydroquinone) as described by Mehta and Singh (1977). After purification on silica, the product in hexane solution was concentrated to 1.1 ml under nitrogen, quantitated by GC, and submitted to polarimetry.

Partial isomerization of **A** to **C** by double-bond migration in acid solution was a step in one method for the chiral analysis of **A**. The isomerization was done by placing about 10 mg of Dowex 50W-X4 (strong cation exchange resin, acid form) into 0.5 ml of hexane solution of **A** and heating to 50°C for 16 hr. Analysis was by GC-MS on the chiral column. Isomerization also occurred when **A** was stored in $CDCl_3$.

Molecular Modeling. Assignment of the relative stereochemistry for the two asymmetric centers in compound **A** was aided by molecular modeling. The energy-minimized, three-dimensional structures for the two possible diastereomers of compound **A** were calculated using the semiempirical AM1 method available in the MOPAC computational package distributed by QCPE (Program Number 506) (Dewar et al., 1985).

Electrophysiology. Coupled GC electroantennographic detection (GC-EAD) was conducted on a Hewlett Packard 6890 GC, interfaced to antennal preparations with equipment and methods generally described by Cossé and Bartelt (2000). Volatiles collections from *A. flava* were analyzed by GC-EAD. Any compounds with actual pheromonal activity would be expected to be readily sensed by the beetle antennae (although GC-EAD activity would not necessarily imply behavioral activity).

RESULTS AND DISCUSSION

Male-Specific Compounds, Polarity, and Mass Spectra. By GC comparison of volatiles collections, males of *P. cruciferae* emitted six compounds, **A–E** and **H**, that were never detected from females (example chromatograms in Figure 1).

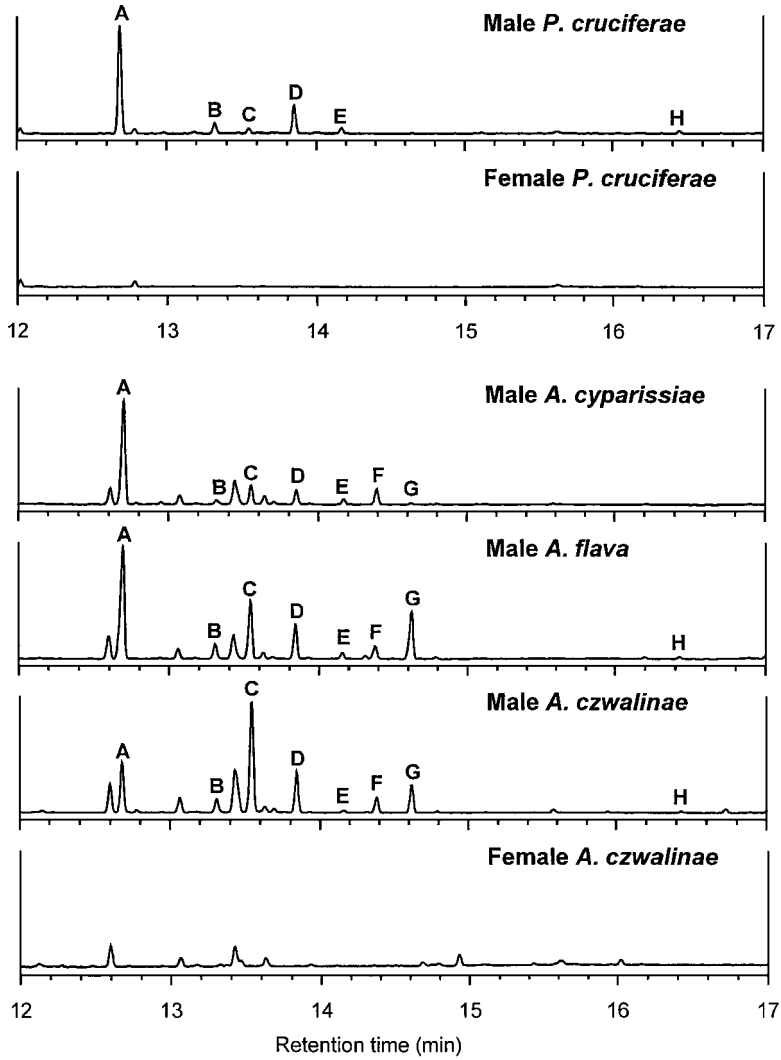


FIG. 1. Typical gas chromatograms of volatiles collected from flea beetles feeding on host material (cabbage chunks for *P. cruciferae* and spurge foliage for *Aphthona* species). Chromatograms for males consistently contained peaks (labeled **A–H**) that females lacked. They were similarly lacking from female *A. cyparissiae* and *A. flava* (chromatograms not shown). Compounds **F** and **G** were never detected from *P. cruciferae*. Compound **H** was detectable in many of the collections from *A. cyparissiae* males but not in the example shown here. In the chromatograms for males, the largest peak in the 12- to 17-min retention time window was scaled to 100%; chromatograms for females were scaled the same as for the corresponding males. Peak **A** in the chromatogram for male *P. cruciferae* represented 6 ng; peak **A** for male *A. cyparissiae*, 65 ng; peak **A** for male *A. flava*, 200 ng; and peak **C** for male *A. czwalinae*, 27 ng.

Similarly, volatiles collections from *A. flava*, *A. czwalinae*, and *A. cyparissiae* revealed eight male-specific compounds (**A–H**, Figure 1). (Only one example chromatogram for *Aphthona* females, for *A. czwalinae*, is shown in Figure 1, but those for *A. cyparissiae* and *A. flava* were similar.) Samples from females of all species were scrutinized by GC-MS for possible trace amounts of compounds **A–H**, but none were revealed. (Amounts from females as small as 1% of the major sex-specific peak from males would have been easily detectable.) The unlabeled peaks in Figure 1 were primarily host volatiles, based on GC-MS comparisons to uninfested plant material.

For all species, the usual pattern was for the male-specific compounds to appear within days of placing new beetles into the collection apparatus, but emission then continued as long as the beetles lived. All groups of male beetles eventually produced male-specific compounds. There were several consistent trends in the compound ratios (Table 1) by which the species could be recognized: *P. cruciferae* differed from the *Aphthona* species by lacking **F** and **G**. Among the *Aphthona* species, *A. cyparissiae* consistently had more **F** than **G**, but the opposite was true for *A. flava* and *A. czwalinae*. In *A. flava*, **A** was far more abundant than **C**, but the pattern in *A. czwalinae* was the reverse.

The total amount of compound emitted per male per day was greatest for *A. flava* (Table 1). *A. flava* was also long-lived, easily handled, and relatively available; therefore, *A. flava* became the major source of the material to be used for structural analysis. Most field-collected *A. flava* males emitted volatiles as long as they survived in the laboratory (usually about two months); volatiles were collected from one individual male for six months.

TABLE 1. MALE-SPECIFIC COMPOUNDS IN FOUR FLEA BEETLE SPECIES[a]

	Production of male-specific compounds			
Compound	*P. cruciferae*	*A. cyparissiae*	*A. flava*	*A. czwalinae*
Relative abundance (% of the total for **A–H** ± SD)				
A	66.3 ± 6.1	68.2 ± 2.9	50.4 ± 5.4	20.1 ± 1.9
B	6.6 ± 0.5	2.8 ± 0.1	4.5 ± 0.4	5.0 ± 0.6
C	2.9 ± 0.3	10.9 ± 2.3	16.9 ± 2.3	38.7 ± 4.3
D	17.6 ± 1.6	7.8 ± 0.1	10.9 ± 0.6	14.7 ± 1.9
E	4.0 ± 1.9	2.4 ± 0.3	1.9 ± 0.5	0.8 ± 0.4
F	0.0	6.7 ± 1.3	3.3 ± 0.9	7.8 ± 2.3
G	0.0	1.0 ± 0.3	11.6 ± 3.9	12.3 ± 5.2
H	2.6 ± 3.7	0.2 ± 0.1	0.4 ± 0.3	0.5 ± 0.3
Rate of production (total μg of **A–H** per male per day ± SD)				
A–H	0.19 ± 0.11	0.50 ± 0.12	2.9 ± 0.90	0.67 ± 0.31

[a] For each species, the data were compiled from ten chromatograms representing 10 different volatiles collections. The collections were randomly selected from among those for which emission of male-specific volatiles had commenced.

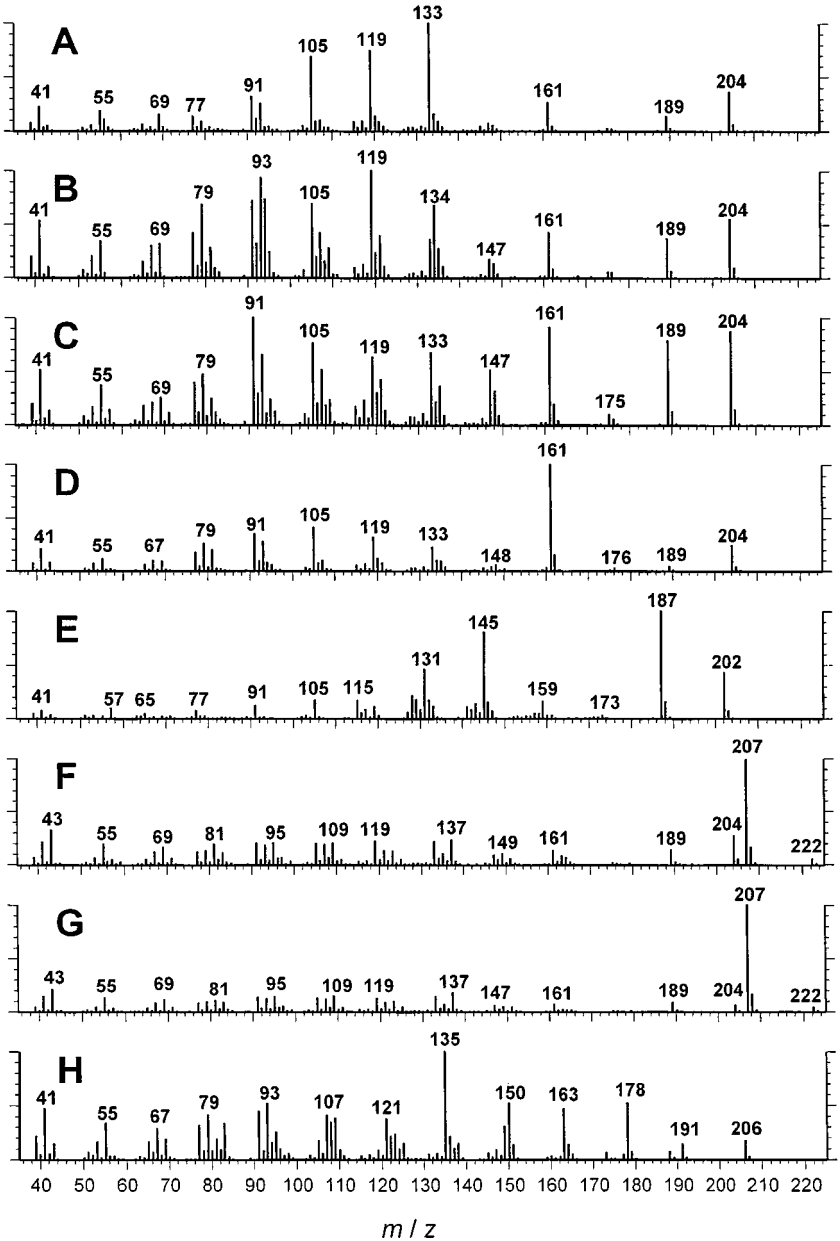


FIG. 2. EI mass spectra of the male-specific flea beetle compounds.

Compounds **A–E** from *P. cruciferae* eluted from the open column of silica with hexane, suggesting they were hydrocarbons. Mass spectra (Figure 2) indicated molecular weights of 204 for **A–D** (probably $C_{15}H_{24}$, with four double-bond equivalents) and 202 for E ($C_{15}H_{22}$, with five double-bond equivalents). Compound **H** eluted from the silica column with 25% ether in hexane. Its polarity indicated that at least one oxygen was present; thus, a molecular ion of m/z 206 could represent a molecular formula of $C_{14}H_{22}O$, with four double-bond equivalents. A fragment ion at m/z 188 ($M - H_2O$) supported the presence of oxygen.

By GC, MS, and column chromatography, six of the compounds from all three *Aphthona* species were identical to **A–E** and **H** from *P. cruciferae*. The two additional compounds (**F** and **G**) were relatively polar and eluted from silica with 25% ether in hexane. Mass spectra of these are shown in Figure 2. Molecular ions were observed at m/z 222 for both **F** and **G**, which suggested a molecular formula of $C_{15}H_{26}O$, with three double-bond equivalents. The base peak was m/z 207 (M-methyl) for both compounds, but a peak was also present at m/z 204 (M – H_2O). Credible matches to the mass spectral library were not found for any of the compounds except **D**, as discussed below.

HPLC Purification. It was clear that NMR spectroscopy and other methods requiring pure compounds would be needed for identification. Hydrocarbons **A–E** could be purified on the silver nitrate HPLC column with 0.5% 1-hexene in hexane as solvent. The retention volumes (ml after injection) were: **A**, 3.7–4.0; **E**, 4.7–4.9; **B**, 11.4–13.0; **C**,14.8–16.4; and **D**, 33.0–35.0; (hold-up volume of the column was 3.0 ml). The silica HPLC column was used for the polar compounds. With 15% ether in hexane as solvent, baseline separation of **F** and **G** was achieved (retention volumes were 15–17 and 17–22 ml, respectively); however, **H** eluted in the tail of the **G** peak (20–22 ml after injection). A second run using 3% acetone in hexane separated **H** from **G** (retention volumes were 11–12 and 17–18 ml after injection, respectively).

*Carbon Skeleton and Double-Bond Positions in Compound **A** from A. flava.* The molecular formula of **A** suggested a sesquiterpene hydrocarbon. Hydrogenation of **A** increased the molecular weight from 204 to 208, indicating that A had two double-bonds and, therefore, two rings. The UV spectrum of **A** had a maximum at 265 nm (hexane, ϵ = 7700), which was consistent with a homoannular conjugated diene system. The observed specific rotation was $[\alpha]_D = +82^\circ$ (c = 0.09%, hexane).

The proton NMR spectrum of **A** ($CDCl_3$) is shown in Figure 3. Key resonances were: δ 0.85 (3 H, d, J = 6.5 Hz), 1.06 (3H, s), 1.11 (3H, s), 1.72 (3H, br s), 5.24 (1H, m), and 5.57 (1H, s), which corresponded to four methyl groups (three aliphatic and one olefinic) and two olefinic protons. (The corresponding shifts in deuterobenzene were: δ 0.91, 1.11, 1.17, 1.80, 5.35, and 5.74, respectively). The aliphatic protons were better separated when deuterochloroform was the solvent, and subsequent analysis and comparisons to the literature

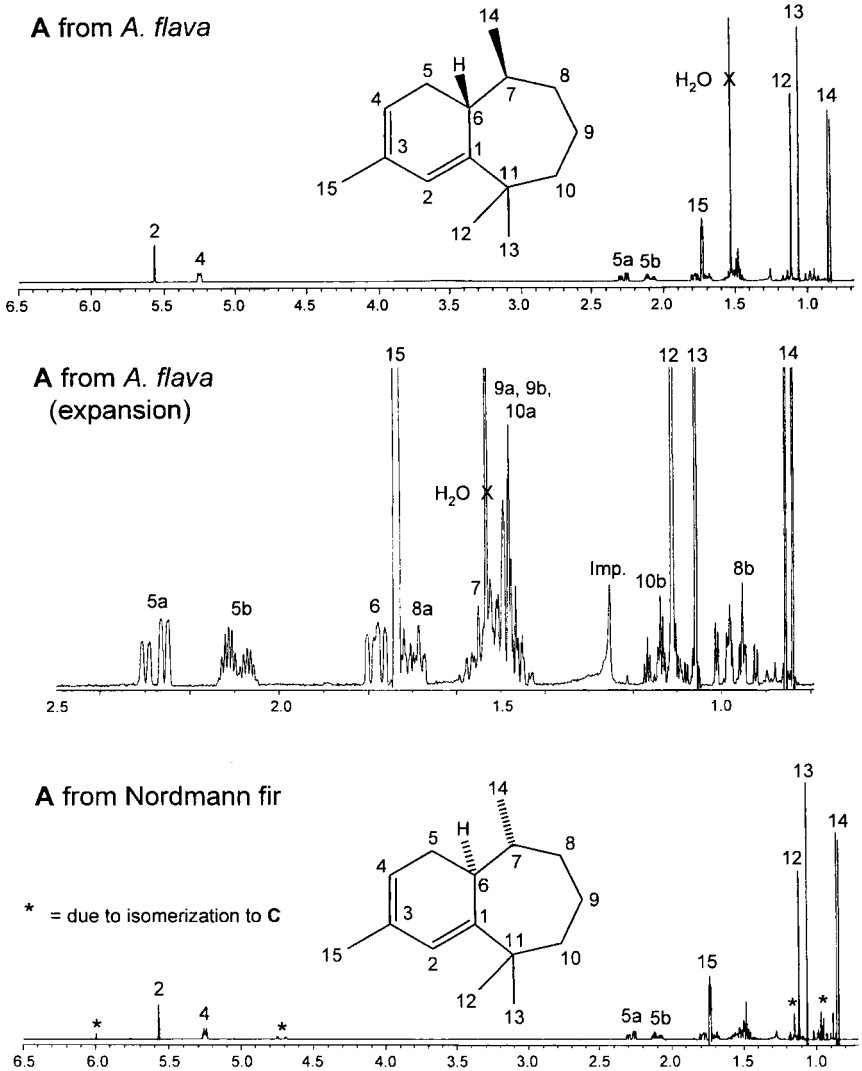


FIG. 3. Proton NMR spectra of compound **A** obtained from *Aphthona flava* and from Nordmann fir in $CDCl_3$. Stereochemistry of structures was determined by additional information (see text). Peaks due to water, an unidentified impurity, or isomerization are indicated on spectra.

TABLE 2. OBSERVED CARBON AND PROTON NMR RESONANCES COUPLING DATA FOR **A** FROM *A. flava*, AND COMPARISONS TO LITERATURE

Carbon resonances			Proton resonances				Proton–proton coupling constants (Hz)
					Literature		
Pos.	Assigned shift[a]	Literature[b]	Pos.	Assigned shift[a]	1[b]	2[c]	
1	153.57	153.45 (0)					$J_{2,4} < 1$
2	119.75	119.69 (1)	2	5.57	5.54	5.73	$J_{4,15} \sim 3$
3	132.69	132.59 (0)					$J_{4,5a} = 6.3$
4	116.06	115.96 (1)	4	5.25	5.23	5.17	$J_{4,5b} \sim 3$
5	28.05	27.99 (2)	5a	2.27			$J_{5b,15} \sim 3$
			5b	2.09			$J_{5a,5b} = 16.8$
6	37.61	37.57 (1)	6	1.78			$J_{5a,6} = 1.5$
7	34.33	34.21 (1)	7	~1.53			$J_{5b,6} = 5.8$
8	39.92	39.76 (2)	8a	1.70			$J_{6,7} = 9.9$
			8b	0.97			$J_{6,14} < 1$
9	23.14	23.04 (2)	9a	~1.45			$J_{7,14} = 6.6$
			9b	~1.45			$J_{8a,8b} \sim 13$
10	40.96	40.91 (2)	10a	~1.45			$J_{10a,10b} \sim 13$
			10b	1.12			
11	38.57	38.47 (0)					
12	26.70	26.60 (3)	12	1.11	1.09	1.15	
13	32.64	32.55 (3)	13	1.06	1.03	1.05	
14	22.47	22.30 (3)	14	0.85	0.83	0.90	
15	21.22	21.10 (3)	15	1.74	1.72	1.67	

[a] Assignments based on COSY, decoupling, DEPT, and HMQC experiments.
[b] The numbers of attached protons reported by Khan et al. (1989) are shown in parentheses; the authors did not assign the shifts to specific structure carbons, but they are listed here matched up with the *A. flava* **A** data.
[c] Mehta and Singh (1977).

were for samples in $CDCl_3$. The observed proton and ^{13}C shifts are summarized in Table 2.

The NMR proton and COSY data led to tentative assignment of the himachalene structure shown in Figure 3 (ignoring stereochemistry, for now), and this allowed a specific search of the literature. Two instances of the proposed gross structure were found. In one (Mehta and Singh, 1977), the structure resulted from acid-induced double-bond migration in a known sesquiterpene, α-himachalene. Their proton NMR data were similar to ours in general features but not in shifts (Table 2). In the other instance, Khan and Pentegova (1988) and Khan et al. (1989) described the structure as a new natural product from European silver fir and Nordmann fir. Their NMR, UV, and mass spectral data matched the beetle-derived compound very closely, and the reported specific rotation ($[\alpha]_D = +42.5^\circ$) had the same sign as that observed for *A. flava* **A**. Khan et al. (1989) attributed the NMR

differences between the tree-derived compound and that of Mehta and Singh (1977) to different relative stereochemistry at the two asymmetric centers, and our results supported this conclusion.

Repeating the conversion of α-himachalene (Mehta and Singh, 1977) yielded a compound with a mass spectrum similar to that of **A** (Figure 2), but the GC retention time was slightly later (by 0.2 min). However, repeating the extractions of both European silver fir and Nordmann fir foliage (Khan and Pentegova, 1988; Khan et al., 1989) afforded a compound that was identical to *A. flava* **A** by proton NMR (Figure 3), carbon NMR, GC retention time, and mass spectrum. Curiously, the observed optical rotation for both tree species was negative ($[\alpha]_D = -106^\circ$, $c = 0.016\%$, hexane, for European silver fir and $[\alpha]_D = -107^\circ$, $c = 0.014\%$, hexane, for Nordmann fir), in contrast to the literature report. Thus, the isolates of **A** from *A. flava* and fir foliage in this study had an enantiomeric relationship to each other. Neither the absolute nor relative stereochemistry could be unambiguously assigned from the literature, as discussed below. Nevertheless, the fact that **A** from *A. flava* and **A** from fir were enantiomers (and must, therefore, share the same relative stereochemistry and NMR properties) was used to advantage: The abundance of **A** from fir allowed the heteronuclear correlation (HMQC) and other key NMR experiments to be run and all proton and carbon assignments to be made (summarized in Table 2). Accurate assignment of shifts was a prerequisite for further investigation of relative stereochemistry and three-dimensional structure.

Independent evidence for the himachalane carbon skeleton of *A. flava* **A** was obtained by comparing its hydrogenation products with those from α-himachalene, a natural product in which the himachalane carbon skeleton had been securely established (Joseph and Dev, 1968a,b) (Figure 4). With four asymmetric centers, saturated "himachalane" has 16 possible stereoisomers (eight pairs of enantiomers), which can be represented by up to eight discrete peaks on an achiral GC column. Hydrogenation of α-himachalene gave four GC peaks (Figure 4) because two new asymmetric centers, denoted by*, were created nonselectively, but the other two centers remained unchanged. Two of the products (at 8.24 and 8.44 min) exactly matched two products from the hydrogenation of *A. flava* **A** by GC retention times and mass spectra, which established that α-himachalene and *A. flava* **A** have the same carbon skeleton. (In fact, exactly two matching peaks were expected because only two products from **A** can have the *cis* ring junction that is present in all the products from α-himachalene). Just three hydrogenation products were observed from **A**, instead of the possible four. Either two of these have the same retention time, or one of the possible products does not form because of steric hindrance. All hydrogenation products of these compounds had nearly identical mass spectra, and only one example is shown in Figure 4. Additional minor GC peaks were seen among the hydrogenation products and were probably due to double-bond migration during the reaction.

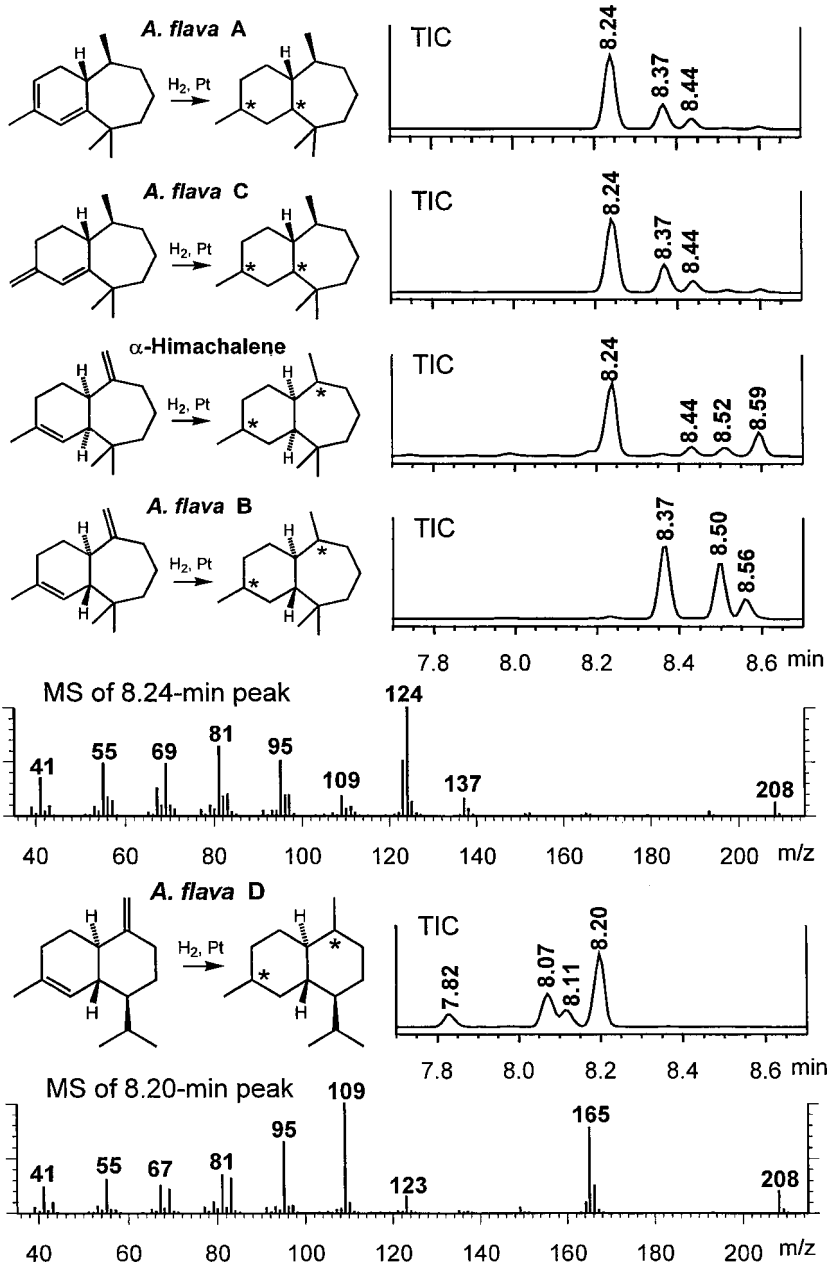
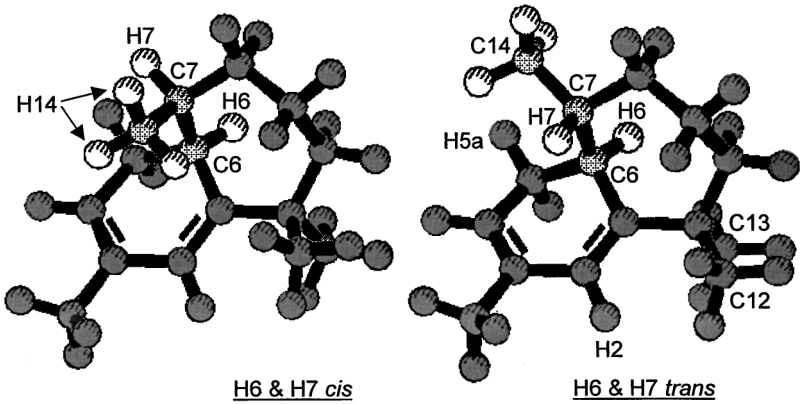


FIG. 4. Hydrogenation products from *A. flava* compounds and standard α-himachalene. Total ion chromatograms (TIC) are shown at right, and an example mass spectrum of the saturated products is shown for each carbon skeleton. Asymmetric centers created nonselectively by hydrogenation are indicated by asterisks.

Relative stereochemistry of **A** by molecular modeling and NMR:



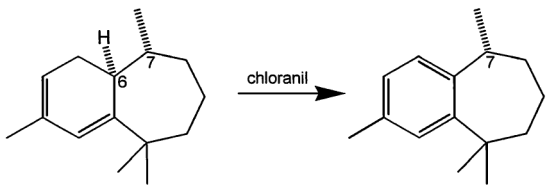
	H6 & H7 *cis*	H6 & H7 *trans*
Calculated dihedral angle H6-C6-C7-H7:	78.9 °	-162.0 °
Expected $J_{6,7}$:	~0 Hz	~9 Hz

Observed $J_{6,7}$ in **A** of 9.9 Hz supports *trans* configuration.

Correspondence between NOE data and calculated distances for *trans* isomer:

Signals	NOE	Distance (A)
Methyl 14 to H6	5%	2.72 (minimum)
Methyl 14 to H5a	8%	2.12 (minimum)
Methyl 13 to H6	7%	2.37 (minimum)
Methyl 12 to H2	17%	2.10 (minimum)
H6 to H7	2% (maximum)	3.08

Absolute configuration at C7 by conversion to known compound:



(-)-**A** from Nordmann fir (-)-7*R-ar*-himachalene

FIG. 5. Relative and absolute stereochemistry of compound **A**. Top: calculated energy-minimized structures for the possible diastereomers of **A**, along with observed NMR data and corresponding dihedral angles and distances (see text). For NOE experiments, the signal listed first was irradiated; calculated distances to methyl protons represent the minimum for the three possibilities. Bottom: Chemical conversion of **A** from Nordmann fir to compound with known absolute configuration.

*Stereochemistry of Compound **A** from A. flava.* The relative stereochemistry of **A** (whether the hydrogens at positions 6 and 7 were *cis* or *trans* to each other) was ambiguous from the literature. Mehta and Singh (1977) tentatively assigned the *cis*-H configuration to the isomer that does not match **A** from *A. flava*, based on "mechanistic considerations." Khan et al. (1989) argued that their fir compound must have the opposite relative configuration because of NMR differences, but for unknown reasons their final structure was again presented as the *cis*-H configuration (according to both their nomenclature and drawn structure). Ambiguity about the sign of rotation for the fir compound made the absolute stereochemistry uncertain also, and the entire stereochemical issue was revisited.

The three-dimensional structures for the *cis*-H and *trans*-H isomers of **A** calculated by molecular modeling are shown in Figure 5. These conformations represent the global energy minima found by the AM1 method (Dewar et al., 1985). The ring structures have remarkably similar shapes; the essential difference is that methyl group 14 is in an axial orientation in the *cis*-H isomer but in an equatorial orientation in the *trans*-H isomer. The *trans*-H structure was energetically favored by about 1 kcal/mol. The calculated dihedral angle involving H-6 and H-7 was 78.9° for the *cis*-H isomer and 162.0° for the *trans*-H isomer. From the Karplus rule (Williams and Fleming, 1980), these values would correspond to NMR coupling constants of about $J_{6,7} = 0$ Hz for the *cis*-H isomer and $J_{6,7} = 9$ Hz for the *trans*-H isomer. The observed $J_{6,7}$ for **A** from *A. flava* was 9.9 Hz, from which we conclude that **A** from *A. flava* and Nordmann fir has the *trans*-H configuration. (With the *cis*-H configuration, the large observed $J_{6,7}$ would have implied a dihedral angle close to 0°; while this conformation might occur within some seven-membered rings, the modeling calculations showed it to be energetically unfavorable in **A**.)

To further test whether the modeled *trans*-H conformation was realistic, certain calculated interatomic distances (especially those that would be strongly affected by conformation of the rings) were considered relative to the nuclear Overhauser enhancements (NOEs) observed for **A**. The NMR data supported the computed three-dimensional structure well; NOEs were detected as expected when the calculated interatomic distances were small (e.g., 2–3 Å) (Figure 5). A small NOE (<2%) was observed for H-7 when H-6 was irradiated; if these hydrogens had been *cis* to each other with a dihedral angle of ~0°, a much larger NOE would have been expected, and this further supports the assigned *trans*-H relative configuration.

The absolute stereochemistry of **A** was determined by aromatizing it to *ar*-himachalene with chloranil (Mehta and Singh, 1977) (Figure 5). The procedure preserved the configuration at carbon 7 but eliminated the asymmetric center at carbon 6; the absolute configuration of *ar*-himachalene was determined previously (Pandy and Dev, 1968), with the (+) optical rotation corresponding to the *S* configuration. Compound **A** from Nordmann fir (which was more abundantly available than that from *A. flava*) gave *ar*-himachalene (by MS, MW = 202; by GC, >99% pure) with a specific rotation of $[\alpha]_D = -2.2°$ ($c = 0.4\%$, hexane).

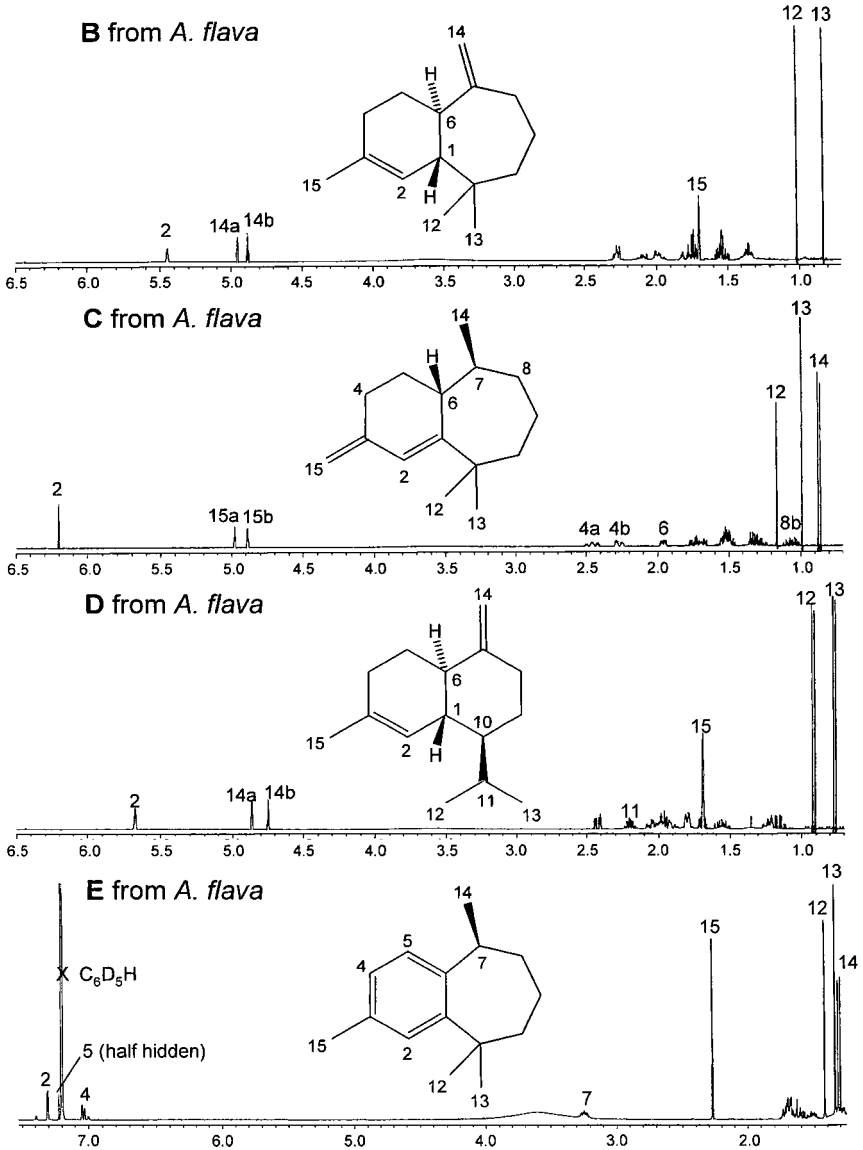


FIG. 6. Proton NMR spectra in benzene-d_6 for hydrocarbons **B–E** and final structures (stereochemistry determined by other considerations; see text).

Thus **A** from the fir samples had the 7*R* configuration, and that from *A. flava* must be 7*S*. Combining this result with the conclusion for relative stereochemistry, (+)-A from *A. flava* must have the 6*R*,7*S* configuration, and (−)-**A** from both fir species, 6*S*,7*R*. An attempt was made to check enantiomeric purity by chiral GC (Cyclodex-B column), but no separation of enantiomers was achieved. It is unknown whether other fir cultivars have different enantiomeric compositions for **A** or whether the sign of rotation reported by Khan et al. (1989) was in error.

*Compound **C** from A. flava.* As shown in Figure 4, hydrogenation of **C** gave the same products as **A**, by both GC and MS; thus, compound **C** also had the himachalane carbon skeleton. The specific rotation of **C** was $[\alpha]_D = +1.6^\circ$ ($c = 0.023\%$, hexane). The UV spectrum indicated an exocyclic conjugated diene system (maximum at 242 nm, hexane, $\epsilon = 15{,}600$). The NMR spectrum (Figure 6) had these key resonances (benzene-d_6): δ 0.87 (3H, d, $J = 6.5$), 0.99 (3H, s), 1.16 (3H, s), 4.89 (1H, br s), 4.98 (1H, br s), 6.20 (1H, s). The NMR data were consistent with a secondary methyl group, two geminal methyl groups, an exocyclic methylene, and one trisubstituted olefin in the ring system. (In $CDCl_3$, the corresponding shifts were: δ 0.95, 0.96, 1.14, 4.69, 4.74, and 5.99, respectively). These data suggested the gross structure shown in Figure 6, and Khan et al. (1989) again reported a compound from the fir species with remarkably similar NMR, UV, and mass spectra. As with **A**, analysis of Nordmann fir foliage confirmed the results of Khan et al. (1989), except with respect to enantiomeric composition.

The enantiomers of **C** were separable by GC on the chiral column. Compound **C** from *A. flava* eluted at 16.49 min and that from Nordmann fir and European silver fir eluted at 16.62 min (with nearly baseline separation). Thus, the isolates of **C** from the two sources were enantiomers, and each source appeared to be enantiomerically pure. (As little as 10% of the opposite enantiomer would have been detectable). This result was inconsistent with the optical rotation reported by Khan et al. (1989), $[\alpha]_D = +2.7^\circ$, which had the same sign as we observed for *A. flava*. Thus, for both **A** and **C**, the polarimetry data of Khan et al. (1989) are incompatible with the fir samples analyzed in this study, and their sign of rotation for **C** is discounted.

Two lines of evidence indicated that **C** and **A** had the same relative stereochemistry at the asymmetric centers. First, their hydrogenation products were exactly the same (Figure 4); *none* of the GC peaks could coincide if **C** and **A** had a diastereomeric relationship at these carbons. Second, **C** slowly isomerized into **A** in $CDCl_3$ (about 85% complete after several days at room temperature, by NMR and GC analysis); this probably was catalyzed by a trace of acid in the NMR solvent and proceeded through a cation intermediate (with the charge at C-3). This conversion would not affect asymmetric centers C-6 and C-7. The conversion did not occur in benzene. The reverse isomerization, of **A** into **C**, also occurred to a minor extent in $CDCl_3$, as shown in the Nordmann fir sample in Figure 3; by GC, the percentage of **C** in the sample before NMR was 0.3%, but this increased to

9.5% in $CDCl_3$. Khan et al. (1989) also noted the isomerization and gave a procedure for converting **C** into **A**, by heating in formic acid solution; the acid-catalyzed equilibration strongly favors **A**.

Compound **C** from Nordmann fir was shown to have the same absolute configuration at carbons 6 and 7 as **A** from Nordmann fir. The partially isomerized NMR sample of **A** from Nordmann fir (Figure 3) was analyzed on the chiral column, and the **C** derived from **A** had the same GC retention as the **C** isolated directly from the foliage (16.62 min). Both (−)-**A** and (−)-**C** from Nordmann fir have the 6*S*,7*R* configuration, and therefore, (+)-**C** from *A. flava* must be 6*R*,7*S*.

*Compound **E** from A. flava.* By GC retention time and MS, *A. flava* **E** was identical to *ar*-himachalene, the aromatization product of **A**. *ar*-Himachalene was first described from Himalayan cedar (Joseph and Dev, 1968a). The proton NMR spectrum (Figure 6) had the following shifts in benzene-d_6: δ 1.31 (3H, d, $J = 6.5$), 1.33 (3H, s), 1.42 (3H, s), 2.27 (3H, s), 3.24 (1H, m), 7.04 (1H, dd, $J = 7.9, 1.6$), 7.21 (1H, d, $J = 7.9$), and 7.31 (1H, d, $J = 1.6$). Two geminal methyls, a secondary methyl adjacent to a benzene ring, an aromatic methyl, and three aromatic protons were indicated, supporting the *ar*-himachalene structure. The corresponding shifts in $CDCl_3$ were 1.33, 1.33, 1.42, 2.31, 3.26, 6.98, 7.11, and 7.18, which were very similar to those reported by Pandy and Dev (1968) in CCl_4. The specific rotation was positive in sign but small in magnitude; thus it could not be measured accurately due to the small amount of isolated material ($[\alpha]_D < +10^\circ$, $c = 0.001\%$, hexane). As noted above, the (+) rotation corresponds to the 7*S* configuration, and **E** from *A. flava* is identical to **E** isolated from Himalayan cedar. **E** was also found in the Nordmann fir extract, but its configuration there was not established. The optical rotation was not measured, and separation of the enantiomers of **E** was not achieved on the chiral GC column.

*Compound **B** from A. flava.* Hydrogenation of **B** afforded a product that was identical in GC retention time (8.37 min) and mass spectrum to one of those from **A** (Figure 4), establishing that **B** also has the himachalane carbon skeleton. The proton NMR spectrum (Figure 6) had key resonances in benzene-d_6 at: δ 0.84 (3H, s), 1.02 (3H, s), 1.70 (3H, br s), 4.88 (1H, br s), 4.95 (1H, br s), and 5.45 (1H, br s). Like **C**, compound **B** had three methyl groups and an exocyclic methylene, but unlike **C**, one of the methyls (1.70 ppm) was olefinic. From the COSY experiment, the single olefinic ring proton (5.45 ppm) was coupled to the olefinic methyl group but not to any ring protons. An olefinic proton adjacent in the ring to a methylene group would be coupled to at least one of the methylene protons, but it would not necessarily be coupled to an adjacent methine proton. Therefore, only the double-bond locations shown for **B** in Figure 6 can be consistent with the NMR data. The corresponding proton shifts in $CDCl_3$ were 0.71, 0.96, 1.68, 4.72, 4.79, and 5.31, and there was no evidence of degradation. The specific rotation was $[\alpha]_D = +50^\circ$ ($c = 0.008\%$, hexane).

Like α-himachalene (Figure 4), the asymmetric centers of **B** are at carbons 1 and 6; however, none of the products of hydrogenation of **B** matched those from α-himachalene (the slightly different GC retention times in the last two products from each source were real and were corroborated by subtly different mass spectra). Thus, all of the hydrogenation products from **B**, and **B** itself, must have the *trans* ring junction. The NMR spectrum of **B** does not match that reported for α-himachalene, but it agrees well with that for *trans*-α-himachalene (Joseph and Dev, 1968c). These authors established that the 1*S*,6*S* configuration has a specific rotation of $[\alpha]_D = -39.5^\circ$. Because of the opposite optical rotation, **B** from *A. flava* must have the 1*R*,6*R* configuration.

*Compound **D** from A. flava.* As with **A**, **C**, and **B**, hydrogenation indicated the presence of two rings, but GC retention times and mass spectra of the products established that the ring system of **D** was very different (Figure 4). The mass spectrum of **D** (Figure 2) was much like the library spectra for γ-cadinene and α-amorphene, both of which have the cadinane carbon skeleton. The specific rotation was $[\alpha]_D = +110^\circ$ ($c = 0.016\%$, hexane). In benzene-d_6, the proton spectrum (Figure 6) had these resonances: δ 0.76 (3H, d, $J = 6.5$), 0.91 (3H, d, $J = 6.5$), 1.68 (3H, br s), 4.74 (1H, br s), 4.86 (1H, br s), and 5.68 (1H, br s). The spectrum was consistent with three methyl groups and one exocyclic methylene, and the COSY experiment confirmed that the aliphatic methyls were part of an isopropyl group. α-Amorphene was eliminated as a possibility because it has four methyl groups. The corresponding proton shifts in $CDCl_3$ were 0.74, 0.93, 1.69, 4.55, 4.66, and 5.56, and the compound was stable in this solvent.

The NMR data in $CDCl_3$ were consistent with literature data for γ-cadinene (Andersen et al., 1977; Cane et al., 1987). Citronella oil is a good source of this compound, and the compound isolated from the commercial oil matched that from *A. flava* **D** exactly by NMR, GC, and MS and agreed fairly well in optical rotation ($[\alpha]_D = +136^\circ$, $c = 0.011\%$, hexane). Carbon and DEPT data were obtained for the more abundant, botanical sample ($CDCl_3$): δ 134.76, 153.39 (0 attached hydrogens); 26.20, 44.18, 45.11, 46.89, 122.42 (1 H); 25.71, 26.52, 30.48, 36.30, 103.15 (2 H); 15.10, 21.55, 23.89 (3 H). The carbon data closely matched those of Cane et al. (1987). Cane et al. (1987) assigned the 1*S*,6*S*,10*R* configuration to the (−) form they encountered; therefore, the (+)-**D** from *A. flava* has the 1*R*,6*R*,10*S* configuration.

*Compounds **F** and **G** from A. flava.* The key to identification of polar compounds **F** and **G** was that both decomposed significantly (up to 50%) when the GC inlet was set to 250°C. HPLC-purified **F** and **G** both produced **A** and **C** (in similar amounts, recognized by mass spectrum and GC retention time). Dehydration of an alcohol was the likely mechanism of decomposition, which would not affect the ring system. Thus **F** and **G** must also have the himachalane carbon skeleton. The specific rotation of **F** was $[\alpha]_D = +72^\circ$ ($c = 0.028\%$, hexane), and that for **G** was $[\alpha]_D = +83^\circ$ ($c = 0.030\%$, hexane). The proton

NMR spectrum of **F** (in benzene-d_6) had these features: δ 0.89 (3H, *d*, $J = 6.7$), 0.96 (3H, s), 1.12 (3H, s), 1.34 (3H, s), and 5.45 (1H, s) (Figure 7). The corresponding NMR data for **G** were: δ 0.91 (3H, d, $J = 6.7$), 0.94 (3H, s), 1.15 (3H, s), 1.26 (3H, s), and 5.41 (1H, s). The data for both compounds support the presence of a hydroxyl group on carbon 3, both with respect to the shift for methyl group 15 (1.34 or 1.26 for **F** and **G**, respectively) and the appearance of this signal (a singlet). An epimeric relationship between **F** and **G** could account for their slightly different spectral and chromatographic properties. **F** and **G** decomposed within hours to **A** and **C** when placed in $CDCl_3$, but there was enough time to obtain the proton shifts (signals in the same order as for

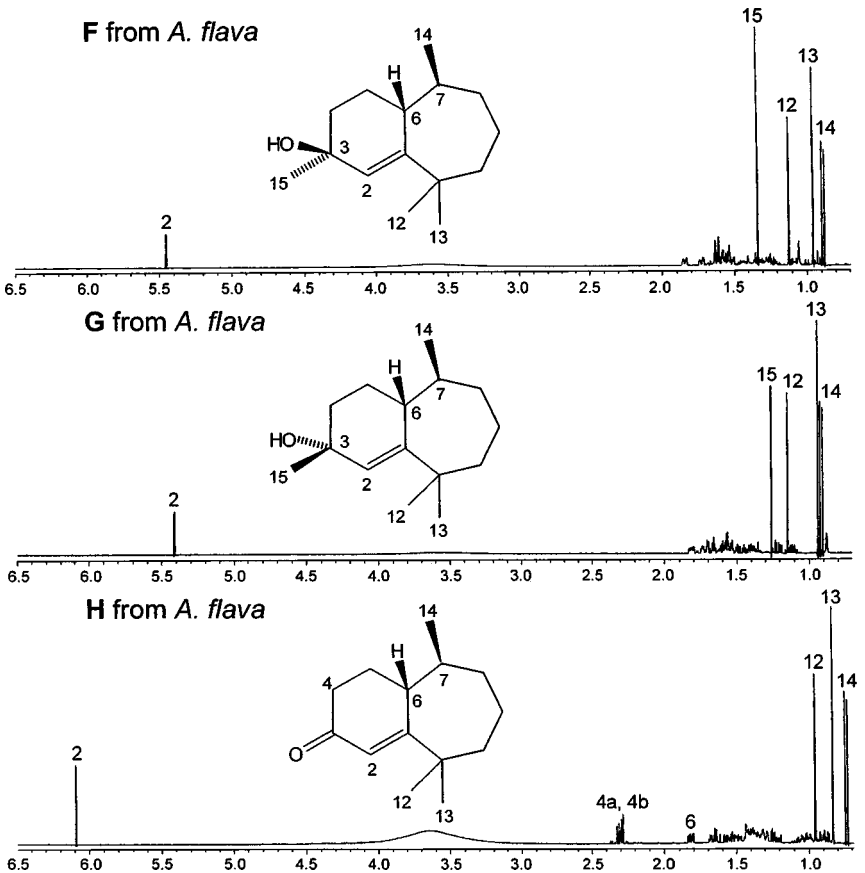


FIG. 7. Proton NMR spectra in benzene-d_6 for polar compounds **F–H** and final structures (stereochemistry determined by other considerations; see text).

benzene-d_6): 0.95, 0.93, 1.10, 1.27, and 5.41 for **F** and 0.97, 0.91, 1.10, 1.23, and 5.33 for **G**.

As tertiary allylic alcohols, **F** and **G** are especially prone to dehydration, but this reaction would not affect the stereochemistry at carbons 6 and 7. When pure **F** or pure **G** was injected onto the chiral GC column, the resulting **C** was only the earlier eluting enantiomer. Thus, (+)-**F** and (+)-**G** both have the same absolute configuration at these two centers as (+)-**A** and (+)-**C** from *A. flava* (6*R*,7*S*). Tentative assignment of configuration at carbon 3 was based on molecular modeling and the NMR shift of methyl group 15. By molecular modeling, methyl group 15 has an axial orientation in one of the epimers and equatorial in the other, and in the axial epimer, methyl group 15 and hydrogen 6 are on the same side of the six-membered ring (i.e., configuration at carbon 3 is *R*). In **G**, the relatively upfield methyl shift (1.26 ppm) would correspond to the axial orientation (3*R*,6*R*,7*S*), while the shift of 1.34 ppm observed for **F** would correspond to the equatorial orientation (3*S*,6*R*,7*S*). The consequent, relatively equatorial orientation of the hydroxyl group in **G** would make it more accessible to adsorbants during liquid chromatography and could explain its longer retention than **F** on the silica HPLC column.

*Compound **H** from A. flava.* Enough **H** was isolated (17 μg) so that a proton NMR spectrum could be acquired (Figure 7). The key resonances (in benzene-d_6) were: δ 0.71 (3H, d, $J = 6.6$), 0.81 (3H, s), 0.93 (3H, s), 6.06 (1H, s), suggesting two geminal methyl groups, a secondary methyl group, and a single olefinic proton. The spectrum of **H** looked much like those of **C**, **F**, and **G**, except that carbon 15 was lacking. A keto group on carbon 3 could account for the number of observed signals, their splittings, and the relatively downfield shifts in the six-membered ring. The UV spectrum had a maximum at 233 nm, which was consistent with a conjugated enone ($\epsilon = 27{,}500$). The micro-Wittig methylenation converted **H** into **C**, by GC retention time and mass spectrum, which established the overall ring structure and the relative stereochemistry at carbons 6 and 7. Injection of this sample onto the chiral GC column gave just one peak for **C**, at the earlier of the possible retention times (16.49 min). Therefore, **H** has the same 6*R*,7*S* configuration as **A**, **C**, **F**, and **G** from *A. flava*. Optical rotation was not measured.

Compound Configurations in Other Beetle Species. By GC on the chiral column, compound **C** from *P. cruciferae, A. cyparissiae, A. czwalinae,* and *A. flava* all have the same absolute configuration. The same is true for **F** and **G** from all *Aphthona* species, by chiral GC analysis of the **C** produced in the hot inlet. Furthermore, **A** from these species has the same configuration as **A** from *A. flava* because purification ($AgNO_3$ HPLC), partial isomerization to **C**, then chiral GC analysis gave only the earlier GC peak for **C**. Compounds **B** and **D** from these species have the same chiral GC retention times as **B** and **D** from *A. flava* (15.50 and 17.92 min, respectively). However, authentic (−) enantiomers of

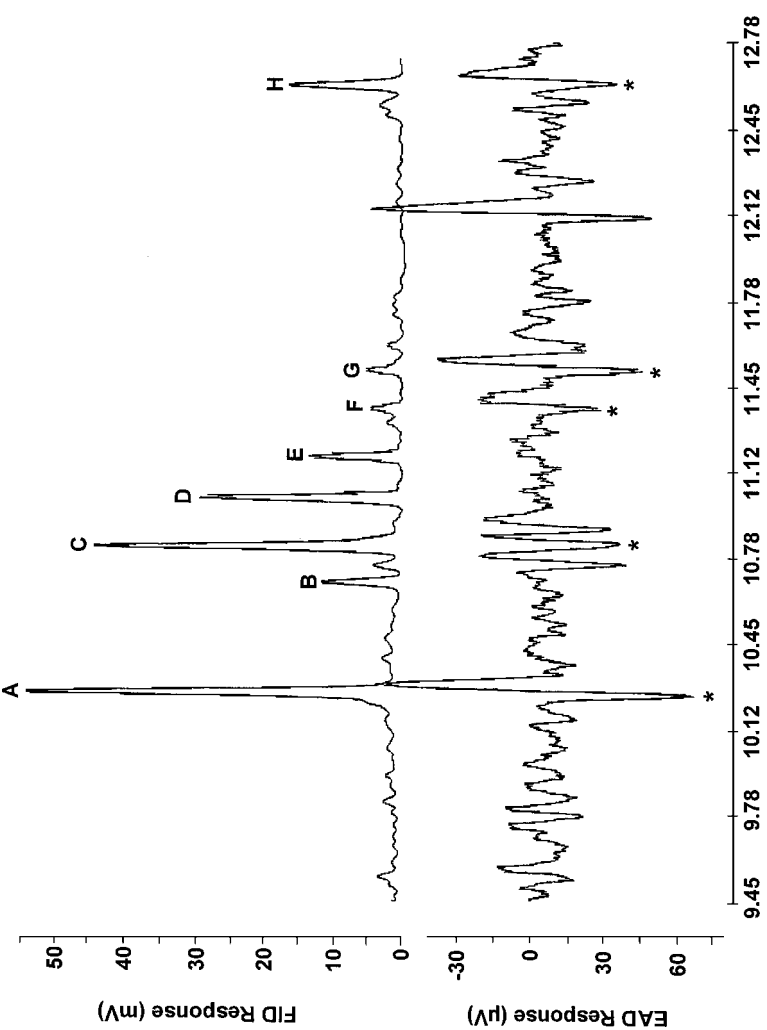


FIG. 8. Simultaneously recorded gas chromatogram (top) and electroantennogram responses (bottom) of typical female *A. flava* antenna to volatiles collection on Super Q from male *A. flava* feeding on leafy spurge. Responses to male-specific compounds are denoted by asterisks.

these compounds were not available for comparison; thus it is unknown whether the enantiomers of **B** and **D** are separable on this column. Differences among the species were not demonstrated with respect to absolute configuration of **B** and **D**, but they cannot be ruled out; there was insufficient material for optical rotation measurements. Configurations of **E** and **H** in the other species remain unstudied.

Electrophysiological Activity. In GC-EAD analyses of volatiles from male *A. flava*, both male and female *A. flava* antennae were stimulated by five of the male-specific compounds (Figure 8). Compounds **A**, **C**, **F**, **G**, and **H** consistently showed EAD activity, whereas compounds **B**, **D**, and **E** showed no EAD activity on at least 10 antennal preparations. Responses did not occur at any of these retention times to volatiles collected from females. In addition to male-specific material, several spurge-specific compounds activated male and female antennae. Initial GC-EAD analyses of compound **A** from *A. flava* with antennae of *A. czwalinae*, *A. cyparissiae*, and *P. cruciferae* indicated that the biological activity of compound **A** is a common feature among the tested flea beetle species. A comprehensive comparative electrophysiological study on male-specific flea beetle compounds is anticipated in the future.

Conclusions and Further Research. Peng et al. (1999) concluded that males of *P. cruciferae* emit an aggregation pheromone that attracts both sexes when the males are feeding on host material, based on behavioral data. The present study demonstrated chemical and electrophysiological patterns that are consistent with this model. First, blends of relatively abundant, male-specific compounds were detected and identified in *P. cruciferae* and also in three other flea beetle species. (Curiously, *P. cruciferae* and the three *Aphthona* species shared many of the same compounds, and ultimately, it was the abundance of the compounds in *A. flava* that made identification of the fairly complicated structures feasible.) Second, the blends had species-specific proportions, which could serve to allow individuals to locate others of their own species even in mixed populations. Third, male-specific compounds were readily sensed by the antennae of both sexes, as would be expected for a pheromone. Clearly, these patterns do not prove a pheromonal function, but the present identification and the anticipated synthesis of these male-specific compounds will allow definitive biological experiments to be run. The function of these compounds in nature may or may not involve attraction, but the existence of such blends is intriguing in any case and invites explanation.

The compounds themselves include several new himachalene sesquiterpenes and new enantiomers of previously identified ones. Only one of the compounds, (+)-γ-cadinene, is known to occur rather widely in nature (in plants). One would not expect compounds used as pheromone components to be abundant in the environment, and those from the flea beetles are generally not. The use of compounds such as himachalenes as pheromone components would not be unprecedented; a homosequiterpene with the himachalene ring system has been previously identified as a pheromone in the sandfly *Lutzomyia longipalpis* (Hamilton et al., 1996).

Acknowledgments—We are grateful to the following individuals for supplying beetles: Dr. Julie Soroka and Dr. Margie Gruber of Agriculture Canada, Saskatoon, Saskatchewan, Canada (*P. cruciferae*); Dr. C. Peng, North Dakota State University, Fargo, North Dakota, USA (*P. cruciferae*); Dr. Anthony Caesar, USDA-ARS-NPARL, Sidney, Montana, USA (*Aphthona* species and leafy spurge plants); and Dr. Robert Nowierski, Montana State University, Bozeman, Montana, USA (*Aphthona* species). Mr. Harold Miller of Harold Miller Landscapes, Jefferson, Oreon, USA, kindly sent us 10 kg of Nordmann fir foliage. Ms. Michele Giovannini grew the leafy spurge plants in the NCAUR greenhouse.

REFERENCES

ANDERSEN, N. H., BISSONETTE, P., LIU, C.-B., SHUNK, B., OTHA, Y., TSENG, C.-L. W., MOORE, A., and HUNECK, S. 1977. Sesquiterpenes of nine European liverworts from the genera, *Anastrepta, Bazzania, Jungermannia, Lepisozia*, and *Scapania*. *Phytochemistry* 16:1731–1751.

ARNETT, R. H., JR. 1993. American Insects: A Handbook of the Insects of America North of Mexico. Sandhill Crane Press, Gainesville, Florida.

BORROR, D. J. and DELONG, D. M. 1964. An Introduction to the Study of Insects, rev. ed. Holt, Rinehart, and Winston, New York.

BURKHARDT, G., REICHLING, J., MARTIN, R., and BECKER, H. 1986. Terpene hydrocarbons in *Pimpinella anisum* L. *Pharm. Weekbl. Sci. Ed.* 8:190–193.

CANE, D. E., RAWLINGS, B. J., and YANG, C.-C. 1987. Isolation of $(-)$-γ-cadinene and aristolochene from *Aspergillus terreus. J. Antibiot.* 9:1331–1334.

COSSÉ, A. A. and BARTELT, R. J. 2000. Male-produced aggregation pheromone of *Colopterus truncatus*: Structure, electrophysiological and behavioral activity. *J. Chem. Ecol.* 26:1735–1748.

DEWAR, M. J. S., ZOEBISCH, E. G., HEALEY, E. F., and STEWART, J. J. P. 1985. AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* 107:3902–3909.

GASSMANN, A., SCHROEDER, D., MAW, E., and SOMMER, G. 1996. Biology, ecology, and host specificity of European *Aphthona* spp. (Coleoptera, Chrysomelidae) used as biocontrol agents for leafy spurge, *Euphorbia esula* (Euphorbiaceae), in North America. *Biol. Control* 6:105–113.

HAMILTON, J. G. C., DAWSON, G. W., and PICKETT, J. A. 1996. 3-Methyl-α-himachalene: Proposed structure for novel homosesquiterpene sex pheromone of *Lutzomyia longipalpis* (Diptera: Psychodidae) from Jacobina, Brazil. *J. Chem. Ecol.* 22:2331–2340.

HEATH, R. R. and SONNET, P. E. 1980. Technique for in situ coating of Ag^+ onto silica gel in HPLC columns for the separation of geometrical isomers. *J. Liq. Chromatogr.* 3:1129–1135.

JOSEPH, T. C. and DEV, S. 1968a. Studies in sesquiterpenes. XXIX. Structure of himachalenes. *Tetrahedron* 24:3809–3827.

JOSEPH, T. C. and DEV, S. 1968b. Studies in sesquiterpenes. XXXI. The absolute stereochemistry of himachalenes. *Tetrahedron* 24:3841–3852.

JOSEPH, T. C. and DEV, S. 1968c. Studies in sesquiterpenes. XXXII. Structure of himachalene monohydrochloride and the preparation of *trans*-himachalenes. *Tetrahedron* 24:3853–3859.

KHAN, V. A. and PENTEGOVA, V. A. 1988. Volatile components of the oleoresin of *Abies alba. Chem. Nat. Comp.* 1988:162–166.

KHAN, V. A., TKACHEV, A. V., and PENTEGOVA, V. A. 1989. New sesquiterpenoids from the oleoresin of *Abies alba. Chem. Nat. Comp.* 1989:606–611.

LAMB, R. J. 1989. Entomology of oilseed *Brassica* crops. *Annu. Rev. Entomol.* 34:211–229.

LESAGE, L. and PAQUIN, P. 1996. Identification keys for *Aphthona* flea beetles (Coleoptera: Chrysomelidae) introduced in Canada for the control of spurge (*Euphorbia* spp, Euphorbiaceae). *Can. Entomol.* 128:593–603.

McDANIEL, B., CLAY, S. H., and SCHOLES, C. 1992. Morphology of three imported *Aphthona* flea beetles used as biological control agents of leafy spurge. Publication TB 98, Agricultural Experiment Station, South Dakota State University, Brookings, South Dakota.

MEHTA, G. and SINGH, B. P. 1977. Terpenes and related systems. 16. Fate of representative bicyclic sesquiterpenes in strong acid medium: A general rearrangement of hydroazulene sesquiterpenes to decalin types. *J. Org. Chem.* 42:632–638.

PANDY, R. C. and DEV, S. 1968. Studies in sesquiterpenes. XXX. Synthesis of *ar*-himachalene and himachalanes. *Tetrahedron* 24:3829–3839.

PENG, C. and WEISS, M. J. 1992. Evidence of an aggregation pheromone in the flea beetle, *Phyllotreta cruciferae* (Goeze) (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 18:875–884.

PENG, C., BARTELT, R. J., and WEISS, M. J. 1999. Male crucifer flea beetles produce an aggregation pheromone. *Physiol. Entomol.* 24:98–99.

SONNET, P. E. 1974. *cis*-Olefins from the Wittig reaction. *Org. Prep. Proc. Int.* 6:269–273.

VIG, O. P., CHUGH, O. P., and MATTA, K. L. 1970. Terpenoids LIII. Synthesis of (±)-γ-cadinene. *Indian J. Chem.* 8:29–32.

WILEY. 1995, Wiley. Specral Library, 6th ed. [CD-ROM]. John Wiley & Sons, New York.

WILLIAMS, M. A., and FLEMING, I. 1980. Spectroscopic Methods in Organic Chemistry, 3rd ed. McGraw-Hill, London.

ZHANG, Z.-Q., and McEVOY, P. B. 1994. Attraction of *Longitarsus jacobaeae* males to cues associated with conspecific females (Coleoptera: Chrysomelidae). *Environ. Entomol.* 23:732–737.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

DOSE-DEPENDENT RESPONSE AND PRELIMINARY OBSERVATIONS ON ATTRACTION RANGE OF *Ips typographus* TO PHEROMONES AT LOW RELEASE RATES

ANNE J. FRANKLIN[1,2,3,*] and JEAN-CLAUDE GRÉGOIRE[1,4]

[1]*Laboratoire de Biologie Animale et Cellulaire*
Université Libre de Bruxelles
CP 160/12, 50 av. F.D. Roosevelt, 1050 Brussels, Belgium

[2]*Centre Luxembourgeois de l'U.L.B*
19 rue de la Fontaine, 6870 Saint-Hubert, Belgium

(Received March 1, 2001; accepted August 10, 2001)

Abstract—Responses of the European spruce bark beetle, *I. typographus* (Coleoptera: Scolytidae), to low release-rate pheromones were investigated in two experiments in a spruce forest at Wellin, southern Belgium. Dose–response of the beetle was first examined in a trapping experiment in August 1999. The major pheromone components of *I. typographus*, *(S)-cis*-verbenol (cV) and 2-methyl-3-buten-2-ol (MB) were released from window traps. Five treatments were replicated five times: (1) blank trap, (2) 0.03 and 1.2, (3) 0.11 and 4.5, (4) 0.34 and 15.2 mg/day of cV and MB, respectively, and (5) Pheroprax. Trap catches increased linearly as a function of increasing release rates of cV and MB. A second study aimed at making preliminary observations on the attraction range of the pheromones as compared to Pheroprax. A release–recapture experiment was carried out in May 2000: four trap-trees located 50 m away from a central release platform were equipped with window traps baited with increasing release rates, tested in separate releases: 0.03 and 1.2; 0.11 and 4.5; 0.34 and 15.2 mg/day of cV and MB, respectively, and Pheroprax. Recaptures increased with the release rate. Trap catches were different at lower release rates, but not between the release rate of 15.2 mg/day of MB and Pheroprax. Variations in captures in relation to wind parameters showed persistently random flight at the lower release rates. Significant upwind flight was observed for Pheroprax only. These results imply that trap interference existed for Pheroprax and suggest that its attraction range may be greater than 50 m.

[3]Supported by Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture.
[4]Supported by Fonds National de la Recherche Scientifique.
*To whom correspondence should be addressed at Royal Belgian Institute of Natural Sciences, Invertebrates Department, Rue Vautier 29, 1000 Brussels, Belgium. E-mail: Anne.Franklin@kbinirsnb.be

0098-0331/01/1200-2425/0 © 2001 Plenum Publishing Corporation

Key Words—*Ips typographus*, Scolytidae, Coleoptera, release rates, dose-response, flight dispersal, Pheroprax®.

INTRODUCTION

Since the development of commercial attractants, pheromone trapping is regularly used to monitor populations of *Ips typographus*, one of the most aggressive and damaging pests of Norway spruce in Europe (Bakke, 1985; Weslien, 1992). This technique is also frequently used in mark–recapture experiments to assess the beetle's dispersal capacities (e.g., Botterweg, 1982; Weslien and Lindelöw, 1990; Zumr, 1992; Duelli et al., 1997; Byers, 1999).

Most dispersal studies have relied on commercial pheromones originally developed for mass trapping, such as Pheroprax, that have high release rates to ensure an efficient attraction of the beetles. However, one of the primary goals of recapturing marked animals is to obtain a good estimate of their flight ability. If traps close to the release point exhibit a strong attraction, most beetles primed to respond to pheromones will end up in these traps and it will not be possible to conclude much about dispersal (Turchin, 1998). Even for species requiring flight exercise, as might be the case for *I. typographus* (Duelli et al., 1997), some individuals might respond to the attractive source when they would have flown further under different conditions.

Our approach to study *I. typographus* dispersal was to recreate a flight environment as natural as possible, using live, standing trap-trees in place of traps, and low release-rate pheromones lures instead of commercial attractants (Franklin et al., 2000). In this paper, we present the design of the lures and an evaluation of their effectiveness in the field.

We first investigated the response of *I. typographus* to several release rates and compared it to Pheroprax. We then studied trap interference for these release rates, in order to make a preliminary evaluation of their attraction range as compared to that of Pheroprax. The range of attraction of pheromone lures is the maximum distance over which insects can be shown to direct their movement to the source (Wall and Perry, 1987). Most studies have been done on Lepidoptera (Wall and Perry, 1987; Elkinton and Cardé, 1988) and some on bark beetles (Schlyter, 1992) or diprionids (Wedding et al., 1995). Probably the most elaborate study on bark beetles was carried out by Turchin and Odendaal (1996), who investigated the effective sampling area of traps capturing newly emerged southern pine beetles (*Dendroctonus frontalis*).

This knowledge is crucial for the development of reliable mark–recapture experiments, but existing information for *I. typographus* is scarce and estimates vary greatly due to the few data available.

METHODS AND MATERIALS

Dispensers and Release Rates. Releases of methyl-3-buten-2-ol (MB) and *(S)-cis*-verbenol (cV) were obtained with semipermeable polyethylene (PE) bags containing the semiochemicals. Each dispenser consisted of two bags, one for MB and one for cV. Ipsdienol was not used, as it did not prove essential for *I. typographus* capture (Schlyter et al., 1987 a,b). The dispensers were built using PE sheets of different thickness sealed into 4×4 cm^2 bags, containing either 200 μl of MB impregnated on 1×1-cm^2 absorbent tissue paper protected from direct sun light by aluminum foil, or 5 mg of cV crystals. Chemicals, sources, PE thickness, and release rates are listed in Table 1. Release rates were determined from weight loss in a wind tunnel, at 0.05 m/sec and $20 \pm 2^\circ$C and calculated as the slope ($\pm$ its 95% confidence limits) of the regression of weight loss versus time for the time period when this relation was linear (i.e., when the release rates were constant) (Table 1).

Dose-Dependent Responses. Experiments with varying release rates were carried out in spruce and beech stands at Wellin, southern Belgium, in August 1999. No trees attacked by *I. typographus* were detected before, during, or after the experiments. There were five treatments, each replicated five times: (1) blank trap, (2) 0.03 and 1.2, (3) 0.11 and 4.5, (4) 0.34 and 15.2 mg/day of cV and MB, respectively, (5) Pheroprax dispenser (35.8 mg/day of the blend). The 15.2 mg/day release

TABLE 1. DISPENSERS TESTED FOR 2-METHYL-3-BUTEN-2-OL (MB) AND (*S*)-*cis*-VERBENOL (CV) AND ESTIMATED RELEASE RATES DURING THEIR CONSTANT RELEASE PHASE AT $20 \pm 2^\circ$C; 200 μL OF MB OR 5 MG OF CV PER DISPENSER[a]

Compound	Source	Chemical purity (%)	PE weight (g/cm^2)	Days with constant release rate (N)	Release rate $\pm$ 95% CI (mg/day)
2-Methyl-3-buten-2-ol	Aldrich	98	2.2	7	11.2 ± 1.0
			4.4	7	8.4 ± 0.7
			7.8	18+	7.6 ± 0.4
			15.6	18+	4.5 ± 1.6
			23.4	18+	3.2 ± 0.9
			31.2	18+	1.2 ± 0.2
(*S*)-*cis*-Verbenol	Aldrich	95	2.2	5	0.43 ± 0.07
			4.4	5	0.34 ± 0.05
			7.8	18+	0.29 ± 0.07
			15.6	18+	0.11 ± 0.03
			23.4	18+	0.04 ± 0.01
			31.2	18+	0.03 ± 0.003
Pheroprax	Cyanamid Agrar			18+	35.8 ± 3.6

[a] $N = 10$ replicates, except for 2.2 and 4.4 g/cm^2 where $N = 20$ and for Pheroprax where $N = 5$.

rate for MB was obtained by using two dispensers each releasing 7.6 mg/day. Ratios of about 40:1 in the release rates of MB and cV were used in order to approach the ideal ratio of 50:1 mentioned by Schlyter et al. (1987c).

Each replication consisted of a line of five live, standing trap-trees spaced 75 m apart, equipped with small transparent PVC barrier traps (13 cm wide × 24 cm high, ending in a funnel connected to a collecting jar) attached to the tree trunks at breast height. MB and cV dispensers were fixed on the traps 10 cm above the collecting funnel. The trees had previously been sprayed with a pyrethroid insecticide (Ripcord 40: 400 g cypermethrin/liter, S.A. Belgian Shell, 25 ml/10 liters water) up to a height of 6 m (Franklin et al., 2000). Each replication was at least 100 m from all others. Traps were emptied, and treatments were rerandomized within each replication on seven occasions between July 24, and September 10, 1999.

Attraction Range. A simple way to establish the attraction range is to study trap interference, i.e., a lowering of catch per trap when traps baited with synthetic pheromones are placed close to each other compared to widely spaced traps (Schlyter, 1992). Another method is to compare catches with high and low release rates in a given trapping grid. We chose this latter approach for a preliminary trial, which could give insight on the range of distances to be tested subsequently in an experimental design involving several trap spacings. A release–recapture experiment was performed at Wellin in May 2000. The setup consisted of four trap-trees located in the four cardinal directions, 50 m away from a central release platform. The distance between two adjacent trap-trees was 71 m. The trapping method was identical to the one described for the dose–response experiment.

Four release rates were tested, each replicated five times, except for the lower release rate, which was replicated four times only due to adverse weather conditions (0.03 and 1.2; 0.11 and 4.5; 0.34 and 15.2 mg/day of cV and MB, respectively, and Pheroprax). Each replicate was carried out as follows: freshly emerging beetles were collected from emergence tents (Lindelöw and Weslien, 1986), counted, marked with fluorescent powder, and allowed to fly off from the release platform. After 45 min, releases were stopped and nonflyers were counted. Fifteen minutes later, traps were emptied and the number of beetles caught was recorded. Short release–recapture periods were chosen in order to maximize the number of beetles caught on their outward dispersing flight and to minimize captures of beetles returning after a long flight as can be expected from simulation models (Byers, 2000). Such release–recapture experiments, in which insects are recovered quickly, were found to provide better attraction range estimates (Wall and Perry, 1987). Short periods were also used in order to have weather conditions as constant as possible during a given replicate.

Several releases were performed during one afternoon (12:00–17:30 PM). A period of 30 min was allowed to elapse between each replicate, and the barrier traps were changed for each treatment in order to prevent the accumulation

of pheromones on the traps and in the vicinity of the trap-trees. Different colors of fluorescent powder were used to differentiate between release rates. Release rates were randomized during the afternoon so as to test the different rates at different times of the day.

Wind direction was assessed with a compass and a thread allowed to flutter freely in the wind. Wind speed was measured with a hand-held anemometer (Testovent 4200, Testoterm S.A.). Both parameters were measured at the start and end of the 60-min flight period; we took five recordings during an interval of 5 min, and an average was then calculated.

Statistical analysis of circular data was carried out with Oriana v. 1.06 software (Kovach Computing Services, Pentraeth, UK). The Rayleigh test (Zar, 1996) was used to determine whether wind direction and catches of marked beetles in the four traps were random or directed. Watson's *F*-test (Zar, 1996) was then used to compare the beetle's flight direction to the wind direction, to determine if their mean angles differed significantly.

RESULTS

Dispensers and Release Rates. Within the range studied, release rates of our PE bag dispensers decreased exponentially as a function of PE thickness (Table 1): for MB, $y = 12.9\mathrm{e}^{-0.07x}$ ($R^2 = 0.97$, $P < 0.001$) and for cV, $y = 0.54e^{-0.10x}$ ($R^2 = 0.97$, $P < 0.001$) with y corresponding to the release rate in milligrams per day and x to the PE thickness in grams per square centimeter. This decrease differed for the two components, as shown by the comparison of the slopes of the regressions on log transformed data ($t\ \mathrm{obs}_{8df} = 2.86$; $P = 0.02$).

During our tests, the release rate of Pheroprax averaged 35.8 mg/day for the blend after the 18-day test period (Table 1). This is comparable to results of Jakus and Simko (2000), who estimated the release rate of the same Pheroprax dispenser at about 27 mg/day after leaving dispensers in the field for seven weeks.

Dose-Dependent Responses. A total of 582 *I. typographus* were caught throughout the season—about 64% with Pheroprax. Trap catches increased with increasing release rates (Figure 1) from an average of 0.2 captures for blank traps to an average of 93 captures for traps baited with Pheroprax.

Analysis of variance on log-transformed counts showed differences between catches at different release rates ($F_{4,16df} = 63.6$, $P < 0.001$); post hoc analyses indicated that Pheroprax caught more beetle's than the other release rates ($P < 0.01$, Figure 1). When regression analysis was used to compare *I. typographus* catches for our PE bag dispensers, excluding Pheroprax as it had different formulation and conditioning, it showed a linear response with increasing release rates (beetle catches as a function of MB release, slope = 1.69, $R^2 = 0.39$, $P = 0.003$).

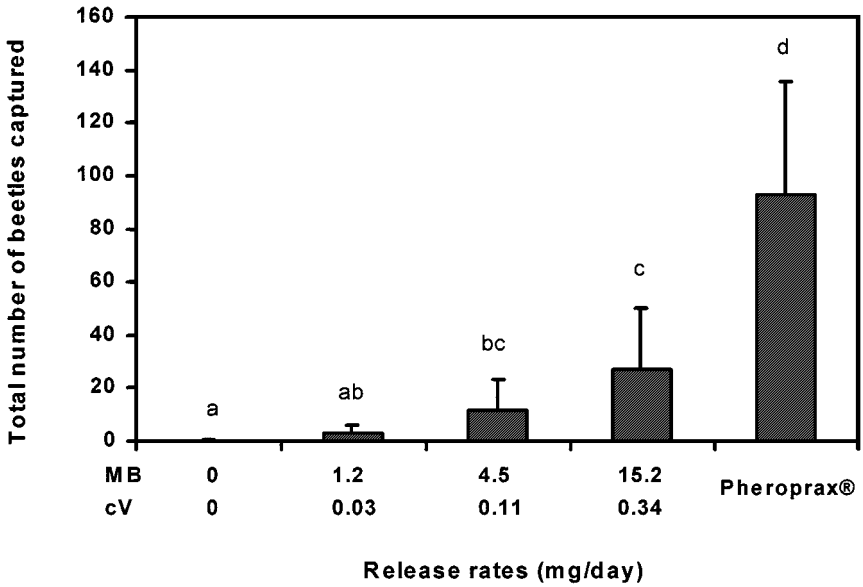


FIG. 1. Mean number of *Ips typographus* caught at different release rates of MB and cV, expressed as MB release rates (±SE). $N = 35$ replications. Pheroprax has a release rate of 35.8 mg/day. Means followed by the same letter are not significantly different, Student Newman-Keuls multiple-range test, $P = 0.01$.

Attraction Range. Results of the release–recapture experiment are shown in Table 2. Recapture of marked beetles increased with the release rate, from an average of 0.2% with the lowest release rate to 4.4% with Pheroprax. Increases in catches tended to stabilize at the two higher release rates. An analysis of variance on arcsine square-root-transformed data ($F_{3,15df} = 24.4$, $P < 0.001$) followed by post hoc tests indicated differences between the release rates, except between 15.2 mg/day of MB and Pheroprax (Student-Neuman-Keuls multiple range test, $P = 0.01$).

Capture patterns of unmarked beetles increased in a similar fashion (Table 2). Differences among average captures rates were significant ($F_{3,15df} = 11.6$, $P < 0.001$) and post hoc tests showed that captures at the two lower release rates differed, while differences were not significant between release rates of 4.5 mg/day and 15.2 mg/day of MB, and between 15.2 mg/day of MB and Pheroprax (Student-Neuman-Keuls test, $P = 0.01$).

Variations in captures in relation to wind parameters are shown in Table 3. Wind speeds and directions were averaged from measures at the start and end of the 60-min flight period. There was no correlation between wind speeds and the occurrence of wind directions ($r = 0.4$, $P = 0.07$). The beetles showed undirected

TABLE 2. *I. typographus* RELEASE–RECAPTURE DATA FOR ATTRACTION RANGE EXPERIMENT[a]

MB release rate (mg/day)	No. Marked *N*	No. flying *N*	Captured (*N*)		Recaptured %
			Marked	Unmarked	
1.2	700	423	0	1	0.0
1.2	700	648	1	8	0.2
1.2	450	443	2	3	0.5
1.2	450	345	1	6	0.3
Total	2300	1859	4	18a	0.2a
4.5	800	702	10	42	1.4
4.5	700	683	18	22	2.6
4.5	400	367	6	8	1.6
4.5	450	410	4	11	1.0
4.5	450	420	5	24	1.2
Total	2800	2582	43	107b	1.6b
15.2	800	763	33	34	4.3
15.2	600	533	13	15	2.4
15.2	400	374	11	34	2.9
15.2	500	453	7	18	1.5
15.2	320	255	11	39	4.3
Total	2620	2378	75	140bc	3.2c
Pheroprax	900	796	46	44	5.8
Pheroprax	900	661	19	35	2.9
Pheroprax	400	377	19	38	5.0
Pheroprax	450	395	18	40	4.6
Pheroprax	400	358	11	44	3.1
Total	3050	2587	113	201c	4.4c

[a] Totals followed by the same letter indicate that mean catches are not significantly different (ANOVA, followed by a Student-Newman-Keuls multiple-range test, $P = 0.01$).

flight during most replicates, whereas the wind direction was significant on 15 of 19 occasions. At the lower pheromone release rates, *I. typographus* flights were always random, even when the wind direction was significant. Upwind flights were observed for Pheroprax replicates only (Table 3), but significant downwind flights were not observed.

DISCUSSION

The making of our own pheromone dispensers provided us with low-release-rate lures, for which we could control the release rates of each component individually. However, there were two shortcomings. First, release rates over several orders of magnitude proved impractical due to the limitation of the dispenser size and to the available PE thickness. Second, for higher release rates, the release of

TABLE 3. FLIGHT DIRECTION OF *I. typographus* IN RELATION TO WIND PARAMETERS DURING RELEASE–RECAPTURE EXPERIMENT AT WELLIN (MAY 2000)[a]

MB release rate (mg/day)	Number of catches in cardinal directions (N, E, S, W)	Flight direction $\theta b \pm 95\%$ CI[b]	Wind direction $\theta w \pm 95\%$ CI[b]	Upwind flight? θb vs. θw[c]
1.2	0, 0, 0, 0	None	$283^\circ \pm 33^\circ$	—
1.2	1, 0, 0, 0	Random	$331^\circ \pm 11^\circ$	—
1.2	0, 1, 0, 1	Random	$057^\circ \pm 38^\circ$	—
1.2	0, 0, 0, 1	Random	$027^\circ \pm 33^\circ$	—
4.5	2, 2, 3, 3	Random	Random	—
4.5	5, 1, 5, 7	Random	$261^\circ \pm 30^\circ$	—
4.5	1, 2, 3, 0	Random	$050^\circ \pm 35^\circ$	—
4.5	2, 0, 1, 1	Random	Random	—
4.5	2, 2, 0, 1	Random	$085^\circ \pm 37^\circ$	—
15.2	11, 8, 3, 11	Random	$232^\circ \pm 13^\circ$	—
15.2	6, 2, 1, 4	Random	Random	—
15.2	4, 0, 1, 6	$297^\circ \pm 36^\circ$	$214^\circ \pm 19^\circ$	No
15.2	3, 0, 0, 4	$307^\circ \pm 44^\circ$	Random	—
15.2	6, 1, 2, 2	Random	$249^\circ \pm 26^\circ$	—
Pheroprax	9, 10, 18, 9	Random	$268^\circ \pm 26^\circ$	—
Pheroprax	6, 2, 2, 9	$300^\circ \pm 41^\circ$	$247^\circ \pm 18^\circ$	Yes
Pheroprax	7, 8, 2, 2	$050^\circ \pm 42^\circ$	$349^\circ \pm 39^\circ$	Yes
Pheroprax	9, 0, 1, 8	$315^\circ \pm 26^\circ$	$004^\circ \pm 36^\circ$	Yes
Pheroprax	5, 2, 2, 2	Random	$043^\circ \pm 35^\circ$	—

[a] Average wind speeds ranged from 0 to 0.3 m/sec.
[b] Random = random distribution as determined by the Rayleigh test ($P > 0.05$).
[c] Comparisons made using Watson's F test. Null hypothesis: mean flight directions are similar. No = no upwind flight ($P \leq 0.05$), Yes = upwind flight ($P > 0.05$).

the semiochemicals was constant over short time periods only five and seven days for cV and MB, respectively). This might not prove too restrictive for release–recapture studies for which daily trap collections are made, as dispensers can then be renewed regularly.

Results of our dose–response experiment follow those of Schlyter (1987c), who observed a linear increase in catches as a function of increasing release rates of cV and MB. If we consider *I. typographus* catches for the PE bag dispensers, we also obtain such a linear response with increasing release rates. This linear increase in beetle response—as opposed to the logarithmic relationship commonly found in pheromone studies—appears to be due to MB, which has an unusually steep dose–response curve in EAGs and field tests and which is the most critical pheromone component in determining the total number of beetles caught (Dickens, 1981; Schlyter, 1987b,c).

Experiments and observations providing reasonable evidence of the range of attraction of insect species to pheromones are not common. The best and most accurate estimates of the attraction range are found to require either direct observation of insects in flight or carefully timed release–recapture experiments in which insects are recovered very quickly (Wall and Perry, 1987). Such experiments for *I. typographus* were performed by Helland et al. (1984). Data were analyzed in terms of attraction radius by Schlyter (1992). In two separate trials, Helland et al. (1984) released *I. typographus* 12 and 20 m away from a central pheromone trap equipped with Ipslure, a commercial attractant similar to Pheroprax (50 mg/day of MB and 1 mg/day of cV) (Schlyter, 1987a,c). They recaptured 60.1% of the beetles within the first 10 min for the 12-m release distance but only 19.1% of those released 20 m away. From these results, Schlyter (1992) estimated the attraction range of the pheromone trap to be between 12 and 20 m, probably closer to 12 m, because the majority of the responding population was caught from this distance.

Our preliminary observations suggest that this attraction range might have been underestimated. In our second experiment, trap catches were not significantly different between the release rate of 15.2 mg/day and Pheroprax (35.8 mg/day of the blend), a fact that was observed both for marked and unmarked beetles. Moreover, if traps are interacting and result in overlapping pheromone plumes, it would be expected that most beetles would be caught in the upwind traps as a consequence of "overshooting" (Wedding et al., 1995). This was indeed observed for Pheroprax, as three of the five replicates showed significant captures upwind. At lower release rates, flight was mostly random (11 of 13 replicates), with no upwind flight for the two significant flight directions.

These results imply that traps interfered for Pheroprax at the distance studied, i.e., 50 m between the trap-trees and the release point. They also suggest that the attraction range of Pheroprax probably extended to 50 m, at least in direction of the wind. Further experiments are needed to confirm our results, but these are in line with data from Schlyter (1992). Estimations of the attraction range of *I. typographus* from regression analyses of trap interactions showed ranges of 17–67 m for lures releasing 0.24–240 mg/day of MB. Variability was extremely high, however, and confidence intervals overlapped for all estimates due to the few data points available.

This variation in experimental results shows that, in order to achieve even a crude estimate of the range of attraction of pheromones, it will be necessary to carry out many observations or experiments. Even so, our preliminary data suggest that the attraction range of commercial pheromones might be higher than is generally thought. If proved correct, this challenges the validity of earlier release–recapture studies in terms of their interpretation of *I. typographus* dispersal capacities. In most experiments, the first trap ring was close to the release platform: traps equipped either with Pheroprax or Ipslure were placed as close as 5 m

(Duelli et al., 1997), 30 m (Zolubas and Byers, 1995) and 50 m (Botterweg, 1982; Zumr 1992). Under these conditions, migrating beetles necessarily had to cross pheromone plumes and might have been primed to respond to the pheromones, while they would have dispersed further in the absence of such pheromones. Absolute dispersal distances might, therefore, need to be reinterpreted, as well as dispersal models, which assumed an exponential or power decrease in recaptures as a function of distance (e.g., Weslien and Lindelöw, 1990; Zolubas and Byers, 1995; Duelli et al., 1997). Ideally, a compromise should be found between the need to recapture enough marked beetles for statistical analysis and the study of the beetles' flight ability and variability in response to pheromones.

Acknowledgments—The authors thank MM. J. Gillissen and M. Wuidar for allowing them to carry out the experiments in the Wellin forest district. Funding for this work was provided by a F.R.I.A. PhD research grant to Anne Franklin and by the F.N.R.S. to J.-C. Grégoire. A. Franklin also thanks Dr. J. Byers and Dr. F. Schlyter for their technical advice on making pheromone dispensers.

REFERENCES

BAKKE, A. 1985. Deploying pheromone-baited traps for monitoring *Ips typographus* populations. *Z. Angew. Entomol*. 99:33–39.

BOTTERWEG, P. F. 1982. Dispersal and flight behaviour of the spruce bark beetle *Ips typographus* in relation to sex, size and fat content. *Z. Angew. Entomol*. 94:466–489.

BYERS, J. A. 1999. Effects of attraction radius and flight paths on catch of scolytid beetles dispersing outward through rings of pheromone traps. *J. Chem. Ecol*. 25:985–1005.

BYERS, J. A. 2000. Wind-aided dispersal of simulated bark beetles flying through forests. *Ecol. Model*. 125:231–243.

DICKENS, J. C. 1981. Behavioural and electrophysiological responses of the bark beetle *Ips typographus*, to potential pheromone components. *Physiol. Entomol*. 6:251–261.

DUELLI, P., ZAHRADNIK, P., KNIZEK, M., and KALINOVA, B. 1997. Migration in spruce bark beetles (*Ips typographus*) and the efficiency of pheromone traps. *J. Appl. Entomol*. 121:297–304.

ELKINTON, J. S. and CARDÉ, R.T. 1988. Effects of intertrap distance and wind direction on the interaction of gypsy moth (Lepidoptera: Lymantriidae) pheromone-baited traps. *Environ. Entomol*. 17:764–769.

FRANKLIN, A. J., DEBRUYNE, C., and GRÉGOIRE, J.-C. 2000. Capture of *Ips typographus* with attractants of low release rates: Localized distribution and environmental influences. *Agric. For. Entomol*. 2:259–270.

HELLAND, I. S., HOFF, J. M., and ANDERBRANT, O. 1984. Attraction of bark beetles (Col., Scolytidae) to a pheromone trap. Experiment and mathematical models. *J. Chem. Ecol*. 10:723–752.

JAKUS, R. and SIMKO, J. 2000. The use of dispensers with different release rates at pheromone trap barriers for *Ips typographus*. *Anz. Schald. Pflanz. Umwelt*. 73:33–36.

LINDELÖLW, W. A. and WESLIEN, J. 1986. Sex-specific emergence of *Ips typographus* L. (Col., Scolytidae) and flight behaviour in response to pheromone sources following hibernation. *Can. Entomol*. 118:59–67.

SCHLYTER, F. 1992. Sampling range, attraction range, and effective attraction radius: Estimates of trap efficiency and communication distance in coleopteran pheromone and host attractant systems. *J. Appl. Entomol*. 114:439–454.

SCHLYTER, F., BIRGERSSON, G., BYERS, J. A., LÖFQVIST, J., and BERGSTRÖM, G. 1987a. Field response of spruce bark beetle, *Ips typographus*, to aggregation pheromone candidates. *J. Chem. Ecol.* 13:701–716.

SCHLYTER, F., LÖFQVIST, J., and BYERS, J. A. 1987b. Behavioural sequence in the attraction of the bark beetle *Ips typographus* to pheromone sources. *Physiol. Entomol.* 12:185–196.

SCHLYTER, F., BYERS, J. A., and LÖFQVIST, J. 1987c. Attraction to pheromone sources of different quantity, quality, and spacing: Density-regulation mechanisms in bark beetle *Ips typographus*. *J. Chem. Ecol.* 13:1503–1523.

TURCHIN, P. 1998. *Quantitative Analysis of Movement. Measuring and Modeling Population Redistribution in Animals and Plants.* Sinauer Associates, Sunderland, Massachusetts.

TURCHIN, P., and ODENDAAL, F .J. 1996. Measuring the effective sampling area of a pheromone trap for monitoring population density of southern pine beetle (Col., Scolytidae). *Environ. Entomol.* 25:582–588.

WALL, C. and PERRY, J. N. 1987. Range of action of moth sex-attractant sources. *Entomol. Exp. Appl.* 44:5–14.

WEDDING, R., ANDERBRANT, O., and JÖNSSON, P. 1995. Influence of wind conditions and intertrap spacing on pheromone trap catches of male European pine sawfly, *Neodiprion sertifer Entomol. Exp. Appl.* 77:223–232.

WESLIEN, J. 1992. Monitoring *Ips typographus* (L.) populations and forecasting damage. *J. Appl. Entomol.* 114:338–340.

WESLIEN, J. and LINDELÖW, A. 1990. Recapture of marked spruce bark beetles (*Ips typographus*) in pheromone traps using area-wide mass trapping. *Can. J. For. Res.* 20:1786–1790.

ZOLUBAS, P. and BYERS, J. A. 1995. Recapture of dispersing bark beetle *Ips typographus* in pheromone baited traps: Regression models. *J. Appl. Entomol.* 119: 285–289.

ZAR, J. H. 1996. *Biostatistical Analysis*, 3 ed. Prentice Hall International, London, United Kingdom.

ZUMR, V. 1992. Dispersal of the spruce bark beetle *Ips typographus* (L) (Col, Scolytidae) in spruce woods. *J. Appl. Entomol.* 114:348–352.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

BEHAVIORAL AND CHEMICAL ANALYSIS OF VENOM GLAND SECRETION OF QUEENS OF THE ANT *Solenopsis geminata*

LEOPOLDO CRUZ-LÓPEZ,[1,*] JULIO C. ROJAS,[1]
RICARDO DE LA CRUZ-CORDERO,[1] and E. DAVID MORGAN[2]

[1]*El Colegio de la Frontera Sur*
Carretera Antiguo Aeropuerto Km. 2.5
Tapachula, Chiapas, México, C.P 30700, AP 36

[2]*Chemical Ecology Group*
Lennard-Jones Laboratory
School of Chemistry and Physics
Keele University, Staffs., ST5 5BG, England

(Received May 26, 2000; accepted August 10, 2001)

Abstract—Bioassays in a Y-tube olfactometer showed that workers of *Solenopsis geminata* (Hymenoptera: Formicidae) were attracted to venom gland extracts of queens. Gas chromatography coupled mass spectrometry analysis of individual glands of queens of *S. geminata* showed that the secretion is composed mainly of a large amount of 2-alkyl-6-methylpiperidine alkaloids and a tiny amount of a δ-lactone and a α-pyrone, which have been earlier identified as components of the queen attractant pheromone of *Solenopsis invicta* Buren. However, additional small amounts of a mixture of sesquiterpenes and pentadecene were found. The possible function of the sesquiterpenoid compounds is discussed.

Key Words—*Solenopsis geminata*, fire ants, venom secretion, sesquiterpenes, queen pheromone recognition.

INTRODUCTION

Solenopsis geminata (F.) (Hymenoptera: Formicidae) is a species that was originally distributed from the southern United States to northern South America, but due to commerce has been introduced into both Asia and Africa (Trager, 1991). In Central America, *S. geminata* is considered a sorghum and maize seed pest (Bhatkap, 1983; del Rio and Casares, 1990). On the other hand, Mackay et al.

*To whom correspondence should be addressed. E-mail: lcruz@tap-ecosur.edu.mx

0098-0331/01/1200-2437/0 © 2001 Plenum Publishing Corporation

(1990) suggested that this ant species could act as an important agent of biological control of pests. For example *S. geminata* is able to decrease the population of *Spodoptera frugiperda* (Smith) larvae, *Agrotis* sp., and *Listronotus* sp. (Lastres, 1990) in corn plantations. Workers of this myrmicine also feed on eggs, nymphs, and adults of the blood sucking bug, *Triatoma gerstaeckeri* (Stål) (Camacho and Garcia, 1981). In spite of its apparent importance, relatively few studies have been carried out on the biology and ecology of these ants. In this contribution we consider one aspect of their biology: the chemical communication between the queen and workers.

Chemical communication between ant queens and their workers has been recognized in several species of ants including *Formica consocians* (Wheeler, 1910), *Lasius alienus* (Foerster), *Pheidole pallidula* (Nylander) (Stumper, 1956), *Neivamyrmex* spp., *Labidus* spp. (Watkins and Cole, 1966), and *Myrmica rubra* Latreille (Brian, 1973), but the most studied species has been *Solenopsis invicta* Buren. Jouvenaz et al. (1974) observed that workers of *S. invicta* and *S. geminata* were attracted to areas of blotting paper to which queens of their species had been confined. Further, Glancey (1980) showed that volatiles released by the queens and extracts of queens were highly attractive to workers. Vander Meer et al. (1980) reported the venom gland of *S. invicta* was the storage site for the attractive pheromone. Later, Rocca et al. (1983a,b) identified the chemical structures of the compounds responsible for activity in *S. invicta* as (*E*)-6-(1-pentenyl)-2*H*-pyran- 2-one and tetrahydro-3,5-dimethyl-6-(1-methylbutyl)-2*H*-pyran-2-one (invictolide). Dihydroactinidiolide was also identified, but no biological activity has been yet described for this compound.

It was previously demonstrated that queens of *S. geminata* behave like those of *S. invicta* in strongly attracting their workers (Jouvenaz et al., 1974; Fierro-Santos, 1995). However, the chemical composition of the pheromone in *S. geminata* remains unknown. This work was undertaken to evaluate the response of *S. geminata* workers to venom gland extract of queens, dealated virgin queens, alate queens captured in nuptial flights, alate virgin queens, and workers and to identify the compounds present in the venom gland secretion of *S. geminata* queens. Additionally, the essential oil of *Melaleuca cajuputi*, which contains two related compounds identified in the venom gland secretions of the queen of *S. geminata*, was tested for attraction to workers.

METHODS AND MATERIALS

Insects. Colonies of *S. geminata* were excavated around Tapachula, Chiapas, Mexico, and transported to El Colegio de la Frontera Sur. The queens, dealated virgin queens, alate virgin queens, and alate queens captured in nuptial flight were placed individually in small plastic bottles (10×6 cm); the

rest of the colony was maintained in plastic bowls (30 × 50 cm), and which were used as nests. The colonies were maintained in the laboratory at 25 ± 3°C and 70 ± 5% reletive humidity, under 8–10 hr of light/day, and fed on dead insects and honey-water.

Gland Dissection. Individual queens, dealated virgin queens, alate virgin queens, alate queens captured in nuptial flights, and workers were cooled in a refrigerator, following which the venom gland together with the sting was dissected under distilled water. The Dufour's gland and sting were removed. Individual venom glands were either solvent extracted in a small tissue grinder with hexane (100 μl) or carefully dropped into thin-walled soda-glass tubes (1.8 mm ID, 20 mm long), previously sealed at one end, and the open end was then sealed in a micro flame (Morgan, 1990).

Chemical Analysis. Gas chromatography–mass spectrometry was performed with a Varian Star 3400 CX gas chromatograph linked to a Varian Saturn 4D mass spectrometer. The samples were analyzed by using a fused silica column (30 m × 0.25 mm ID) coated with poly (5% diphenyl–95% dimethylsiloxane). The over temperature was programmed from 50°C to 250°C at 15°C/min. Helium was used as carrier gas. The injector port temperature was held at 200°C, and the sample was heated in the injector for 4 min before crushing the capillary, as described by Morgan and Wadhams (1972).

Olfactometer Bioassay. Venom gland extracts of queens, dealate virgin queen, alate virgins, alate queens captured in nuptial flight, and workers were tested for worker attraction in a Y-tube olfactometer as described by Vander Meer et al. (1988). The olfactometer consisted of two 24/40 ground glass joints sealed to one of the arms of a 5-cm Y tube so that 1 cm of each Y tube arm extended through half the ground joints. Extracts were placed in one of the choice chambers. All extracts were applied to filter paper strips (3 cm × 1 cm) and evaluated at a concentration of one gland equivalent. The other choice chamber was left blank. Pure hexane was used as blank. Compressed air was split into two streams, each stream being independently controlled by flowmeters. Airflow was regulated at 0.2 liters/min into each choice chamber. Approximately 50–70 ants from laboratory colonies were confined in a 2.5-cm piece of 0.9-cm-ID Tygon tubing sealed at one end with wire gauze. The open end of the tubing was attached to the entrance stem. The initial choice of the first 20 ants that walked down the entrance tube and into one of the arms of the Y tube was recorded. Ants that were not trapped in a choice chamber and came back to the entrance stem were not counted if they made another choice. Test samples were retested with worker ants from the same colony, but the choice chamber in which the sample control were placed was reversed. This procedure eliminated any bias that was inherent in the individual choice chambers. A complete replicate was the sum of the results from the two tests. The worker response to the extract and control was compared by using a *t* test. The level of probability considered significant was $P < 0.05$. The response of

28 individual workers to the queen gland secretion extracts was bioassayed as described above.

RESULTS

Results of the Y-tube olfactometer bioassays are shown in Table 1. Venom gland extract of queens, dealated virgin queens, alate queens captured in nuptial flights, and alate virgin queens elicited a signficant response for *S. geminata* workers. The response of workers to extract of worker gland secretion was not different from the response to control. When the response of individual workers to the queen venom extract was tested, a significant response was observed. Of the 28 workers tested, only four did not respond.

Analysis by GC-MS of individual samples of excised venom glands of *S. geminata* queens revealed that the secretion is composed mainly of 2-methyl-6-alkylpiperidines. The secretion is largely dominated by *cis*-2-methyl-6-undecylpiperidine, which comprises 55% of the secretion (Figure 1). The alkaloid components were identified from the mass spectra provided by Brand et al. (1972). The secretion of the queens also contained a small amount of 11 terpenoid compounds with apparent molecular ions at m/z 204 and m/z 218, suggesting molecular formulas of $C_{15}H_{24}$ and $C_{15}H_{22}O$. Pentadecene was also found in small amounts (Figure 1). The first terpenoid eluting, present in the smallest quantity, had a mass spectrum close to that of elemene (Adams, 1989). The other two, more abundant and in a concentration ratio of 1:2, had mass spectra close to β-selinene and α-selinene, respectively, as given in Adams (1989). We compared the spectra and

TABLE 1. RESPONSE OF *Solenopsis geminata* WORKERS TO VENOM GLAND EXTRACT OF QUEENS AND TO ESSENTIAL OIL OF *Melaleuca cajuputi*[a]

Extract source	Workers attracted to [N mean[a] ($\pm$ SD)]	
	Extract	Control
Queen (1 QE)	20.2 ± 5.8	$5.2 \pm 2.8^{***}$
Dealated virgin queen (1 QE)	18.4 ± 2.19	$5.6 \pm 2.9^{***}$
Queen captured in nuptial flight (1 QE)	20.4 ± 5.4	$5.8 \pm 3.9^{***}$
Alated virgin queen (1 QE)	17.4 ± 6.1	$5.4 \pm 4.0^{***}$
Queen (1 QE)	16.4 ± 5.3	$4.4 \pm 6.2^{***b}$
Worker (1 WE)	9.6 ± 7.5	7.0 ± 4.0 NS
Melaleuca cajuputi essential oil	14.8 ± 5.1	11 ± 2.9 NS

[a] Average of five tests. QE = Queen equivalent, WE = worker equivalent. NS = no significant difference; $^{***}P < 0.001$ (t test).
[b] Pure hexane as blank.

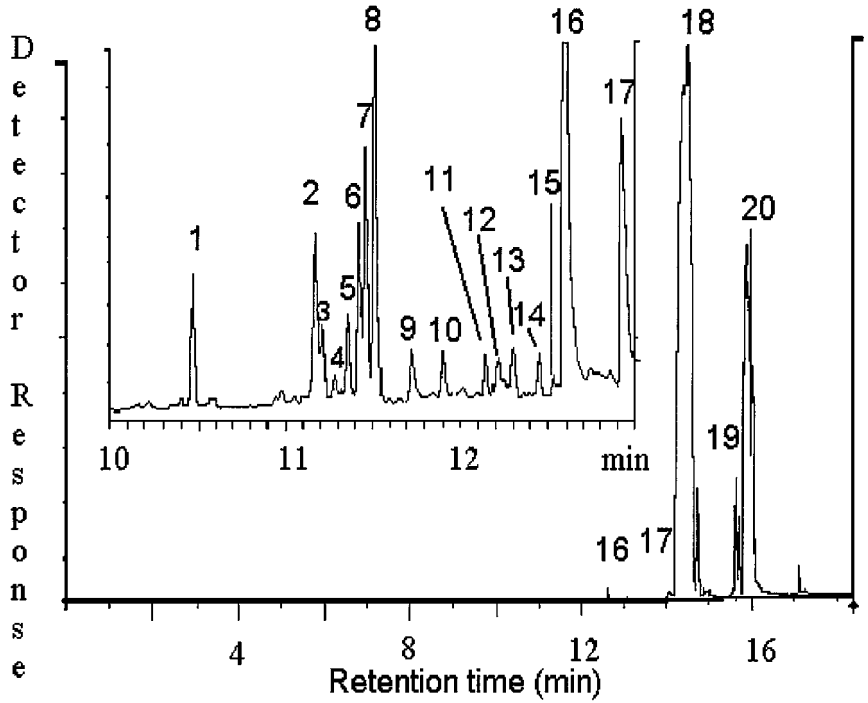


FIG. 1. Gas chromatogram of the venom gland secretion of *Solenopsis geminata* queen. The inset is an expansion for the region of 10–13 minutes. Numbers above the peaks correspond to the following substances: 1, elemene; 2, pentadecene; 3 and 4, unidentified sesquiterpenes; 5, tetrahydro-3,5-dimethyl-6-(1-methylbutyl)-2*H*-pyran-2-one (isomer); 6, tetrahydro-3,5-dimethyl-6-(1-methylbutyl)-2*H*-pyran-2-one (isomer); 7, β-selinene (isomer); 8, α-selinene (isomer); 9, (*E*)-6-(1-pentenyl)-2*H*-pyran-2-one; 10–15, unidentified sesquiterpenes; 16 and 17, 2-methyl-6-alkylpiperidines; 18, *cis*-2-methyl-6-undecylpiperidine; 19 and 20, 2-methyl-6-alkylpiperidines.

retention times of these with authentic α- and β-selinene contained in the essential oil of *M. cajuputi* (provided by J. Brophy). There were small differences in the intensities of some ions between the spectra of the *M. cajupiti* essential oil and the venom compounds, and we concluded there must be small differences in their structures. In testing the workers responses, the essential oil showed no activity (Table 1).

The two remaining compounds (peaks 6 and 9 in Figure 1) had mass spectra identical to two of the three components of the queen recognition pheromone of *S. invicta* (Rocca et al., 1983a,b): (*E*)-6-(1-pentenyl)-2*H*-pyran-2-one and tetrahydro-3,5-dimethyl-6-(1-methylbutyl)-2*H*-pyran-2-one (invictolide). One queen's venom gland contained approximately 1–5 ng of both of these compounds, but the

proportions varied considerably from one individual to another. The identifications were confirmed by injecting pure samples of the two compounds (supplied by R. K. Vander Meer). Dihydroactinidiolide, the inactive compound of *S. invicta*, was not detected in *S. geminata*. The same compounds were found in the venom gland contents of dealated virgin queens, alated queens captured in nuptial flights, and alated virgin queens, but not in workers.

DISCUSSION

Fierro-Santos (1995) demonstrated that workers of *S. geminata* were attracted to venom glands extracts of their queens. By using an Y-tube olfactometer, we confirmed those results. We also found that the venom gland extract of virgin alate queens was attractive to the workers. The suspected effect of trail laying by the first exploring ant was eliminated in assays considering the first ants, since individual workers showed a positive response to the queen venom gland extract.

In *S. invicta*, it was found previously that virgin alate queens did not attract workers (Glancey et al., 1981). The difference between species may be due to the fact that the process of production of the pheromone is faster in *S. geminata* than in *S. invicta*. However, other factors may be involved. For example, the degree of maturity of queens before leaving the nests may vary during the year and so may the onset of pheromone production. In *S. invicta*, it has been found that if queens are reared in the spring or summer, there seems to be a high probability that they will leave the nest for a mating flight before fully mature oocytes are present in their ovarioles, but if they overwinter in the parental nest, their ovaries continue to develop slowly and they begin to oviposit before they leave in early spring (Fletcher and Blum, 1983). The fundamental processes of dealation and oogenesis are usually prevented from occurring while virgin queens are still in the parental nest by the presence of one or more primer pheromones secreted by the mother queen (Willer and Fletcher, 1986). In *S. invicta*, it has been shown that immediately after the nuptial flight, queens are weakly attractive (Glancey et al., 1981).

We found that the venom gland extract of virgin delated queens of *S. geminata* was attractive to the workers. A similar phenomenon had been reported in *S. invicta* (Glancey et al., 1981). These authors found that alate queens of *S. invicta* begin to produce pheromone between six and nine days after removal of the wings, reaching their maximum activity 12 days later. A correlation between attractiveness and histolysis of wing muscles, and the presence of large number of eggs was found in this species (Glancey et al., 1981). The production of pheromone may, therefore, be linked to the biochemical, physiological, or hormonal changes associated with histolysis of wing muscles and production of eggs (Glancey et al., 1981). In other

ant species, it has been shown that virgin queens are not attractive to the workers, independent of the presence or absence of wings. For example, Carr (1962) found that virgin queens of the genus *Myrmica* were unattractive to workers whether they were alates or dealates.

By analyzing individual venom gland of *S. geminata* with the solid sampling technique (Morgan, 1990), we were able to identify (*E*)-6-(1-pentenyl)-2*H*-pyran-2-one and tetrahydro-3,5-dimethyl-6-(1-methylbutyl)-2*H*-pyran-2-one (invictolide), which have been identified as components of the queen recognition pheromone of *S. invicta*. However, we did not find dihydroactinidiolide, which is the third component of the *S. invicta* queen pheromone. Additionally, we report here for first time a mixture of 11 sesquiterpene compounds as components of the venom secretion of the queen *S. geminata*. The two more abundant sesquiterpene components had similar mass spectra of α- and β-selinene, respectively, but when they were compared with the mass spectra of the authentic selinenes from the oil of *M. cajuputi*, they showed small differences in the intensities some ions. Thus, we considered them as isomers of α- and β-selinene. In order to know whether these tentatively identified selinene isomers had any biological activity, the essential oil was tested with workers in the same way the extracts of venom glands were tested, but the ants did not exhibit any behavioral response. Sesquiterpenes have been identified from other *Solenopsis* species and their pheromonal activities documented (Vander Meer et al., 1988). The same mixture of compounds was found in the venom gland contents of dealated virgin queen and alated queen captured in nuptial flight and alated virgin queens. These results might explain why workers were attracted to the venom gland extract of virgin alated queens.

On the other hand, Jouvenaz et al. (1974) reported that *S. invicta* and *S. geminata* workers aggregated within squares on absorbent paper on which their queens had been confined. Bioassays showed that *S. invicta* workers were more attracted to squares that had held conspecific queens, but species specificity was also noted as *S. invicta* workers were attracted to areas previously occupied either by their own or *S. geminata* queens, but *S. geminata* workers were attracted only to areas occupied by queens of their own species. In field tests, Glancey et al. (1984) observed that *S. geminata*, *S. xyloni*, and *S. richteri* did not respond either to live queen extracts or synthetic compounds of *S. invicta*. Our results, and the observations obtained by Jouvenaz et al. (1974) and Glancey et al. (1984), suggest several possible explanations. The compounds responsible for attraction of *S. geminata* worker ants to their queens may be the same as those of *S. invicta*, but they are present in different proportions. The venom of *S. invicta* may contain a repellent compound (e.g., dihydroactinidiolide) for *S. geminata* workers and *S. invicta* may not detect the absence of that compound in *S. geminata*.

We know now the reason that workers of *S. invicta* are attracted to the queens of *S. geminata* is because the queen of *S. geminata* contains two components

of their queen pheromone. We suggest that a mixture of the two components of the queen pheromone of *S. invicta* plus a mixture of sesquiterpenes may act as queen pheromone of *S. geminata*. We also conclude that this mixture of volatiles is produced exclusively in the queen's venom gland, since no evidence was found of these compounds in the venom gland of workers. Further experiments are needed to explain to function of the sesquiterpene compounds.

Acknowledgments—We thank J. J. Brophy of the University of New South Wales for the gift of *M. cajuputi* oil; R. K. Vander Meer of U.S. Department of Agriculture, Gainesville, Florida, for the gift of (*E*)-6-(1-pentenyl)-2*H*-pyran-2-one, tetrahydro-3,5-dimethyl-6-(1-methyl)-2*H*-pyran-2-one, and dihydroactinidiolide; and Armando Virgen, Antonio Santiesteban, and Alvaro García Ballinas for technical assistance. L.C.L. thanks CONACYT for the award of a research studentship during which this work was begun.

REFERENCES

ADAMS, R. P. 1989. Identification of Essential Oils by Ion Trap Mass Spectroscopy. Academic Press, San Diego, California.

BHATKAP, A. P. 1983. Supresión natural de las plagas del frijol (*Phaseolus vulgaris* L.) en el sistema agrohidráulico de Bancales, pp 78–79, *in* Proceeding, XVIII Congreso Nacional de Entomología. Sociedad Mexicana de Entomología, México.

BRAND, J. M., BLUM, M. S, FALES, H. M., and MACCONNELL, J. G. 1972. Fire ant venoms: Comparative analysis of alkaloid components. *Toxicon* 10:259–271.

BRIAN, M. V. 1973. Queen recognition by brood-rearing workers of the ant *Myrmica rubra* L. *Anim. Behav.* 21:691–698.

CAMACHO, T. R. and GARCIA, P. J. 1981. Observaciones sobre la depredación de *Triatoma gerstaeckeri* (Hemiptera: Reduviidae) por *Solenopsis geminata* (Hymenoptera: Formicidae) en condiciones de laboratorio, pp. 179–180, *in* Proceedings, XXVI Congreso Nacional de Entomología, Veracruz, México.

CARR, C. A. H. 1962. Further studies on the influence of the queen ants of the genus *Myrmica*. *Insectes Soc*. 9:197–211.

DEL RIO, L. E. and CASARES, O. 1990. Identificación de factores de mortalidad en frijol de primera postrera en Honduras. Publicaciones Manejo Integrado de Plagas de Honduras.

FIERROS-SANTOS, F. 1995. Conducta de la hormiga de fuego *Solenopsis geminata* (Fabricius) (Hymenoptera: Formicidae) a extractos de glándulas de veneno en condiciones de laboratorio. Thesis. Universidad Nacional Autonoma de México, 74 pp.

FLETCHER, D. J. C. and BLUM, M. S. 1983. The inhibitory pheromone of queens fire ants: Effects of disinhibition on dealation and oviposition by virgin queens. *J. Comp. Physiol.* 153:467–475.

GLANCEY, B. M. 1980. Biological studies of the queen pheromone of the red imported fire ant, pp. 149–154, *in* Proceedings, Tall Timbers Conference on Ecology, Animal Control and Habitat Manage, Tallahassee. Florida.

GLANCEY, B. M., GLOVER, A., and LOFGREN, C. S. 1981. Pheromone production by virgin queens of *Solenopsis invicta* Buren. *Sociobiology* 6:119–127.

GLANCEY, B. M., ROCCA, J., LOFGREN, C. F., and TUMLINSON, J. H. 1984. Field tests with synthetic components of the queen recognition pheromone of the red imported fire ant *Solenopsis invicta* Buren. *Sociobiology* 9:19–30.

JOUVENAZ, D. P., BANKS, W. A., and LOFGREN, C. S. 1974. Fire ants: Attraction of workers to queen secretions. *Ann. Entomol. Soc. Am.* 67:442–444.

LASTRES, M. L. 1990. The role of two predators, *Duro taeniatum* and *Solenopsis geminata* F., as control agents of *Spodoptera frugiperda* in Honduras. M S thesis. Texas A&M University, College Station, Texas, 297 pp.

MACKAY, W. P., MACKAY, E. E., and VINSON, S. B. 1990. La biología de *Solenopsis invicta* (Hymenoptera: Formicidae). *Fol. Entomol. Mex.* 78:209–240.

MORGAN, E. D. 1990. Preparation of small-scale samples of insects for chromatography. *Anal. Chim. Acta* 236:227–235.

MORGAN, E. D. and WADHAMS, L. J. 1972. Gas chromatography of volatile compounds in small samples of biological material. *J. Chromatogr. Sci.* 10:528–529.

ROCCA, J. R., TUMLINSON, J. H., GLANCEY, B. M., and LOFGREN, C. S. 1983a. The queen recognition pheromone of *Solenopsis invicta*: Preparation of (*E*)-6-1(1-pentyl)-2*H*-pyran-2 one. *Tetrahedron Lett.* 24:1889–1892.

ROCCA, J. R., TUMLINSON, J. H., GLANCEY, B. M., and LOFGREN, C. S. 1983b. Synthesis and stereochemistry of tetrahydro-3,5-dimethyl-6(1-methylbutyl)-2*H*-pyran-2-one, a component of the queen recognition pheromone of *Solenopsis invicta*. *Tetrahedron Lett*. 24:1893–1896.

STUMPER, R. 1956. Etudes myrmécologiques LXXVII. Les sécretions attractives des reines des fourmis. *Mitt. Schweiz. Entomol. Ges.* 29:373–380.

TRAGER, J. C. 1991. A revision of the fire ants *Solenopsis geminata* group (Hymenoptera: Formicidae: Myrmicinae). *J. N.Y. Entomol. Soc.* 99:141–198.

VANDER MEER, R. K., GLANCEY, B. M., LOFGREN, C. S., GLOVER, A., TUMLINSON, J. H., and ROCCA, J. R. 1980. The poison sac of red imported fire ant queens: Source of a pheromone attractant. *Ann. Entomol. Soc. Am.* 73:609–612.

VANDER MEER, R. K., ALVAREZ, F., and LOFGREN, C. S. 1988. Isolation of the trail recruitment pheromone of *Solenopsis invicta. J. Chem. Ecol.* 14:825–838.

WATKINS, J. F. and COLE, T. W. 1966. The attraction of army ant workers to secretions of their queens. *Tex. J. Sci.* 18:254–265.

WHEELER, W. M. 1910. Ants, Theirs Structures Development and Behavior. The Columbia University Press, New York.

WILLER, D. E. and FLETCHER. D. J. C. 1986. Differences in inhibitory capability among queens of the ant *Solenopsis invicta*. *Physiol. Entomol.* 11:475–482.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

IDENTIFICATION OF FEMALE SEX PHEROMONE OF THE RICE LEAF BUG, *Trigonotylus caelestialium*

MASASHI KAKIZAKI[1,*] and HAJIME SUGIE[2]

[1]*Hokkaido Central Agricultural Experiment Station Naganuma, Hokkaido 069-1301, Japan*

[2]*National Institute of Agro-Environmental Sciences Kannondai 3-1-1, Tsukuba, Ibaraki 305-0856, Japan*

(Received August 3, 1999; accepted August 6, 2001)

Abstract—The female sex pheromone of the rice leaf bug, *Trigonotylus caelestialium*, was analyzed by GC-EAD and GC-MS. Ten EAD active compounds—*n*-hexyl *n*-hexanoate, (*E*)-2-hexenyl *n*-hexanoate, *n*-hexyl (*E*)-2-hexenoate, *n*-octyl *n*-butyrate, *n*-octyl *n*-hexanoate, (*E*)-2-octenyl *n*-hexanoate, *n*-pentyl *n*-hexanoate, *n*-hexyl *n*-butyrate, (*E*)-2-hexenyl *n*-butyrate, and *n*-hexyl (*E*)-2-butenoate—were detected in the ratio of 1000:414–491:trace–5:5–11:55–71:50–63:trace–3:225:90:32 from female body extracts, and in the ratio of 1000:271–342:10–43:1–3:58–78:14–19:trace:178:36:26 from male body extracts. Field trapping tests with these synthetic compounds indicated that *n*-hexyl *n*-hexanoate, (*E*)-2-hexenyl *n*-hexanoate, and *n*-octyl *n*-butyrate are pheromone components, and mixtures in ratios of 1000:400–500:10–100 were more attractive to males. Doses ranging from 4.29 μg to 14.3 μg of the three-component mixture in the ratio of 1000:400:30 loaded into glass capillary tubes were most attractive to males.

Key Words—Female sex pheromone, Miridae, rice leaf bug, *Trigonotylus caelestialium*, *n*-hexyl *n*-hexanoate, (*E*)-2-hexenyl *n*-hexanoate, *n*-octyl *n*-butyrate.

INTRODUCTION

Males of the true bug (Heteroptera) families Pentatomidae, Alydidae, Rhopalidae, Reduviidae, and Coreidae often secrete aggregation pheromones or sex pheromones (Aldrich, 1988). Some of these pheromones have been chemically identified (aggregation pheromone: Kochansky et al., 1989; Aldrich et al., 1991,

*To whom correspondence should be addressed. Present address: Hokkaido Ornamental Plants and Vegetables Research Center, Higashi-Takikawa 735, Takikawa, Hokkaido 073-0026, Japan.

0098-0331/01/1200-2447/0

1997; Leal et al., 1995, 1996, 1998; Sugie et al., 1996; sex pheromone: Brézot et al., 1994). On the other hand, in 12 species of Miridae, females attract males of the same species (Scales, 1968; Boivin and Stewart, 1982; Thistlewood et al., 1989; McBrien and Millar, 1999), and female sex attractant pheromones have been identified for *Campylomma verbasci* (Smith et al., 1991), *Phytocoris relativus* (Millar et al., 1997), and *P. californicus* (Millar and Rice, 1998).

The rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy) (Miridae), is a major pest causing pecky rice, and also injuring wheat, maize, and gramineous forage grasses in the northern part of Japan. Males are attracted and sexually stimulated by virgin females, indicating that females posses a sex pheromone (Kakizaki and Sugie, 1997). Identification of the female sex pheromone of *T. caelestialium* is reported in this paper.

METHODS AND MATERIALS

Insects and Pheromone Extraction. Adult bugs of the second generation of *T. caelestialium* were collected in an insect collecting tube (3 cm diam., 8 cm long) near a light from 20:00 hr to 24:00 hr at Hokkaido Central Agricultural Experiment Station (HCAES), Naganuma, in mid-August 1992, 1993, and 1994. Females and males were placed in separate tubes. Whole bodies of about 100–200 bugs were dipped into 30 ml of hexane and extracted for one to two days at room temperature.

Column Chromatography. After evaporation of the solvent, extracts were placed on a column packed with the Florisil containing 7% H_2O (Carroll, 1961), and successively eluted with hexane, 5%, 15%, 25%, and 50% ether in hexane.

Bioassay by Coupled Gas Chromatography–Electroantennographic Detector (GC-EAD). The responses of antennae of *T. caelestialium* males to each fraction from the Florisil column chromatography were recorded with a GC-EAD system (Struble and Arn, 1984), and the compounds in the extracts were monitored by a gas chromatograph (Hewlett-Packard HP5890) equipped with an HP-INNOWAX capillary column (Hewlett-Packard: 30 m $\times$ 0.53 mm ID $\times$ 0.25-μm film thickness) and coupled to a thermal conductivity detector (TCD). The oven temperature was programmed at 5°C/min from 60°C (1.5 min) to 220°C (30 min).

Chemical Analysis. Each EAD active compound in the fraction from Florisil column chromatography of female and male body extracts was collected from a vent of the TCD by a glass capillary tube. EAD activities were confirmed by GC-EAD, and the compounds were analyzed by gas chromatography–mass spectrometry (GC-MS). GC-MS analysis (EI mode) was performed on a Hewlett-Packard HP5971A with an HP-INNOWAX capillary column (60 m $\times$ 0.33 mm ID $\times$ 0.25-μm film thickness). The oven temperature was programmed as above. The interface temperature was 250°C. Structures of ester compounds were estimated by the characteristic fragment ions, corresponding to the C_m carboxylic moiety

(e.g., $C_{m-1}H_{2m-1}COOH_2^+$ and $C_{m-1}H_{2m-1}CO^+$), and the C_n alcohol moiety (e.g., $C_nH_{2n}^+$), and the molecular ion (M^+). Double-bond positions in unsaturated compounds were determined by adduction of dimethyl disulfide (DMDS) (Buser et al., 1983). Geometric isomerism was determined by comparing GC retention times for synthetic compounds with the polar column noted above.

Chemical Synthesis. Esters were synthesized by using dicyclohexylcarbodiimide (DCC) and aminopyridine catalyst (Hassner and Alexanian, 1978) and the same equivalent of the corresponding carboxylic acids and the alcohols. The compounds were purified by silica gel (Wakogel C-200) column chromatography with a solvent system of hexane–ethyl acetate (10–20:1). Geometric isomers were separated by silica gel–silver nitrate (16.7%) column chromatography. The purity of each compound was greater than 99.8%, and the isomeric purity greater than 99.9% by GC.

Baits. Solutions containing test samples in 1 μl hexane were loaded in the center of glass capillary tubes (Duran ringcaps 5 μl, 0.021 mm ID, 125 mm long, bent into a V shape at the center by heating with a gas burner) (Kozai and Wakamura, 1982). The hexane was evaporated at room temperature overnight, and they were used as lure in all experiments. Two virgin females in a steel net cage (soup basket; 6 cm diam. $\times$ 6.5 cm high and 1.0 mm mesh) also were used in the test for comparison to the synthetic sex pheromone.

Field Experiments. Field experiments were carried out during the second (August–September) and third (October) adult generations of *T. caelestialium* in fields of Italian rye grass at HCAES (22,500 m^2, in 1995–1997), and at Hokkaido Ornamental Plants and Vegetables Research Center (6000 m^2, Takikawa, in 1998). Water-pan traps (white, 35 cm diam. $\times$ 6 cm deep) were used. The baits were hung 20 cm above the pans with wire. Blank traps without baits were used as controls. Three to six replicates were conducted for each experiment. The traps were placed in a line 20–25 m apart on the ground in fields. The number of bugs captured by each trap was counted at intervals of one to three days, and the location of traps was rotated at the same time.

In test I, attraction activity of each of the 10 EAD active compounds, a six-component mixture of *n*-hexyl *n*-hexanoate (5 μg), (*E*)-2-hexenyl *n*-hexanoate (2.5 μg), *n*-hexyl (*E*)-2-hexenoate (5 ng), *n*-octyl *n*-butyrate (150 ng), *n*-octyl *n*-hexanoate (275 ng), (*E*)-2-octenyl *n*-hexanoate (275 ng), and a 10-component mixture of *n*-pentyl *n*-hexanoate (5 ng), *n*-hexyl *n*-butyrate (1.15 μg), (*E*)-2-hexenyl *n*-butyrate (450 ng), and *n*-hexyl (*E*)-2-butenoate (150 ng) mixed with the above mentioned six-component mixture were tested. In test II, one of the compounds of the six-component mixture was deleted to evaluate the necessity of the compound. In test III, one of the compounds of a mixture of *n*-hexyl *n*-hexanoate, (*E*)-2-hexenyl *n*-hexanoate, *n*-hexyl (*E*)-2-hexenoate, *n*-octyl *n*-butyrate, and *n*-octyl *n*-hexanoate, which were selected by test II, was deleted and the necessity of the compounds was evaluated. In test IV, attraction activity

of a three-component mixture of *n*-hexyl *n*-hexanoate (3 μg), (*E*)-2-hexenyl *n*-hexanoate (1.5 μg), and *n*-octyl *n*-butyrate (90 ng), which were selected by test III, was evaluated. Effect of the addition of either *n*-pentyl *n*-hexanoate, *n*-hexyl *n*-butyrate, (*E*)-2-hexenyl *n*-butyrate, *n*-hexyl (*E*)-2-butenoate, or all of these compounds to the three-component mixture was evaluated in tests V and VI. In test VII, effect of the addition of *n*-octyl *n*-butyrate in variable doses of 0.015–1.5 μg in a mixture of *n*-hexyl *n*-hexanoate (5 μg) and (*E*)-2-hexenyl n-hexanoate (2.5 μg) was evaluated. Effect of the addition (*E*)-2-hexenyl *n*-hexanoate in variable doses of 0.5–5 μg on a mixture of *n*-hexyl *n*-hexanoate (5 μg) and *n*-octyl *n*-butyrate (0.15 μg) were evaluated in test VIII. In test IX, optimum doses of the three-component mixture in a ratio of 1000:400:30 ranging from 1.43 μg to 143 μg were evaluated with glass capillary tubes. In test X, attraction activity of the sex pheromone, *n*-hexyl n-hexanoate (5 μg), (*E*)-2-hexenyl *n*-hexanoate (2.5 μg), and *n*-octyl *n*-butyrate (150 ng) loaded into the glass capillary tube was compared with that of traps baited with two virgin females.

Trap data (X) were transformed to square root ($X + 0.5$) before analysis of variance, and were compared by Tukey's test.

RESULTS

EAD Active Peaks in Extracts. All EAD active peaks by male antennae were contained in the 5% ether in hexane fraction from the Florisil column chromatography (Table 1). In the extracts of one female equivalent (1 FE), there were two stronger EAD peaks (about 1.05–0.95 mV), three intermediate EAD peaks (about 0.3–0.15 mV), and five weaker EAD peaks (about 0.05–0.025 mV). EAD peaks with identical retention times to those of above peaks were also detected in male body extracts.

Identification of EAD Active Compounds. EAD active compounds were identified as in Table 1: Two stronger EAD active compounds are *n*-hexyl *n*-hexanoate {H:H, abbreviation in all tables; the retention time (R_t) = 30.67 min; m/z 117 [relative intensity: 100% (base peak)], 99 (52%), 84 (40%), 200 (M^+, 0.3%)}, and (*E*)-2-hexenyl *n*-hexanoate [*E*2H:H; $R_t = 32.38$ min; m/z 117 (3%), 99 (100%), 82 (20%), 198 (M^+, 2.5%); DMDS adduct: m/z 292 (M^+), 103 ($C_3H_7CH{=}S^+CH_3$), 189 ($CH_3S^+{=}CHCH_2OCOC_5H_{11}$); synthetic *E* isomer, $R_t = 32.38$ min, *Z*-isomer, $R_t = 32.17$ min]. Three intermediate EAD active compounds are *n*-hexyl (*E*)-2-hexenoate [H:*E*2H; $R_t = 34.46$ min; m/z 115 (100%), 97 (36%), 84 (5%), 198 (M^+, 0.1%); DMDS adduct: m/z 292 (M^+), 189 ($C_6H_{13}OCOCH{=}S^+CH_3$), 103 ($CH_3S^+{=}CHC_3H_7$); synthetic *E* isomer, $R_t = 34.46$ min], *n*-octyl *n*-hexanoate [O:H; $R_t = 36.45$ min; m/z 117 (100%), 99 (40%), 112 (38%), 228 (M^+, 0.2%)], and (*E*)-2-octenyl *n*-hexanoate [*E*2O:H; $R_t = 32.38$ min; m/z 117 (3%), 99 (100%), 110 (10%), 226 (M^+, 2.3%); DMDS adduct: m/z 320 (M^+), 131

TABLE 1. EAD ACTIVE COMPOUNDS IN 5% ETHER IN HEXANE FRACTION FROM FLORISIL COLUMN CHROMATOGRAPHY TO MALE ANTENNAE IN *Trigonotylus caelestialium*

Compound[a]	Retention time (min)[b]	Relative ratio in extracts[c]		EAD activity in one female extract (mV)
		Female	Male	
H:H	30.67	1000	1000	1.05
*E*2H:H	32.38	414–491	271–342	0.95
H:*E*2H	34.46	tr.–5	10–43	0.15
O:B	30.82	5–11	1–3	0.05
O:H	36.45	55–71	58–78	0.2
*E*2O:H	38.10	50–63	14–19	0.3
P:H	27.65	tr.–3	tr.	0.05
H:B	24.91	225	178	0.05
*E*2H:B	26.70	90	36	0.05
H:*E*2B	28.98	32	26	0.025

[a] Abbreviations: H:H, *n*-hexyl *n*-hexanoate; *E*2H:H, (*E*)-2-hexenyl *n*-hexanoate; H:*E*2H, *n*-hexyl (*E*)-2-hexenoate; O:B, *n*-octyl *n*-butyrate; O:H, *n*-octyl *n*-hexanoate; *E*2O:H, (*E*)-2-octenyl *n*-hexanoate; P:H, *n*-pentyl *n*-hexanoate; H:B, *n*-hexyl *n*-butyrate; *E*2H:B, (*E*)-2-hexenyl *n*-butyrate; H:*E*2B, *n*-hexyl (*E*)-2-butenoate.

[b] GC: one female equivalent of 5% ether in hexane fraction from the Florisil column chromatography was injected and separated on an HP-INNOWAX column, programmed at 5°C/min from 60°C (1.5 min) to 220°C (30 min).

[c] Investigations were performed for first six compounds in 1992 and 1993, and for all ten compounds in 1994.

($C_5H_{11}CH{=}S^+CH_3$), 189 ($CH_3S^+{=}CHCH_2OCOC_5H_{11}$); synthetic *E* isomer, R_t = 38.10 min, *Z* isomer, R_t = 37.11 min]. Five weaker EAD active compounds are *n*-octyl *n*-butyrate [O:B; R_t = 30.82 min; *m*/*z* 89 (100%), 71 (98%), 112 (24%), 201 (M+1, 0.5%)], *n*-pentyl: *n*-hexanoate [P:H; R_t = 27.65 min; *m*/*z* 117 (100%), 99 (62%), 70 (59%), 186 (M^+, 0.3%)], *n*-hexyl *n*-butyrate [H:B; R_t = 24.91 min; *m*/*z* 89 (100%), 71 (85%), 84 (45%), 172 (M^+, 0.1%)], (*E*)-2-hexenyl *n*-butyrate [*E*2H:B; R_t = 26.70 min; *m*/*z* 89 (4%), 71 (100%), 82 (15%), 170 (M^+, 1.0%); DMDS adduct: *m*/*z* 264 (M^+), 103 ($C_3H_7CH{=}S^+CH_3$), 161 ($CH_3S^+{=}CHCH_2OCOC_3H_7$); synthetic *E* isomer, R_t = 26.70 min, *Z* isomer, R_t = 26.50 min], and *n*-hexyl (*E*)-2-butenoate [H:*E*2B; R_t = 28.98 min; *m*/*z* 87 (83%), 69 (100%), 84 (4%), 171 (M+1, 1.0%); DMDS adduct: *m*/*z* 264 (M^+), 189 ($C_6H_{13}OCOCH{=}S^+CH_3$), 75 ($CH_3S^+{=}CHCH_3$); synthetic *E* isomer, R_t = 28.98 min].

These EAD active compounds were identified from whole body extracts of both sexes, and female-specific compounds showing EAD activity could not be detected.

Ratio of Compounds in Extracts. The ratios of the 10 compounds in female and male body extracts were quantified as Table 1. The relative ratios of

(*E*)-2-hexenyl *n*-hexanoate, (*E*)-2-octenyl *n*-hexanoate, and *n*-octyl *n*-butyrate to *n*-hexyl *n*-hexanoate tended to be high in female extracts, and that of *n*-hexyl (*E*)-2-hexenoate to *n*-hexyl *n*-hexanoate in male extracts.

Mean amounts of *n*-hexyl *n*-hexanoate in whole-body extracts were 127 ng per female ($N = 10$) and 27 ng per male ($N = 10$) at seven days after emergence.

Attraction Activity of EAD Active Compounds in the Field. None of the ten EAD active compounds alone attracted males, but the six- and 10-component mixtures were both equally attractive to males (test I in Table 2). When *n*-hexyl *n*-hexanoate or (*E*)-2-hexenyl *n*-hexanoate were deleted from the six- or five-component mixture (tests II and III in Table 3), the numbers of males captured were as low as those in blank traps. By adding *n*-octyl *n*-butyrate to the mixture of *n*-hexyl *n*-hexanoate and (*E*)-2-hexenyl *n*-hexanoate (test IV), the number of captured males increased. However, the deletion of (*E*)-2-octenyl *n*-hexanoate, *n*-octyl *n*-hexanoate, or *n*-hexyl (*E*)-2-hexenoate from the six- and five-component

TABLE 2. ATTRACTIVENESS OF EAD ACTIVE COMPOUNDS AND THEIR MIXTURES TO *T. caelestialium* IN THE FIELD (TEST I, NAGANUMA, AUGUST 11–25, 1995)

Baits[a]	Amount (μg)	Bugs caught/trap[b]		
		Males	Females	Nymphs
H:H	5	2.7 b	0.7 a	0.3 a
H:H	1	2.7 b	0.3 a	0.3 a
*E*2H:H	5	4.3 b	0.3 a	0 a
*E*2H:H	1	6.3 b	0.7 a	0.7 a
H:*E*2H	1	1.7 b	1.3 a	0.7 a
O:B	1	3.0 b	1.0 a	0.7 a
O:H	1	3.0 b	0.3 a	0.3 a
*E*2O:H	1	5.3 b	0.7 a	0.3 a
P:H	1	2.3 b	0.7 a	1.0 a
H:B	1	4.7 b	1.0 a	0.3 a
*E*2H:B	1	2.7 b	0.7 a	0.7 a
H:*E*2B	1	3.3 b	0.7 a	0.7 a
6-component[c]	8.205	13.3 a	0.3 a	0.7 a
10-component[d]	9.960	12.7 a	0.7 a	1.7 a
Blank		4.0 b	0.7 a	0.3 a

[a] These compounds and mixtures were loaded in glass capillary tubes. Abbreviations are same as in Table 1.

[b] Values are means from three traps during the period of the investigation. The means followed by the same letter in the same column are not different at the 5% level by Tukey's test.

[c] Mixture of 5 μg H:H, 2.5 μg *E*2H:H, 5 ng H:*E*2H, 150 ng O:B, 275 ng O:H and 275 ng *E*2O:H.

[d] Mixture adding of 5 ng P:H, 1.15 μg H:B, 450 ng *E*2H:B and 150 ng H:*E*2B on the above six-component mixture.

TABLE 3. ATTRACTIVENESS OF 6-, 5-, 4-, 3-, AND 2-COMPONENT MIXTURES OF EAD ACTIVE COMPOUNDS TO *T. caelestialium* IN THE FIELD AT NAGANUMA

Baits[a]						Bugs caught/trap[b]		
H:H (μg)	*E*2H:H (μg)	O:B (ng)	H:*E*2H (ng)	O:H (ng)	*E*2O:H (ng)	Males	Females	Nymphs
Test II, August 8–15, 1995								
5	2.5	150	5	275	275	15.3 ab	2.0 a	0.3 a
5	2.5	150	5	275		17.0 a	2.2 a	0.2 a
5	2.5	150	5		275	12.7 abc	1.7 a	0 a
5	2.5	150		275	275	11.8 abc	1.7 a	0.3 a
5	2.5		5	275	275	13.0 ab	1.8 a	0.3 a
5		150	5	275	275	7.8 cd	2.5 a	0.7 a
	2.5	150	5	275	275	7.8 cd	2.5 a	0.5 a
	Blank					5.5 d	1.7 a	0.5 a
Test III, August 13–27, 1996								
5	2.5	150	5	275		16.5 ab	1.2 a	1.3 a
5	2.5	150	5			22.7 a	1.2 a	2.0 a
5	2.5	150		275		23.2 a	1.7 a	2.3 a
5	2.5		5	275		16.5 ab	1.8 a	1.7 a
5		150	5	275		7.2 c	2.3 a	1.0 a
	2.5	150	5	275		9.8 bc	2.3 a	0.8 a
	Blank					3.7 c	1.0 a	0.8 a
Test IV, August 9–23, 1996								
3	1.5	90				32.3 a	0 a	0 a
3	1.5					20.7 b	0.3 a	0 a
	Blank					0.7 c	0 a	0 a

[a] Mixtures were loaded in glass capillary tubes. Abbreviations are same as in Table 1.
[b] Values are means from six traps (test II and III) or three traps (test IV) during the period of the investigation. The means followed by the same letter in the same column in each test are not different at the 5% level by Tukey's test.

mixtures (tests II and III), and the addition of each compound of *n*-pentyl *n*-hexanoate, *n*-hexyl *n*-butyrate, (*E*)-2-hexenyl *n*-butyrate, and *n*-hexyl (*E*)-2-butenoate (test V in Table 4), and of a mixture of these four compounds (test VI) to the three-component mixture, did not affect male attraction. No females or nymphs of *T. caelestialium* were attracted to these mixtures in any of the experiments.

Optimum Ratio and Doses of the Three Compounds for Male Attraction. The addition of 0.05–0.5 μg *n*-octyl *n*-butyrate in a two-component mixture of 5 μg *n*-hexyl *n*-hexanoate and 2.5 μg (*E*)-2-hexenyl *n*-hexanoate (in ratios of 10–100:1000:500) (test VII in Table 5), and the addition of 2–2.5 μg (*E*)-2-hexenyl *n*-hexanoate in a two-component mixture of 5 μg *n*-hexyl *n*-hexanoate and 0.15 μg *n*-octyl *n*-butyrate (in ratios of 400–500:1000:30) (test VIII), led to more males being captured than in other ratios.

TABLE 4. EFFECT ON ATTRACTIVENESS TO *T. caelestialium* BY ADDING FOUR EAD ACTIVE COMPOUNDS TO 3-COMPONENT MIXTURES IN THE FIELD AT NAGANUMA

Baits[a]							Bugs caught/trap[b]		
H:H (μg)	*E*2H:H (μg)	O:B (ng)	P:H (ng)	H:B (μg)	*E*2H:B (ng)	H:*E*2B (ng)	Males	Females	Nymphs
Test V, August 15–28, 1997									
5	2.5	150	5				12.6 a	1.8 a	0 a
5	2.5	150		1.15			10.4 a	0.6 a	0.2 a
5	2.5	150			450		11.0 a	0.2 a	0.6 a
5	2.5	150				150	12.4 a	0.8 a	0.6 a
5	2.5	150					11.2 a	0.6 a	0.4 a
	Blank						1.2 b	0.8 a	0.2 a
Test VI, August 15–25, 1997									
5	2.5	150	5	1.15	450	150	15.3 a	0 a	0.3 a
5	2.5	150					16.0 a	0.7 a	0.3 a
	Blank						0 b	0.3 a	0 a

[a] Mixtures were loaded in glass capillary tubes. Abbreviations are same as in Table 1.
[b] Values are means from five traps (test V) and three traps (test VI) during the period of the investigation. The means followed by the same letter in the same column in each test are not different at the 5% level by Tukey's test.

Doses greater than 42.9 μg of the three-component mixture captured smaller numbers of males than doses within the range 1.43–14.3 μg (test IX in Table 6), indicating that attraction was inhibited by excessive amounts. At all doses used, glass capillary tubes containing doses of 4.29 μg and 14.3 μg caught the largest numbers of males.

Comparison of Numbers of Bugs Captured by Synthetic Sex Pheromone and Two Virgin Females. The numbers of male bugs captured by traps baited with 7.65 μg of the three-component mixture were greater than those by traps baited with two virgin females (test X in Table 7).

DISCUSSION

The 10 compounds identified stimulated a response from male antennae. However, *n*-hexyl (*E*)-2-hexenoate, *n*-octyl *n*-hexanoate, (*E*)-2-octenyl *n*-hexanoate, *n*-pentyl *n*-hexanoate, *n*-hexyl *n*-butyrate, (*E*)-2-hexenyl *n*-butyrate, and *n*-hexyl (*E*)-2-butenoate did not affect the attraction activity, and these compounds were shown not to be necessary for attraction. In conclusion, only three compounds are responsible for male attraction: *n*-hexyl *n*-hexanoate and (*E*)-2-hexenyl *n*-hexanoate were the essential components, and *n*-octyl *n*-butyrate enhanced the male attraction in combination with the two essential components. Neither females

TABLE 5. ATTRACTIVENESS OF 3-COMPONENT MIXTURE WITH VARIABLE RATIOS TO *T. caelestialium* IN THE FIELD AT NAGANUMA

Baits[a]			
H:H (μg)	*E*2H:H (μg)	O:B (μg)	Male bugs caught/trap[b]
Test VII, October 2–16, 1997			
5	2.5	0	5.3 b
5	2.5	0.015	5.7 b
5	2.5	0.05	10.0 a
5	2.5	0.15	6.7 ab
5	2.5	0.5	6.0 ab
5	2.5	1.5	4.7 b
	Blank		0 c
Test VIII, October 1–12, 1997			
5	0.5	0.15	8.0 d
5	1	0.15	8.7 cd
5	1.5	0.15	8.7 cd
5	2	0.15	16.0 a
5	2.5	0.15	12.7 abc
5	3	0.15	8.7 cd
5	3.5	0.15	7.3 d
5	4	0.15	8.0 d
5	4.5	0.15	10.7 bcd
5	5	0.15	14.7 ab
	Blank		0.7 e

[a] Mixtures were loaded in glass capillary tubes. Abbreviations are same as in Table 1.

[b] Values are means from three traps during the period of the investigation. The means followed by the same letter in the same column in each test are not different at the 5% level by Tukey's test.

nor nymphs were attracted to the three-component mixture. Therefore, the three-component mixture appears to be the sex pheromone of female *T. caelestialium*. Although a clear optimum ratio of *n*-hexyl *n*-hexanoate and (*E*)-2-hexenyl *n*-hexanoate was not shown, the mixtures of *n*-hexyl *n*-hexanoate (*E*)-2-hexenyl *n*-hexanoate, *n*-octyl *n*-butyrate in ratios of 1000:400–500:10–100 were attractive to males.

In Miridae, the mixture of *n*-butyl *n*-butyrate and (*E*)-2-butenyl *n*-butyrate in *Campylomma verbasci* (Smith et al., 1991), the mixture of *n*-hexyl acetate and (*E*)-2-octenyl *n*-butyrate in *Phytocoris relativus* (Millar et al., 1997), and the mixture of *n*-hexyl acetate and (*E*)-2-octenyl acetate in *P. californicus* (Millar and Rice, 1998) were reported as attractant pheromones. The pheromones in these species

TABLE 6. ATTRACTIVENESS OF 3-COMPONENT MIXTURE WITH VARIABLE AMOUNTS TO *T. caelestialium* IN THE FIELD (TEST IX, TAKIKAWA, OCTOBER 9–23, 1998)

Baits[a]				
H:H (μg)	*E*2H:H (μg)	O:B (μg)	Total amounts (μg)	Male bugs caught/trap[b]
100	40	3	143	10.7 b
30	12	0.9	42.9	12.3 b
10	4	0.3	14.3	23.7 a
3	1.2	0.09	4.29	32.3 a
1	0.4	0.03	1.43	15.0 b
	Blank			0.7 c

[a] Mixtures were loaded in glass capillary tubes. Abbreviations are same as in Table 1.
[b] Values are means from three traps during the period of the investigation. The means followed by the same letter are not different at the 5% level by Tukey's test.

contain at least one female-specific compound. On the other hand, in the case of *T. caelestialium*, the components of the attractant pheromone were common in body extracts of both sexes. However, traps baited with *T. caelestialium* males do not attract conspecific males (Kakizaki and Sugie, 1997), and there may be other factors disturbing the attraction by the males.

In a number of true bugs, many esters have been detected from extracts or secretions (Aldrich, 1988). *n*-Hexyl *n*-hexanoate and/or (*E*)-2-hexenyl *n*-hexanoate

TABLE 7. ATTRACTIVENESS OF SYNTHETIC SEX PHEROMONE AND TWO VIRGIN FEMALES IN *T. caelestialium* IN THE FIELD (TEST X, TAKIKAWA, OCTOBER 12–16, 1998)

	Bugs caught/trap[a]		
Baits	Males	Females	Nymphs
Sex pheromone[b]	15.7 a	0 a	0 a
2 females[c]	8.0 b	0 a	0 a
Blank	0 c	0 a	0 a

[a] Values are means from three traps during the period of the investigation. The means followed by the same letter in the same column are not different at the 5% level by Tukey's test.
[b] Mixture of 5 μg H:H, 2.5 μg *E*2H:H and 150 ng O:B was loaded in glass capillary tube. Abbreviations are same as in Table 1.
[c] Two virgin females were kept in a steel net cage with the germinal forage grasses.

were reported in secretions of *Blepharidopterus angulatus* (Miridae) (Knight et al., 1984) and *Homoeocerus unipunctatus* (Coreidae) (Kitamura et al., 1984), and (*E*)-2-hexenyl *n*-hexanoate was reported to be an alarm pheromone in *Riptortus clavatus* (Alydidae) (Leal and Kadosawa, 1992). However, *n*-octyl *n*-butyrate has not been reported from other true bugs. The existence of *n*-hexyl *n*-butyrate or (*E*)-2-hexenyl *n*-butyrate was reported in other mirids (Gueldner and Parrott, 1978; Knight et al., 1984; Smith et al., 1991; Millar et al., 1997).

This pheromone would be useful for monitoring *T. caelestialium* adult populations. The attraction period of the lures with the glass capillary tubes was less than two weeks, and a more long-lived pheromone dispenser should be developed for monitoring.

Acknowledgments—We thank Dr. Yukihiro Fukushi of Laboratory of Ecological Chemistry, Faculty of Agriculture, Hokkaido University, for instruction in the syntheses of the chemicals, and Dr. Kenjiro Kawasaki of the National Institute of Sericultural and Entomological Sciences for advice on GC-EAD. Dr. Tetsuo Gotoh of Laboratory of Applied Entomology and Zoology, Faculty of Agriculture, Ibaraki University, Dr. Hiroshi Noguchi of National Institute of Agro-Environmental Sciences; and Dr. Yoichi Kajino of Hokkaido Central Agricultural Experiment Station are thanked for their comments and advice. The manuscript was critically read by Dr. Sadahiro Tatsuki, Laboratory of Applied Entomology, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

REFERENCES

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J. R., HOFFMANN, H. P., KOCHANSKY, J. P., LUSBY, W. R., EGER, J. E., and PAYNE, J. A. 1991. Identification and attractiveness of a major pheromone component for nearctic *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae). *Environ. Entomol.* 20:477–483.

ALDRICH, J. R., LEAL, W. S., NISHIDA, R., KHRIMIAN, A. P., LEE, C. J., and SAKURATANI, Y. 1997. Semiochemistry of aposematic seed bugs. *Entomol. Exp. Appl.* 84:127–135.

BRÉZOT, P., MALOSSE, C., MORI, K., and RENOU, M. 1994. Bisabolene epoxides in sex pheromone in *Nezara viridula* (L.) (Heteroptera: Pentatomidae): Role of *cis* isomer and relation to specificity of pheromone. *J. Chem. Ecol.* 20:3133–3147.

BOIVIN, G. and STEWART, R. K. 1982. Attraction of male green apple bug, *Lygocoris communis* (Hemiptera: Miridae), to caged females. *Can. Entomol.* 114:765–766.

BUSER, H. R., ARN, H., GUERIN, P., and RAUSHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

CARROLL, K. K. 1961. Separation of lipid classes by chromatography on florisil. *J. Lipid Res.* 2:135–141.

GUELDNER, R. C. and PARROTT, W. L. 1978. Volatile constituents of the tarnished plant bug. *Insect Biochem.* 8:389–391.

HASSNER, A. and ALEXANIAN, V. 1978. Direct room temperature esterification of carboxylic acids. *Tetrahedron Lett.* 46:4475–4478.

KAKIZAKI, M. and SUGIE, H. 1997. Attraction of males to females in the rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy) (Heteroptera: Miridae). *Appl. Entomol. Zool.* 32:648–651.

KITAMURA, C., WAKAMURA, S., and TAKAHASHI, S. 1984. Identification and functions of ventral glands secretion of some Heteroptera. *Appl. Entomol. Zool.* 19:33–41.

KNIGHT, D. W., ROSSISTER, M., and STADDON, B. W. 1984. Ester from the metathoracic scent gland of two capsid bugs, *Pilophorus petplexus* Douglas and Scott and *Blepharidopterus angulatus* (Fallen) (Heteroptera: Miridae). *Comp. Biochem. Physiol.* 78B:237–239.

KOCHANSKY, J. P., ALDRICH, J. R., and LUSBY, W. R. 1989. Synthesis and pheromone activity of 6,10,13-trimethyl-1-tetradecanol for predatory stink bug, *Stiretrus anchorago* (Heteroptera: Pentatomidae). *J. Chem. Ecol.* 15:1717–1728.

KOZAI, S. and WAKAMURA, S. 1982. Sex pheromone of the turnip moth, *Agrotis segetum* Denis et Schiffermüller (Lepidoptera: Noctuidae): Glass capillary formulation of synthetic sex pheromone. *Jpn. J. Appl. Enotmol. Zool.* 26:262–265 (in Japanese with English summary).

LEAL, W. S. and KADOSAWA, T. 1992. (*E*)-2-Hexenyl hexanoate, the alarm pheromone of the bean bug *Riptortus clavatus* (Heteroptera: Alydidae). *Biosci. Biotech. Biochem.* 56:1004–1005.

LEAL, W. S., HIGUCHI, H., MIZUTANI, N., NAKAMORI, H., KADOSAWA, T., and ONO, M. 1995. Multifunctional communication in *Riptortus clavatus* (Heteroptera: Alydidae): Conspecific nymphs and egg parasitoid *Ooencyrtus nezarae* use the same adult attractant pheromone as chemical cue. *J. Chem. Ecol.* 21:973–985.

LEAL, W. S., UEDA, Y., and ONO, M. 1996. Attractant pheromone for male rice bug, *Leptocorisa chinesis*: semiochemicals produced by both male and female. *J. Chem. Ecol.* 22:1429–1437.

LEAL, W. S., KUWAHARA, S., SHI, X., HIGUCHI, H., MARINO, C. E. B., ONO, M., and MEINWALD, J. 1998. Male-released sex pheromone of the stink bug *Piezodorus hybneri*. *J. Chem. Ecol.* 24:1817–1829.

MCBRIEN, H. L. and MILLAR, J. G. 1999. Phytophagous bugs, pp. 277–304, *in* J. Hardie and K. Minks (eds.). *Non-Lepidopteran Insects Associated with Agricultural Plants*. CABI Publishing, New York.

MILLAR, J. G. and RICE, R. E. 1998. Sex pheromone of the plant bug *Phytocoris californicus* (Heteroptera: Miridae). *J. Econ. Entomol.* 91:132–137.

MILLAR, J. G., RICE, R. E., and WANG, Q. 1997. Sex pheromone of the mirid bug *Phytocoris relativus*. *J. Chem. Ecol.* 23:1743–1754.

SCALES, A. L. 1968. Female tarnished plant bugs attract males. *J. Econ. Entomol.* 61:1466–1467.

SMITH, R. F., PIERCE, H. D., and BORDEN, J. H. 1991. Sex pheromone of mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *J. Chem. Ecol.* 17:1437–1447.

SUGIE, H., YOSHIDA, M., KAWASAKI, K., NOGUCHI, H., MORIYA, S., TAKAGI, K., FUKUDA, H., FUJIIE, A., YAMANAKA, M., OHIRA, Y., TSUTSUMI, T., TSUDA, K., FUKUMOTO, K., YAMASHITA, M., and SUZUKI, H. 1996. Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Appl. Entomol. Zool.* 31:425–431.

STRUBLE, D. L. and ARN, H. 1984. Combined gas chromatography and electro-antennographic recording in insect olfactory responses, pp.161–178., *in* H. E. Hummel and T. A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, Tokyo, Japan.

THISTLEWOOD, H. M. A., BORDEN, J. H., SMITH, R. F., PIERCE, H. D., and MCMULLEN, R. D. 1989. Evidence for a sex pheromone in the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *Can. Entomol.* 121:737–744.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

VOLATILE COMPONENTS IN DORSAL GLAND SECRETIONS OF THE WHITE-LIPPED PECCARY, *Tayassu pecari*, FROM BOLIVIA

JOHN S. WATERHOUSE,[1,*] MARK HUDSON,[1] JOHN A. PICKETT,[2] and PAUL J. WELDON[3]

[1]*Environmental Sciences Research Centre*
Anglia Polytechnic University
East Road Cambridge CB1 1PT, UK

[2]*IACR Rothamsted*
Harpenden, Herts AL5 2JQ, UK

[3]*Conservation and Research Center*
Smithsonian Institution
1500 Remount Road
Front Royal, Virginia, 22630

(Received January 1, 2001; accepted July 15, 2001)

Abstract—Secretions from the dorsal gland of male and female free-ranging adult white-lipped peccaries (*Tayassu pecari*) from Bolivia were analyzed by gas chromatography–mass spectrometry. Fifty compounds were identified, some provisionally. Secretions of both sexes contain saturated and unsaturated C_5–C_{18} carboxylic acids as well as isomers of the diterpene springene. The females' secretions uniquely possess farnesyl esters of C_8 and C_{10} carboxylic acids, whereas the males' secretions uniquely showed two compounds provisionally identified as monounsaturated C_{16} lactones. Saturated high-molecular-weight esters of C_{10} carboxylic acids are more abundant in the females' secretions than in those of the males. The secretions of both sexes also contain monoalkanoate esters of methylhydroquinone as major components.

Key Words—Dorsal gland, white-lipped peccary, *Tayassu pecari*, gas chromatography, mass spectrometry, springene, farnesyl esters, phenols.

INTRODUCTION

Peccaries (Tayassuidae) are New World artiodactyl mammals represented by three extant species: the collared (*Tayassu tajacu*), the white-lipped (*T. pecari*), and the

*To whom correspondence should be addressed. E-mail: J.S.Waterhouse@apu.ac.uk

0098-0331/01/1200-2459/0

Chacoan peccary (*Catagonus wagneri*) (Sowls, 1984). All three species possess a prominent skin gland, known as the dorsal gland, located in the middle of the rump. This organ produces strongly odorous secretions that are thought to repel predators (Epling, 1956) or to have pheromonal activity (e.g., Sowls, 1984). An analysis of the dorsal gland secretions of free-ranging *T. tajacu* from Texas revealed squalene, geranylgeraniol, and several isomers of springene—a diterpene homolog of farnesene (Waterhouse et al., 1996). High-molecular-weight, straight- and branched-chain aliphatic esters were prominent in females but were not observed in males.

We report here the results of an analysis of the volatile components of dorsal gland secretions of free-ranging *T. pecari* from Bolivia. This species, which normally travels in herds of from 50 to hundreds of individuals (Kiltie and Terborgh, 1983, and references therein), inhabits rain forests from southern Mexico to northeastern Argentina. *T. pecari* rubs its dorsal gland primarily against conspecifics during alloanointing. In this situation, peccaries stand side-by-side, facing in opposite directions, and rub their glands against each other (Sowls, 1984) (Figure 1). In the field, alloanointing has been observed among as many as four individuals simultaneously (Hernandez et al., 1995). Sowls (1984) asserts that *T. pecari* emits the strongest scented dorsal gland secretions of the three peccary species, a feature that may render it less preferred for human consumption.

METHODS AND MATERIALS

Sample Collection

Dorsal gland secretions were collected during February 1997 from adult *T. pecari* in free-ranging herds in the Noel Kempff Mercado National Park, Santa Cruz, Bolivia, at the base of the northern tip of the Huanchaca escarpment. Peccary herds were captured in a large pen constructed around a frequently used salt lick in the forest. Once in the pen, animals received 120–150 mg of equal parts of tiletamine hydrochloride and zolazepam (Telazol) in plastic darts delivered by a CO_2-powered pistol. Individuals not sufficiently immobilized for safe handling received 100 mg doses of ketamine hydrochloride injected intramuscularly. Secretions from eight males and 12 females were collected by placing the mouth of a glass vial over the duct opening of the gland and then manually applying pressure to the body of the gland. Secretions were pooled according to sex and several milliliters of hexane were added to each vial before it was sealed with a polyethylene-lined cap. Vials were shipped on solid CO_2 and stored at -70°C.

FIG. 1. A pair of *Tayassu pecari* alloanointing (photograph by O. Amaral Ferraz).

Analytical Methods

Secretion samples were diluted with further volumes of hexane. Samples (1 μl) of the resulting solutions were analyzed by gas chromatography–mass spectrometry (GC–MS) as described previously (Waterhouse et al., 1996). Identities of compounds were indicated by their mass spectra. For most compounds, identities were confirmed by comparisons of retention times with those of authentic compounds, which were obtained commercially (Sigma-Aldrich, Gillingham, UK) or synthesized. Synthesized farnesyl esters were analyzed using a second set of conditions: a Hewlett-Packard 5890 gas chromatograph fitted with a HP 1 column (50 m $\times$ 0.32 mm ID, 0.25-μm film thickness) coupled to a VG Autospec mass spectrometric detector using the following temperature program: 30–250°C, beginning at 30°C for 5 min, raised at 5°C/min, and finally held at 240°C.

Synthetic Methods

Farnesyl Esters. A mixture of (*Z*,*Z*)-, (*E*,*Z*)-, (*Z*,*E*)-, and (*E*,*E*)-farnesyl decanoates was prepared by standard methods (Khorana, 1953; Brown, 1963) using molecular equivalent amounts of farnesol and decanoic acid with a threefold molecular equivalent of dicyclohexylcarbodiimide and a catalytic amount of dimethylaminopyridine in dichloromethane at room temperature.

Methylhydroquinone Esters. Reaction in tetrahydrofuran of a 1:1 molar ratio of methylhydroquinone (Sigma-Aldrich) and either 2-methylpropanoyl chloride or 2-methylbutanoyl chloride (Sigma-Aldrich) in the presence of a molar excess of the strongly basic ion exchange resin SRA 65 (Sigma-Aldrich) in its OH form yielded products containing mono esters of methylhydroquinone. The esters were not isolated, but their identities were confirmed by their mass spectra, which display the following m/z (%) values: 2-methyl-4-hydroxyphenyl 2-methylbutanoate: 208(M^+, 4), 125(10), 124(100), 95(3), 85(3), 77(5), 57(18), 55(4), 41(13), 39(8); 3-methyl-4-hydroxyphenyl 2-methylbutanoate: 208(M^+, 5), 125(7), 124(100), 94(5), 85(3), 77(6), 67(6), 57(27), 55(5), 41(15), 39(7); 2-methyl-4-hydroxyphenyl 2-methylpropanoate and 3-methyl-4-hydroxyphenyl 2-methylpropanoate (indistinguishable mass spectra): 194(M^+, 5), 125(8), 124(100), 123(19), 95(8), 77(6), 67(6), 55(7), 43(39), 41(15), 39(14).

RESULTS AND DISCUSSION

Chromatograms obtained from male and female pooled samples are shown in Figure 2, in which peak numbers correspond to compound numbers identified in Table 1. Our analysis of *T. pecari* reveals some components in common with *T. tajacu*, its sole extant congener. We identified the following three isomers of springene in male *T. pecari*: (3*Z*,6*E*,10*E*)-α-springene, (3*E*,6*E*,10*E*)-α-springene,

and (6*E*,10*E*)-β-springene. The identities of these compounds were confirmed by comparison of their mass spectra and retention times with those of synthesized compounds (Waterhouse et al., 1996). Unlike *T. tajacu*, however, where both sexes contain all three springenes, we observe only (3*E*,6*E*,10*E*)-α-springene in female *T. pecari*. These isomers of springene were first reported from the dorsal gland of another artiodactyl, the springbok (*Antidorcas marsupialis*)

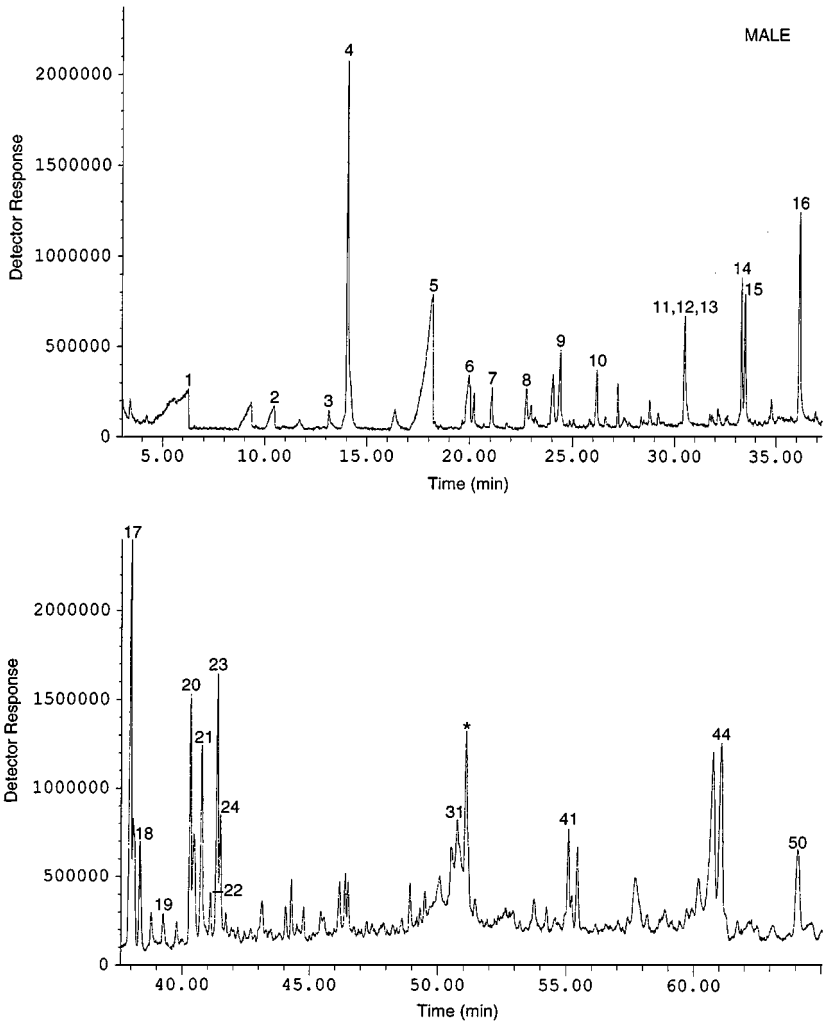


FIG. 2. Gas chromatograms of hexane extracts of dorsal gland secretions of eight male and 12 female *Tayassu pecari*.

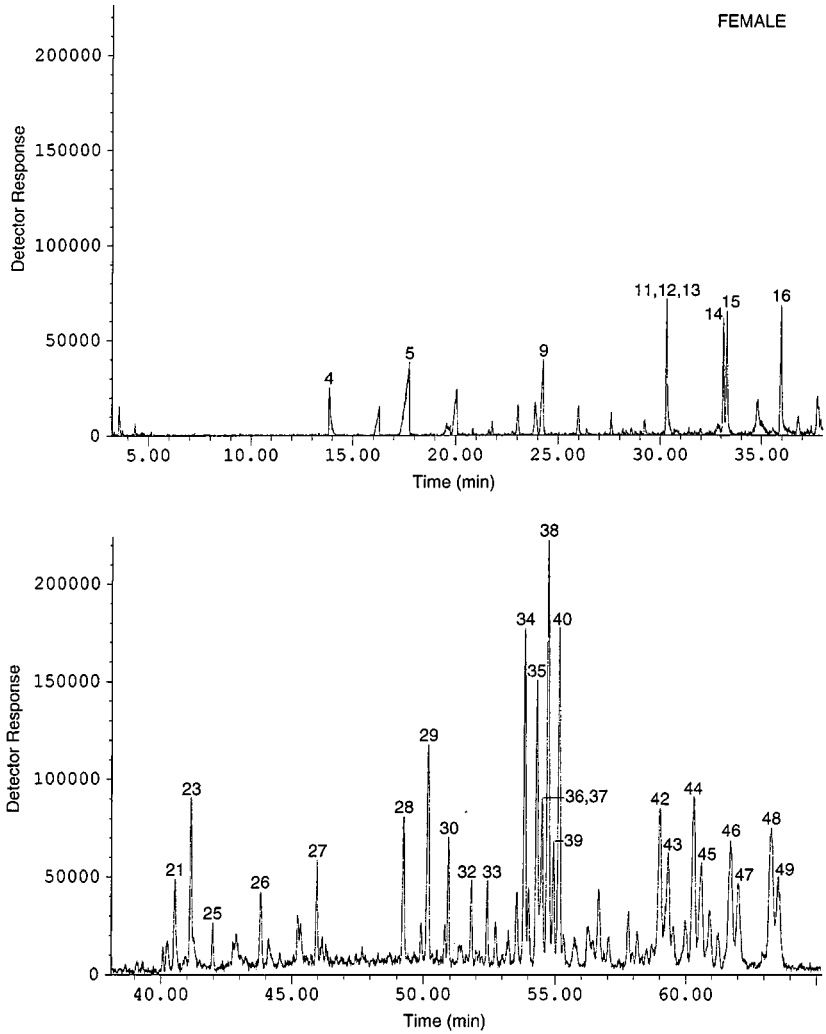


FIG. 2. Continued.

(Burger et al., 1978, 1981). Squalene (all *E* isomer) was detected in male *T. pecari*; this and another unidentified isomer of squalene were reported in both sexes of *T. tajacu* (Waterhouse et al., 1996).

We observed a variety of esters in *T. pecari*, which were largely confined to females. A total of 13 farnesyl esters were indicated in the females' secretions. The (*Z*,*Z*)-, (*E*,*Z*)-, (*Z*,*E*)-, and (*E*,*E*)-farnesyl decanoates in the secretions (**34**, **36**, **37**, and **40**, respectively) were resolved and identified by comparison of retention times

TABLE 1. COMPOUNDS IDENTIFIED IN DORSAL GLAND SECRETIONS OF MALE AND FEMALE *Tayassu pecari*

Compound no.[a]	Compound	Male	Female
Carboxylic acids			
1	2-Methylbutanoic acid	+[c]	–[c]
2	Hexanoic acid[b]	+	–
5	Octanoic acid[b]	+	+
6	Phenylacetic acid[b]	+	–
7	Nonanoic acid[b]	+	–
8	3-Phenylpropanoic acid	+	–
9	Decanoic acid[b]	+	+
10	2-Methyldecanoic acid	+	–
11	Dodecanoic acid[b]	+	+
16	Tetradecanoic acid[b]	+	+
19	Pentadecanoic acid	+	–
21	9-Hexadecenoic acid	+	+
23	Hexadecanoic acid[b]	+	+
26	Monounsaturated C_{18} carboxylic acid	–	+
27	9-(*Z*)-Octadecanoic acid (oleic acid)[b]	–	+
Alcohol			
4	2-Phenylethanol	+	+
Phenolics			
3	2-Methoxyphenol[b]	+	–
12	3-Methyl-4-hydroxyphenyl 2-methylpropanoate[b]	+	+
13	2-Methyl-4-hydroxyphenyl 2-methylpropanoate[b]	+	+
14	3-Methyl-4-hydroxyphenyl 2-methylbutanoate[b]	+	+
15	2-Methyl-4-hydroxyphenyl 2-methylbutanoate[b]	+	+
Lactones			
17	Monounsaturated C_{16}-lactone?	+	–
18	Monounsaturated C_{16}-lactone?	+	–
Hydrocarbons			
20	(6*E*,10*E*)-β-Springene[b,d]	+	–
22	(3*Z*,6*E*,10*E*)-α-Springene[b,d]	+	–
24	(3*E*,6*E*,10*E*)-α-Springene[b,d]	+	+
50	Squalene (all *E* isomer)[b,d]	+	–
Esters			
25	Ethyl nonanoate	–	+
28	Farnesyl C_8-alkanoate	–	+
29	Farnesyl C_8-alkanoate	–	+
30	Farnesyl C_8-alkanoate	–	+
31	Farnesyl C_8-alkanoate	–	+
32	Farnesyl alkanoate	–	+
33	Farnesyl alkanoate	–	+
34	(*Z*,*Z*)-Farnesyl decanoate[a]	–	+
35	Farnesyl C_{10}-alkanoate	–	+
36	(*Z*,*E*)- Farnesyl decanoate[b]	–	+
37	(*E*,*Z*)-Farnesyl decanoate[b]	–	+

TABLE 1. CONTINUED

Compound no.[a]	Compound	Male	Female
38	Farnesyl C_{10}-alkanoate	–	+
39	Farnesyl C_{10}-alkanoate	–	+
40	(*E*,*E*)-Farnesyl decanoate[b]	–	+
41	Alkyl C_{10}-alkanoate	+	–
42	C_{16}-Alkyl C_{10}-alkanoate	–	+
43	Hexamethyl 8-methylnonanoate[b,d]	–	+
44	C_{16}-Alkyl C_{10}-alkanoate	+	+
45	Hexadecyl decanoate[b,d]	–	+
46	Alkyl C_{10}-alkanoate	–	+
47	Alkyl C_{10}-alkanoate	–	+
48	Alkyl C_{10}-alkanoate	–	+
49	Alkyl C_{10}-alkanoate	–	+

[a] Compound numbers correspond to peak numbers shown in Figure 2.
[b] Identities confirmed by comparison of retention time with that of authentic compound.
[c] +, compound observed; –, compound not observed.
[d] Compounds also reported in *T. tajacu* (Waterhouse et al., 1996).

and mass spectra with those of the synthesized materials using CG-MS (second conditions). However, in both the synthesized mixture and in the secretion, the (*E*,*Z*)- and (*Z*,*E*)- isomers coeluted on GC analysis (Figure 2). Their mass spectra all show an acyl fragment at m/z 155 ($C_9H_{19}CO^+$). Compounds **35**, **38**, and **39** are also provisionally identified as farnesyl esters of C_{10} carboxylic acids, as there are very close similarities between their mass spectra and those of the synthetic farnesyl decanoates and they each show an acyl fragment at m/z 155 ($C_9H_{19}CO^+$). Full identification was not possible, but it is likely that these compounds have branching in the acid residues. Likewise, **28–31** are provisionally identified as farnesyl esters of C_8 carboxylic acids. They have similar mass spectra to those of the synthesized farnesyl esters and they show an acyl fragment at m/z 127 ($C_7H_{15}CO^+$). There is no acyl fragment observable in the mass spectra of **34** and **35**, but the similarities of their mass spectra to those of the other farnesyl esters suggests that they are also farnesyl alkanoates.

At least nine saturated esters (**41–49**) are indicated in *T. pecari.* Only two of them, hexyl 8-methylnonanoate and hexadecyl decanoate, were reported in *T. tajacu*; they were positively identified by comparison of retention times with those of the synthesized compounds (Waterhouse et al., 1996). The others were tentatively identified from their mass spectra alone, which indicate them to be alkyl C_{10}-alkanoates. Evidence for this is provided by an acyl $C_9H_{19}CO^+$ fragment at m/z 155 and a peak at m/z 173, which is in many cases the base peak and represents $C_9H_{19}CO_2H_2^+$ resulting from a double hydrogen transfer. The lack of identifiable molecular ions in the mass spectra of **41** and **46–49** does not allow the

identification of the alkyl group. Compound **42**, with a molecular ion at m/z 396 and a $C_9H_{19}CO^+$ fragment at m/z 155, is provisionally identified as a C_{16}-alkyl C_{10}-alkanoate. Similar high-molecular-weight saturated esters have been previously identified in the dorsal gland secretion of *T. tajacu* (Waterhouse et al., 1996), and esters **43** and **45** are common to both species. In *T. tajacu*, the esters are exclusive to females. Although saturated esters are not exclusive to females in *T. pecari*, they are more prominent in this sex.

We were unable to identify positively compounds **17** and **18** in the male secretions. The mass spectra of both compounds exhibit molecular ions at m/z 252 and are indicative of monounsaturated C_{16} lactones. The mass spectrum of **17** exhibits the following m/z (%) values: 252(4, M^+), 196(15), 111(11), 110(11), 109(15), 97(21), 96(24), 95(30), 83(25), 82(27), 81(36), 79(14), 69(40), 68(35), 67(46), 55(87), 54(27), 43(45), 42(25), 41(100). The mass spectrum of **18** exhibits the following m/z (%) values: 252(8, M^+), 196(6), 110(11), 109(17), 96(36), 95(37), 82(67), 81(63), 80(19), 79(24), 69(25), 68(45), 67(82), 55(68), 54(36), 43(31), 42(18), 41(100). This mass spectrum is nearly identical to that of isoambrettolide [(*Z*)-oxacycloheptadeca-10-en-2-one], a commercially available isomer of ambrettolide [(*Z*)-oxacycloheptadeca-8-en-2-one]; the latter naturally occurring C_{16} lactone is known from the ambrette seed (*Abelmoschus moschatus*) and is used as a fragrance in perfumery (Mookhergee et al., 1972; Sanz and Seoane, 1982). Isoambrettolide, however, had a retention time 1.5 min longer than that of **18**. Thus, while our data are consistent with the identification of 18 as an unsaturated C_{16} lactone, we suspend judgement on its detailed structure and that of the apparently related **17**.

Significant components of both the male and female secretion are four esters of methylhydroquinone: 2-methyl-4-hydroxyphenyl 2-methylpropanoate (**12**), 3-methyl-4-hydroxyphenyl 2-methylpropanoate (**13**), 3-methyl-4-hydroxyphenyl 2-methylbutanoate (**14**), and 2-methyl-4-hydroxyphenyl 2-methylbutanoate (**15**) (Figure 3). To the best of our knowledge, these acylated methylhydroquinones have not been reported previously. Although these compounds could be pheromonal, they could offer reductive protection to the readily oxidizable springenes, since they represent hydroquinones protected as the readily hydrolyzable monoesters. Other naturally occurring anti-oxidants based on the hydroquinone structure have been reported. For example, α-tocopherol hydroquinone (a metabolite of α-tocopherol containing a trimethylated hydroquinone unit) has been shown to be a more effective antioxidant scavenger than α-tocopherol itself (Shi et al., 1999). Phenolic compounds are known to occur in the skin gland secretions, urine, and other discharges of a variety of mammals (Albone, 1984). Burger et al. (1999) identified phenol, 3-methylphenol, 3-ethylphenol, and 3-propylphenol in the interdigital secretions of the bontebok (*Damaliscus dorcas dorcas*). More than 20 phenolics have been reported in castoreum, from the castor (anal) sacs of beavers (*Castor* sp., Rodentia) (Lederer, 1946; Tang et al., 1993). These compounds, which may function as pheromones (Müller-Schwarze, 1992), are thought to be derived from

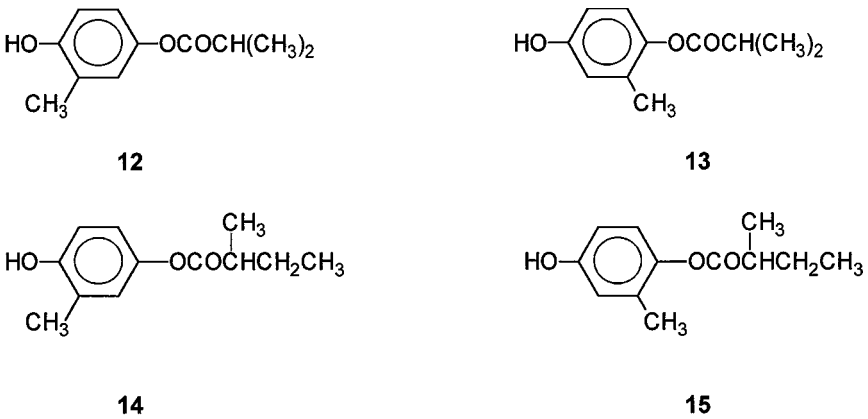


FIG. 3. Structures of monoalkanoyl esters of methylhydroquinone observed in dorsal gland secretions of *Tayassu pecari*.

woody plants, the dietary staple of beavers (Lederer, 1946; Tang et al., 1993). The phenolics that we have identified in the dorsal gland secretions of *T. pecari* may also be derived from plants. This species, like *T. tajacu*, subsists primarily on fruits and seeds (Barreto et al., 1997). *T. pecari*, however, consumes larger and harder seeds than does *T. tajacu*, a feature attributed to its greater bite force (Kiltie, 1982). The possibility that the different diets of these two peccaries underlie apparent differences in their dorsal gland chemistry needs to be examined.

Acknowledgments—We wish to thank W. B. Karesh, R. L. E. Painter, and W. B. Wallace for obtaining secretion samples; N. Tagua and J. Chuviqa for assisting in the field; D. Perrine and T. Spande for providing library searches for compounds; and Y.-Q. Li and K. Nakanishi for translations of papers. The Bolivian National Secretariat for Protected Areas granted permission to work at Noel Kempff Mercado National Park, and the National Directorate for Protection of Biodiversity issued permits. L. K. Sowls provided the photograph of alloanointing. We are grateful for gifts of isoambrettolide from J. Stephen (Cotswold Perfumery) and B. Narayanaswamy (Vinayak Impex Agencies, India). IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

REFERENCES

ALBONE, E. S. 1984. Mammalian Semiochemistry: The Investigation of Chemical Signals between Mammals. John Wiley & Sons, New York.

BARRETO, G. R., HERNANDEZ, O. E., and OJASTI, J. 1997. Diet of peccaries (*Tayassu tajacu* and *T. pecari*) in dry forest of Venezuela. *J. Zool. London* 241:279–284.

BROWN, D. M. 1963. Phosphorylation. *Adv. Org. Chem.* 3:115–147.

BURGER, B. V., LE ROUX, M., SPIES, H. S. C., TRUTER, V., and BIGALKE, R. C. 1978. Mammalian pheromone studies—III. (*E,E*)-7,11,15-Trimethyl-3-methylenehexadeca-1,6,10,14-tetraene, a

new diterpene analogue of β-farnesene from the dorsal gland of the springbok, *Antidorcas marsupialis. Tetrahedron Lett.* 52:5221–5224.

BURGER, B. V., LE ROUX, M., SPIES, H. S. C., TRUTER, V., and BIGALKE, R. C. 1981. Mammalian pheromone studies, IV. Terpenoid compounds and hydroxy esters from the dorsal gland of the springbok, *Antidorcas marsupialis. Z. Naturforsch.* 36C:340–343.

BURGER, B. V., NELL, A. E., SPIES, H. S. C., LE ROUX, M., BIGALKE, R. C, and BRAND, P. A. J. 1999. Mammalian exocrine secretions. XII: Constituents of interdigital secretions of bontebok, *Damaliscus dorcas dorcas*, and blesbok, *D. d. phillipsi. J. Chem. Ecol.* 25:2057–2084.

EPLING, G. P. 1956. Morphology of the scent gland of the javalina. *J. Mammal.* 37:246–248.

HERNANDEZ, O. E., BARRETO, G. R., and Ojasti, J. 1995. Observations of behavioural patterns of white-lipped peccaries in the wild. *Mammalia* 59:146–148.

KHORANA, H. G. 1953. The chemistry of carbodiimides. *Chem. Rev.* 53:145–168.

KILTIE, R. A. 1982. Bite force as a basis for niche differentiation between rain forest peccaries (*Tayassu tajacu* and *T. pecari*). *Biotropica* 14:188–195.

KILTIE, R. A. and TERBORGH, J. 1983. Observations on the behavior of rain forest peccaries in Perú: Why do white-lipped peccaries form herds? *Z. Tierpsychol.* 62:241–255.

LEDERER, E. 1946. Chemistry and biochemistry of the scent glands of the beaver (*Castor fiber*). *Nature* 157:231–232.

MOOKHERJEE, B. D., TRENKLE, R. W., and PATEL, R. R. 1972. Synthesis of Δ^9-isoambrettolide and its isomers from 1,9-cyclohexadecadiene. *J. Org. Chem.* 37:3846–3848.

MÜLLER-SCHWARZE, D. 1992. Castoreum of beaver (*Castor canadensis*): Function, chemistry and biological activity of its components, pp. 457–484, *in* R. L. Doty and D. Müller-Schwaze (eds.). Chemical Signals in Vertebrates VI. Plenum Press, New York.

SANZ, V. and SEOANE, E. 1982. Synthesis of ambrettolide from phloionolic acid. *J. Chem. Soc. Perkin Trans I* 1982:1837–1839.

SHI, H. L., NOGUCHI N., and NIKI, E. 1999. Comparative study on dynamics of antioxidative action of α-tocopherol hydroquinone, ubinol and α- tocopherol, against peroxidation. *Free Radic. Biol. Med.* 27:334–346.

SOWLS, L. K. 1984. The Peccaries. University of Arizona Press, Tucson, Arizona.

TANG, R., WEBSTER, F. X., and MÜLLER-SCHWARZE, D. 1993. Phenolic compounds from male castoreum of the North American beaver (*Castor canadensis*). *J. Chem. Ecol.* 19:1491–1500.

WATERHOUSE, J. S., KE, J., PICKETT, J. A., and WELDON, P. J. 1996. Volatile components in dorsal gland secretions of the collared peccary, *Tayassu tajacu* (Tayassuidae, Mammalia). *J. Chem. Ecol.* 22:1307–1314.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

ODORANT SOURCE USED IN EURASIAN BEAVER TERRITORY MARKING

FRANK ROSELL[1,2,*] and LARS JØRAN SUNDSDAL[1]

[1]*Faculty of Arts and Sciences*
Department of Environmental and Health Studies
Telemark University College
N-3800 Bø i Telemark, Norway

[2]*Department of Zoology*
Norwegian University of Science and Technology
N-7491 Trondheim, Norway

(Received March 28, 2001; accepted August 17, 2001)

Abstract—Mammals use urine, feces, or the secretion of specialized skin glands to mark their territories. These sources can carry different information and, thus, have different functions. Presently it is not known if beavers (*Castor* spp.) deposit castoreum (primarily a mixture of secondary metabolites from urine) from the castor sacs and secretion from the anal glands (AGS) together or alone when scent marking their territories. We hypothesized that castoreum would be the main scent signal used in the defense of beaver territories during winter and predicted that castoreum would be deposited more often than AGS. A total of 96 scent marks on snow were collected from January 1 to March 31, 1997–1999 in the Bø River, Telemark County, Norway. In order to obtain control material, we chemically analyzed AGS and castoreum from 60 dead beavers collected during January–May 1997–1999. We compared the compounds found in the dead beavers with compounds found in the scent marks on snow. Samples were analyzed by using gas chromatography–mass spectrometry (GC-MS). All 96 scent marks contained compounds from castoreum, whereas compounds from AGS were found in only four scent marks. This suggests that beavers do not specifically deposit AGS on scent mounds as they do with castoreum and that the AGS compounds we found probably were remnants of AGS from the feet or fur following pelt lubrication or coprophagy behavior. We conclude that castoreum is the main scent signal used in the defense of beaver territories during winter.

Key Words—Beaver, *Castor fiber*, anal gland secretion, castor sacs, castoreum, skin glands, snow-secretion.

* To whom correspondence should be addressed. E-mail: Frank.Rosell@hit.no

INTRODUCTION

Communication and social recognition in many mammals are based on olfactory signals (Wynne-Edwards, 1962; Ralls, 1971; Schulte et al., 1994), and they use urine, feces, or the secretion of specialized skin glands to mark their territories (e.g., Müller-Schwarze, 1983; Gorman, 1984). Recent studies have demonstrated that scent types can carry different information and, thus, have different functions (Johnston et al., 1993). For instance, the study by Gorman et al. (1978) on otters (*Lutra lutra*) showed that deposits of spraints and urine may be used in the maintenance of otter territories, while the deposits of anal gland secretion (AGS) sometimes found at the latrines appear to have another function. The primary roles of skin glands of carnivores are the maintenance of the pelage and thermoregulation (Gorman and Trowbridge, 1989). The same scent may also code for different information and, thus, serve multiple functions (e.g., Quay and Müller-Schwarze, 1971; Epple et al., 1979; Johnston, 1985), while different scents may carry the same information (Baldwin and Meese, 1977; Roeder, 1980; Martin and Beauchamp, 1982).

Eurasian beaver (*Castor fiber*) and North American beaver (*C. canadensis*) possess two pairs of organs: castor sacs and anal glands (Svendsen, 1978; Walro and Svendsen, 1982). Both are suspected to be used for scent marking during territory defense (e.g., Rosell and Bergan, 1998). These are located in two cavities between the pelvis and base of the tail (Walro and Svendsen, 1982; Valeur, 1988). The anal gland is a holocrine secretory gland, but the castor sac is only a pocket lined with a layer of nonsecretory epithelium. They both open into the urogenital pouch (cloaca) (Svendsen, 1978). The castor sac is used to store what is believed to be a mixture of secondary metabolites from urine, collectively called castoreum (Walro and Svendsen, 1982). Copious amounts of castoreum deposited on scent mounds result from a process not dissimilar to urination except that the urine flushes through the contents of the castor sacs. This material can be deposited on the scent mound without the animal contacting the substrate with the cloacal region. The anal gland papillae, however, must be rubbed on the substratum in order to deposit the exudates (Wilsson, 1971; Svendsen, 1978). All age classes (except kits younger than 5 months) and both sexes defend their territories by scent marking (Wilsson, 1971; Svendsen, 1980; Welsh and Müller-Schwarze, 1989; Nolet and Rosell, 1994; Buech, 1995). Scent is usually secreted by the Eurasian beaver onto small piles of mud and debris scraped together and placed close to the water's edge and at the borders of the territory throughout the entire year (Rosell and Nolet, 1997; Rosell et al., 1998).

Both beaver species are suspected to use castoreum more frequently than AGS when scent-marking the territories (Schulte et al., 1994, 1995; Rosell and Bergan, 1998). Schulte (1993) discovered by chemical analysis that many compounds in the North American beaver scent mounds ($N = 4$) were common to castor sacs and castor fluid collected from live beavers. The presence of AGS compounds

was not examined. Field observations indicate that beavers scent mark using only castoreum, only AGS, or sometimes both (Rosell and Bergan, 1998). The odor of beaver scent marks, as detected by humans, varies greatly within site over time. Whether this is due to different beavers or different scents (castoreum and AGS smell different), or to a change in the compounds, is uncertain (Rosell and Bergan, 1998).

Castoreum consists of phenolic, neutral, basic, and acidic components (Tang et al., 1995). No clear sex difference has been detected so far (Pedersen, 1999; Sun and Müller-Schwarze, 1999). Chemical analyses of the AGS of Eurasian beavers have revealed that AGS contains free fatty acids, fatty alcohols, coprostanone, cholestenols, cyclic triterpenes, sterol esters, and wax esters. Wax esters are found only in males, whereas females possess fatty acids (Grønneberg, 1978–1979; Grønneberg and Lie, 1984). Most of the compounds in castoreum have a low molecular weight (Tang et al., 1993, 1995; Pedersen, 1999), while most of the lipids in AGS have a molecular weight above 300 (Grønneberg, 1978–1979; Grønneberg and Lie, 1984; Sun, 1996). The upper size limit for airborne pheromones is a molecular weight of about 300 (e.g., Wilson, 1963; Bradbury and Vehrencamp, 1998). Thus, AGS will normally not be volatile enough to act as an effective chemical messenger through air. One function suggested for AGS is that it serves to waterproof the fur (Walro and Svendsen, 1982). Excision of the anal glands seems to reduce the ability of the pelage to repel water (Walro and Svendsen, 1982). Prohibition of autogrooming in Eurasian beaver produces similar results (Wilsson, 1971). Both the glands that produce a secretion that waterproofs and maintains the fur and the motor patterns associated with application of the secretion would be expected to develop early in semiaquatic animals. Such is the case in beavers (Walro and Svendsen, 1982). However, several researchers have found that AGS can elicit territorial responses, similar to those shown to castoreum (Hodgdon, 1978; Müller-Schwarze et al., 1986). The secretion could be well suited to long-term signaling and may be deposited around the territory borders for the purpose of territory defense.

Castoreum may be an ideal substance for scent marking the territory because it has a minimal energetic cost to the signaler. Selection for effective signal-sending behavior harnesses odors that are already available at no extra cost (Müller-Schwarze, 1999). The large number of phenolics and terpenes in castoreum (Tang et al. 1993, 1995), undoubtedly diet-derived, may, therefore, constitute an honest signal, advertising the physical condition of the individual and, indirectly, the food supply in the territory (Müller-Schwarze, 1999). As such, beavers may have evolved a unique organ to store and excrete the secondary defense compounds produced by plants, which may in turn be used in territorial scent marking.

Presently it is not known if beavers deposit castoreum and AGS together or alone when scent marking their territories. Neither is it known how often beavers deposit castoreum compared with AGS. The aim of this study was to investigate and

to search for characteristic chemical compounds from the castor sacs and the anal glands in scent deposited on snow in an effort to resolve this issue. We hypothesized that castoreum is the main scent signal used in the defense of beaver territories during winter, and predicted that castoreum is deposited more often than AGS.

METHODS AND MATERIALS

Study Area and Animals. The study was conducted on two distinct sections of the Bø River in the municipalities of Bø and Sauherad (59°25′N, 09°03′–04′E) in Telemark County, Norway. Section 1 was 13 km long, section 2 was 11 km, and the sections were 5 km apart. The sections of the river studied averaged 35 m in width, and most of the river was ice-free during winter due to hydroelectric regulation further upstream. This provided us with the opportunity to study scent-marking behavior of beaver uninhibited by the usual constraints of winter ice (Rosell et al., 1998). Rosell et al. (1998) and Rosell and Bergan (2000) observed that scent was deposited on snow during winter in our study area. Snow, in contrast to the mud and debris normally used to build scent mounds, provides a suitable substrate for determining the deposition frequency of castoreum and AGS on scent mounds. Beavers have occupied the river since the 1920s (Olstad, 1937), and colony density was believed to be near maximum during our field studies (0.54–0.73 colonies/stream km in 1998). During October–December 1996–1998, the study area was ground-censused for active colonies by recording food caches, new mud on the lodges, and fresh feeding sites. Eight active colonies were found on section 1 in 1996 and 1997, and 7 in 1998. Eight active colonies were present on section 2 in 1998 (section 2 was not studied in 1996 and 1997). During autumn 1995, the average colony size on the Bø River (section 1) was 4.0 ± 0.6 (SD) (Rosell et al., 1998). Territorial boundaries were drawn based on the location of scent mound concentrations (Rosell and Nolet, 1997; Rosell et al., 1998) and from sight observations of animals moving up- and downstream of the lodge (Rosell et al., 1998).

Sample Collection. We collected a total of 96 scent samples on snow (hereafter called snow-scent sample, SSS) between January 1 and March 31, 1997–1999 during 14 trips in the study area. Eighteen samples were collected during 1997, 35 during 1998, and 43 during 1999. A SSS was defined as a scent mark directly on snow or in the ice (frozen), situated on a snow-covered tussock or snow mound scraped together where fluid or secretion from the castor sacs and/or anal glands had been deposited (Rosell et al., 1998). All scent marks had a detectable odorant to the human nose at 2 cm or more. Minimum distance between two different scent marks was 10 cm (Rosell and Bergan, 2000). All scent marks were collected between 08:00 and 13:00 hr and within 24 hr after the last snowfall. Beavers usually had one night without snowfall during which they scent-marked before

the marks were collected. A binocular was used to spot the scent marks from a canoe. All tracks from the water in the snow and a range of colors in the snow were investigated.

Samples from 1997 and 1998 were collected using a plastic bag as a glove to prevent contamination of the samples from skin contact. As much of the affected snow as possible was collected, judging from the light yellow to red colored stains seen at depths of 1–3 cm. Frozen samples were loosened with a clean axe. None of the samples contained debris or organic compounds (e.g., soil, mud, twigs, grass, leaves, or conifer needles) normally used to build scent mounds. The plastic bags were sealed and samples thawed at room temperature in the laboratory, emptied into sterile 100 ml plastic or glass vials with caps to minimize bacterial contamination, and then swirled thoroughly to assure a homogeneous mixture. In 1999, samples were collected directly into a plastic or glass cup with airtight caps and swirled thoroughly. The samples were then immediately frozen and stored at -20°C until analyzed. On each sampling day, we also collected control samples of scent-free snow ($N = 14$) into one vial in all territories where we found SSS with the same methods in order to check for contamination from the plastic and glass equipment used to collect the samples.

We collected castor sacs and anal glands from 60 animals shot locally during the normal hunting season from January 28 to May 6, 1997–1999 (Table 1) (Parker and Rosell, 2001), and used the castoreum and AGS from these animals as the basis for comparison with castoreum and AGS found in the SSS. We opened the castor sacs with a surgical blade and scraped the castoreum from the inside surface with a metal scapula. AGS was collected from the glands by cutting off the last 2–3 mm of the papillae and squeezing out the secretion (Rosell and Sun, 1999; Rosell et al., 2000). Sun and Müller-Schwarze (1998a) found no significant variation in the chemical compounds from the right and left gland of the North American beaver, and we, therefore, collected all secretion in the same glass vial. All samples were stored at -20°C until analyses. We sexed the animals by checking for the presence

TABLE 1. CONTROL SAMPLES OF CASTOREUM AND ANAL GLAND SECRETION (AGS) FROM DEAD MALE (M, $N = 30$) AND FEMALE (F, $N = 30$) EURASIAN BEAVERS OF DIFFERENT AGE[a]

Age[b]	Male		Female	
	Castoreum	AGS	Castoreum	AGS
<1 year (6 M, 7 F)	2	5	5	5
≥1 to 2 year (10 M, 6 F)	9	8	5	5
2.5–14 year (14 M, 17 F)	8	7	10	10
$\sum$	19	20	20	20

[a] Samples were collected from January 28 to May 6, 1997–1999.
[b] See methods and materials.

or absence of the os penis (Osborn, 1955). Age was determined by examining tooth root closure and annual cementum and dentine layers of the first molar (van Nostrand and Stephenson, 1964).

Control Experiment. We conducted a control experiment during April 2000 to check whether our methods could detect the compounds from the castor sacs and the anal glands in the SSS. We simulated beaver scent-marking activity by constructing artificial scent mounds of snow, on snow, and applied castoreum and AGS to them. We used scent material from the same animals (all adults 2.5–14 years old plus the number of male subadults necessary to reach a sample size of $N = 10$, see Table 1) as described above. A sample was taken from a castor sac or an anal gland, divided in two, and distributed randomly to the snow control experiment (SCE) and the lab control experiment (LCE). Ten mounds were treated with castoreum (0.5 g) from males, 10 with castoreum (0.5 g) from females, 10 with AGS (0.25 ml) from males, and 10 with AGS (0.25 g) from females. AGS from females has a high viscosity, so we used a spatula to take out the secretion from the vials and weighed it on an electronic balance. We collected the artificial scent marks 20 hr after being made using the same methods as described above for collection.

*Sample Preparation.*The SSS, including the scent-free controls, and the SCE were thawed by putting the bottles into a water bath at 35–40°C for 10 min. Afterwards, the samples were transferred to a 250-ml Erlenmeyer flask with ground joint. We added 10 ml of toluene, and the solution was swirled using a magnetic stirrer at high velocity (about 1400 rpm) for 10 min. The solution was then transferred to a separatory funnel, where it remained for 15–20 min. to allow the phases to separate. The lower water phase was tapped out and the upper organic phase filtered through a filter paper (Schleicher and Schuell no. 595, Dassel, Germany) into an evaporating flask. A PTFE sleeve for ground joints was set in the flask neck to avoid locking. The sample was evaporated in a Heidolph WB 2001 rotary evaporator to a volume of variable size (therefore, we only carried out qualitative analyses), and 1 ml of toluene was added. The solution was transferred to a vial with a snap cap that was kept in a refrigerator (<24 hr) at 4°C until the analysis was done. We also analyzed the water phase. Some of the compounds from the organic phase were found in the water phase as well, but in lower concentrations. However, we found no new compounds in the water phase.

AGS and Castoreum. From the control material (the dead animals), we transferred 0.25 ml AGS from the males and 0.25 g from the females into smaller glass vials before adding 7.5 ml of a mixture of toluene–methanol 3:1. The solutions were filtered through a filter paper (Schleicher and Schuell no. 595) and kept in a refrigerator (< 24 hr) at 4°C until analyses.

We weighed 0.5 g castoreum and added 10 ml of toluene–methanol 3:1. The sample was extracted in a Milestone MLS 1200 Mega microwave oven. The extraction program had the following cycle: 5 min at 250 W and 100°C, 5 min at

400 W and 130°C, and 5 min at 400 W and 160°C. All compounds were dissolved. The sample was then treated the same way as the AGS solution.

Chemical Analysis. We injected 1 μl of the resulting solution into a Hewlett-Packard 6890 Series II gas chromatograph equipped with a nonpolar HP-5 MS 5% phenyl-methyl-siloxane column (30.0 m long $\times$ 0.25 mm ID $\times$ 0.25-μm film thickness) connected to a Hewlett-Packard 5973 Series mass spectrometer detector with a split/splitless inlet used in the splitless mode. Helium was used as the carrier gas at a constant flow of 0.7 ml/min. The following temperature program was employed for all analyses: 130°C to 310°C at 4°C/min and kept at 310°C for 15 min. To avoid that the solvent damaged the detector, a delay of 2 min was set for every run. We used the first 60 min as the collection time because few compounds were detected after 60 min and most of these peaks stemmed from the column material. The column was regularly baked out at 315°C to remove any remaining low-boiling-point compounds. The instrument was regularly calibrated to detect possible changes in the sensitivity of the instrument. This prevented unstable conditions during the work. We characterized each compound in a sample by its GC retention time and mass spectrum, and we determined the structures of some of the compounds with a computer-aided compound search of the Wiley 275 Library, which contains about 70,000 known compounds. In addition, all mass spectra from the samples and from the library were visually compared to see if the suggestions from the computer were reasonable. We also made our own library of the compounds not found in the Wiley library, i.e., we saved 12 compounds from the castor sacs and 128 compounds from the anal glands from the dead individuals. We could, therefore, recognize many compounds from individual to individual, and also from individuals to the SSS. Since the main focus of our study was to determine if the beaver used castoreum and/or AGS in their scent-marking behavior, positive identification of these compounds was not attempted, i.e., we did not compare the compounds with a known standard. All major peaks were analyzed, but excessively small peaks were not, i.e., peaks less than twice the noise level were not analyzed. After numerous injections, we could see a small shortening in the retention times of the peaks because the column aged a little. We, therefore, presented the retention time found when the column was new. Peak retention times were rounded off to the first decimal.

Statistics. Since the data did not fit assumptions of distribution and homogeneity of variance for parametric analysis (Sokal and Rohlf, 1995), we used nonparametric statistics (Siegel and Castellan, 1988). A Mann-Whitney U test was used to compare the median number of peaks between two independent groups. We corrected for compounds in the plastic and glass equipment used to collect the samples when counting the number of castoreum and AGS compounds and omitted the smallest peaks (see above). Probability values are two-tailed and 5% was used as the level of significance. All data were treated in Minitab version 12.1 for Windows.

RESULTS

Castoreum. Chemical analysis of the 96 SSS revealed that castoreum compounds were present in all samples ($\bar{X} \pm \text{SD} = 6.75 \pm 4.18$, range = 1–17). Figure 1a is a typical example. We found 24 different compounds from castoreum in the SSS (Table 2). None of the compounds was found in all SSS, and 17 of the compounds were found in 10 or more of the SSS (Table 2). Forty-seven of the 50 (94.0%) compounds detected in the castoreum from the dead animals had a molecular weight below 300 (Table 3).

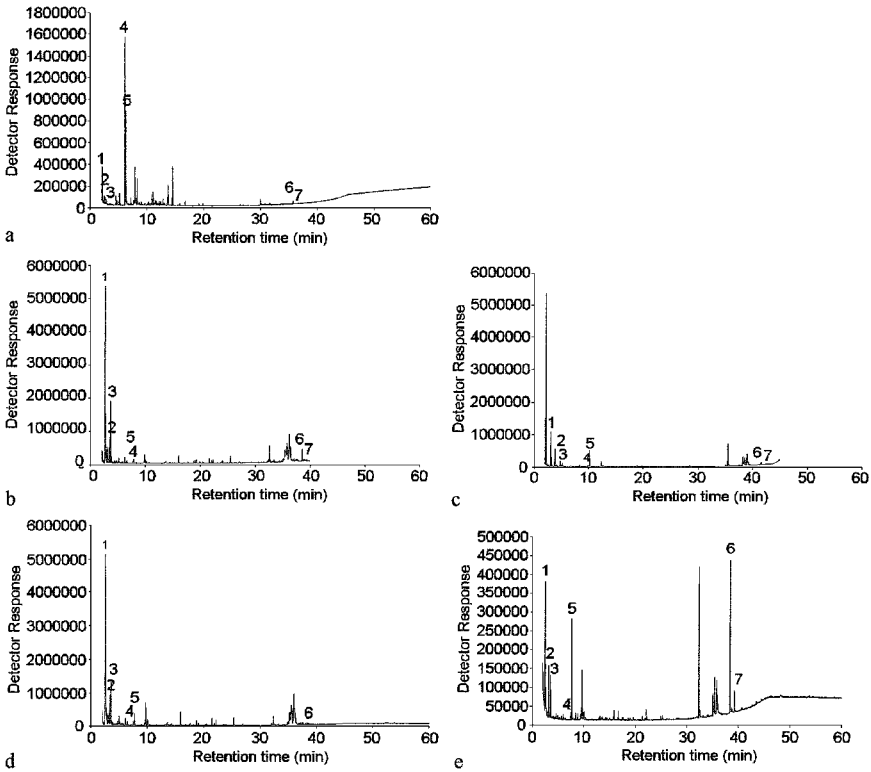


FIG. 1. Typical chromatograms of castoreum from (a) a beaver snow-scent sample, (b) a dead adult male, (c) a dead adult female, (d) from the snow control experiment with the same male as in b and (e) the same female as in c. Compound marked 1–7 in the chromatograms are tentatively identified as: 1, ethylphenol; 2, prophylphenyl; 3,4-ethyl-2-methoxyphenol; 4,4-(4-hydroxyphenyl)-2-butanone; 5, 4-(4-hydroxyphenyl)-2-butanol; 6, cholest-5-ene-3-ol; and 7 cholest-7-ene-3-ol (3β,5-α). Note that identities of the compounds have not been verified with known samples (see Methods and Materials). The *x* axis is the time in minutes, and the *y* axis is in arbitrary units.

TABLE 2. TENTATIVELY IDENTIFIED COMPOUNDS FOUND IN 96 SNOW-SCENT SAMPLES FROM EURASIAN BEAVERS

No.	Tentatively identified compound[a]	Retention time (min)	Samples (*N*)	Source[b] (Sex)[c]
1	Ethylphenol	2.2	75	c
2	Borneol	2.3	3	c
3	α-Terpineol	2.4	5	c
4	Myrthenol	2.4	12	c
5	Methoxymethylphenol	2.4	6	c
6	Verbenone	2.5	35	c
7	3-phenylpropanol	2.6	34	c
8	Propylphenol	2.7	66	c
9	2-Hydroxybenzylalcohol	2.8	29	c
10	4-Ethyl-2-methoxyphenol	2.9	33	c
11	Unknown	3.6	7	c
12	4-Hydroxyacetophenone	4.6	33	c
13	Unknown	5.0	12	c
14	4-Hydroxy-3-metoxyacetophenone	5.3	54	c
15	4-Hydroxy-3-methoxybenzoic acid, methylester	5.7	38	c
16	4-(4-Hydroxyphenyl)-2-butanone	6.3	49	c
17	4-(4-Hydroxyphenyl)-2-butanol	6.5	44	c
18	4-(4-Hydroxy-3-methoxyphenyl)-2-butanone	8.0	13	c
19	Unknown	8.1	10	c
20	4-(4-Hydroxy-3-methoxyphenyl)-2-butanol	8.5	15	c
21	Unknown	14.0	4	c
22	Unknown	14.7	10	c
23	Hexadecanoic acid	16.5	1	a (F)
24	Unsaturated C_{14} hydrocarbon	18.3	3	a (M)
25	Double unsaturated C_7–C_7 ester	25.1	2	a (M)
26	Double unsaturated C_7–C_8 ester	26.3	2	a (M)
27	Unknown	30.3	7	c
28	Hydrocarbon	31.7	2	a (F)
29	Unsaturated C_{30} hydrocarbon	32.3	3	a (F)
30	C_{29} wax ester	37.2	1	a (M)
31	A steroid	37.8	1	a (M, F)
32	Cholestan-3-ol (3-β,5-α)	37.8	1	a (M, F)
33	Unknown	38.0	1	a (M, F)
34	Saturated C_{15}–C_{15} wax ester	38.1	1	a (M)
35	Unsaturated C_{31} wax ester	38.4	3	a (M)
36	A steroid	38.5	1	a (M, F)
37	Cholest-7-ene-3-ol (3-β,5-α)[d]	39.2	4	a (F) or c
38	4-α-Methylcholest-8(14)-ene-3-β	39.8	1	a (M, F)
39	Saturated C_{31} wax ester	40.3	1	a (M)
40	A steroid	41.4	1	a (F)
41	A steroid	42.0	1	a (F)
42	Unknown	44.8	1	a (M)
43	A steroid	45.8	1	a (M, F)

[a] The identities have not been verified with known samples.
[b] Also found in the castoreum (c) or anal gland secretion (a) samples from dead beavers (control material) (see Tables 3 and 4).
[c] M = male and F = female.
[d] Note that this compound was found in both castoreum and anal gland secretion of females (no. 50 in Table 3 and no. 107 in Table 4).

TABLE 3. TENTATIVELY IDENTIFIED COMPOUNDS FOUND IN CASTOREUM FROM DEAD MALE ($N = 19$) AND FEMALE ($N = 20$) EURASIAN BEAVER[a]

No.	Tentatively identified compound[b]	Retention time (min)	Samples (N)	
			Male	Female
1	Cyclohexandiol	1.8	6	7
2[c]	2-Methylphenol	1.8	13	14
3	Undecane	1.9	1	0
4[c]	Benzenemethanol	2.0	17	19
5[c]	Ethylphenol (1)	2.2	19	20
6[c]	Benzoic acid	2.2	2	5
7[c]	Borneol	2.3	12	12
8[c]	1,2-Benzenediol	2.3	4	7
9[c]	α-Terpineol	2.4	11	11
10[c]	Myrthenol	2.4	14	14
11	2-Hydroxybenzoic acid	2.4	1	0
12	Methoxymethylphenol	2.4	2	3
13[c]	Verbenone	2.5	16	16
14[c]	3-Phenylpropanol	2.6	9	13
15[c]	Propylphenol (2)	2.7	18	18
16[c]	2-Hydroxybenzylalcohol	2.8	13	17
17[c]	Myrthanol	2.8	3	1
18[c]	1,4-Benzenediol	2.8	1	0
19[c]	4-Ethyl-2-methoxyphenol (3)	2.9	17	16
20[c]	Unknown	3.2	1	2
21[c]	Benzenepropanoic acid	3.4	3	4
22[c]	Aromatic carboxylic acid	3.4	3	5
23	Phenyl propanoic acid	3.4	3	1
24	Unknown	3.6	3	2
25[c]	2-Methoxy-4-propylfenol	3.6	6	9
26[c]	5-Methyl-1,3-benzenediol	3.6	2	6
27[c]	4-Ethyl-1,3-benzenediol	3.8	3	8
28[c]	3,4-Dihydro-2H-1-benzophyrane-2-one	4.1	9	9
29[c]	4-Methoxy benzoic acid	4.4	5	5
30[c]	2-(4-Hydroxybenzene)-ethanol	4.4	9	12
31[c]	4-Hydroxyacetophenone	4.6	9	12
32[c]	Unknown	5.0	14	15
33[c]	3-Hydroxy benzoic acid	5.2	8	7
34[c]	4-Hydroxy-3-methoxy acetophenone	5.3	13	11
35	4-Hydroxy-3-methoxybenzoic acid, methylester	5.6	3	1
36[c]	4-Hydroxy-3-methoxymethanoic acid	5.7	6	10
37[c]	4-(4-Hydroxyphenyl)-2-butanone (4)	6.3	19	20
38[c]	4-(4-Hydroxyphenyl)-2-butanol (5)	6.5	19	20
39	Unknown N-compound	7.2	2	3
40[c]	4-(4-Hydroxy-3-methoxyphenyl)-2-butanone	8.0	2	4
41	4-Hydroxy-3-methoxy-benzoacetic acid	8.0	0	1

TABLE 3. CONTINUED

No.	Tentatively identified compound[b]	Retention time min	Samples (*N*) Male	Female
42[c]	Unknown	8.1	14	18
43[c]	4-(4-Hydroxy-3-methoxyphenyl)-2-butanol	8.5	7	7
44[c]	unknown	14.0	11	14
45[c]	Unknown	14.7	10	11
46[c]	Unknown	30.3	13	18
47[c]	Unknown	33.5	17	18
48[c]	Unknown	33.8	19	20
49[c]	cholest-5-ene-3-ol (6)	36.1	11	14
50[c]	cholest-7-ene-3-ol (3-beta, 5-alpha) (7)	39.2	5	9

[a] Numbers in parentheses match those in Figure 1.
[b] The identities have not been verified with known samples.
[c] Compounds were found both in the snow control experiment and in the dead animals used in the control experiment.

We found no difference in median number of castoreum compounds between dead males and females (males: $\bar{X} \pm \text{SD} = 22.53 \pm 2.86$, range = 18–29, $N = 19$; females: $\bar{X} \pm \text{SD} = 23.25 \pm 3.95$, range = 12–29, $N = 20$) ($W = 338.0$, $P = 0.241$). Figure 1b and 1c show typical chromatograms from a dead adult male and female. Forty-six (92%) of the detected compounds were found in both sexes. Three compounds were detected in only males and one in only females (Table 3). However, they were found in only one animal (Table 3). Forty-one (82%) compounds were found both in the SCE and in the LCE (Table 3) (compare Figure 1b and d, and 1c and e). This shows that our method could detect the compounds from the castor sacs in the SSS.

AGS. Chemical analysis of the 96 SSS revealed that AGS compounds were present in only four samples ($\bar{X} \pm \text{SD} = 7.50 \pm 4.93$, range = 2–14). Figure 2a is a typical example. These four scent marks were found on January 19, 1997; February 5 and March 13, 1998; and on March 15, 1999; they were inside four different territories. Three of the SSS were deposited directly on snow, while one was deposited on a snow-covered tussock. We found that two of the scent marks (January 19, 1997, and March 15, 1999) contained compounds from only females, and two contained compounds that were typical for both sexes. We found 20 different compounds from AGS in the SSS (Table 2).

More compounds were found in the AGS from dead males ($\bar{X} \pm \text{SD} = 55.40 \pm 10.81$, range = 29–69, $N = 20$) compared with females ($\bar{X} \pm \text{SD} = 22.70 \pm 4.93$, range = 14–30, $N = 20$) ($W = 607.5$, $P = 0.0001$). Figure 2b and c show typical chromatograms from a dead adult male and female. Overall, 56 different compounds were found in the female AGS and 126 compounds in the

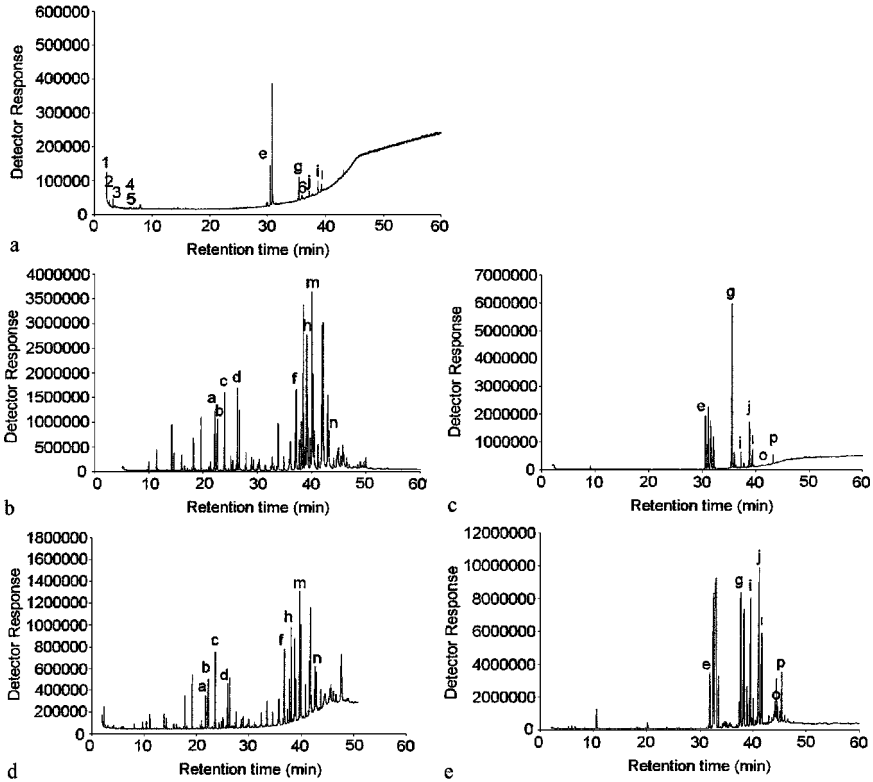


FIG. 2. Typical chromatograms of anal gland secretion from (a) a beaver snow-scent sample deposited by a female (contains both compounds form castoreum and AGS), (b) a dead adult male, (c) a dead adult female, (d) from the snow control experiment with the same male as in b and (e) the same female as in c. Compunds marked form a–p in the chromatograms are tentatively identified as: a, hexadecadiene; b, unknown; c, unknown; d, long hydrocarbon chain; e, unsaturated C_{30} hydrocarbon; f, C_{29} wax ester; g, unknown; h, usaturated wax ester; i, a steroid; j, 4-α-methylcholest-8(14)-ene-3-β; k, unknown; p, a steroid; m, unsaturated C_{32} wax ester; n, saturated C_{33} wax ester; o, a steroid; and p, a steroid. Note that the identities of the compounds have not been verified with known samples (see Methods and Materials). The *x* axis is the time in minutes, and the *y* axis is in arbitray units.

male AGS (Table 4). Nineteen compounds were found in both males and females (Table 4). Only 7 (12.5%) of the compounds detected in AGS of females and 41 (32.5%) of the compounds in the males had a molecular weight below 300 (Table 4). One-hundred thirty-three (89.3%) compounds were found both in the SCE and in the LCE (both sexes combined) (Table 4) (compare Figure 2b and d, and 2c and e). Therefore, our method detected the compounds from the anal gland in the SSS.

TABLE 4. TENTATIVELY IDENTIFIED COMPOUNDS FOUND IN ANAL GLAND SECRETION OF DEAD MALE ($N = 20$) AND FEMALE ($N = 20$) EURASIAN BEAVER[a]

No.	Tentatively identified compound[b]	Retention time (min)	Samples (N)	
			Male	Female
1[c]	Dodecanoic acid	7.6	4	0
2[c]	1,13-Tetradecadiene	9.8	8	0
3[c]	Tetradecanoic acid, methyl ester	9.8	5	0
4[c]	Unsaturated or cyclic hydrocarbon	10.0	12	0
5[c]	1-Tetradecene	10.0	2	0
6	3-Tetradecene	10.0	1	0
7[c]	Unknown	10.3	1	17
8[c]	Pentadecanoic acid, methyl ester	11.2	5	0
9[c]	Pentadecene	11.5	7	0
10[c]	Unknown	11.7	7	0
11	Unknown	11.7	0	2
12[c]	Tetradecanoic acid	11.9	8	3
13[c]	Pentadecanoic acid	13.4	5	3
14[c]	Hexadecanoic acid, methyl ester	13.8	6	0
15[c]	Hexadecadiene (a)	14.2	11	0
16	Methyl hexadecanate	14.2	0	3
17	Hexadecanoic acid	14.3	1	0
18[c]	1-Hexadecene or cyclohexadecane	14.7	9	0
19[c]	Unknown	16.1	5	0
20	Heptadecadiene	16.1	1	0
21	1-Heptadecene	16.1	1	0
22[c]	Unsaturated C_{16} fatty acid ($C_{15}H_{29}COOH$)	16.2	6	0
23	Hexadecanoic acid	16.5	0	3
24[c]	Unknown	16.7	11	0
25	Unknown	17.2	3	0
26[c]	Unknown hydrocarbon	18.0	8	0
27[c]	Unsaturated C_{14} hydrocarbon	18.3	20	0
28[c]	Octadecanoic acid, methyl ester	18.7	5	3
29[c]	Unknown (b)	19.6	13	0
30[c]	Unknown	19.7	4	0
31	Nonadecanoic acid, methyl ester	20.0	2	0
32[c]	Unknown	20.5	0	14
33[c]	Unknown	21.2	9	0
34[c]	N-compound	21.4	18	0
35	Unknown	22.0	1	0
36[c]	Hydrocarbon	22.5	12	0
37[c]	Unknown	22.7	15	0
38[c]	Unknown (c)	24.0	18	0
39[c]	Unknown	24.2	2	0
40	Aromatic compound	24.3	1	0
41[c]	Double unsaturated C_7–C_7 ester	25.1	12	0
42[c]	Unknown	25.2	20	0
43[c]	N-compound	25.6	19	0

TABLE 4. CONTINUED

No.	Tentatively identified compound[b]	Retention time (min)	Samples (*N*)	
			Male	Female
44[c]	Unknown	26.1	16	0
45[c]	Double unsaturated C_7–C_8 ester	26.3	5	0
46[c]	Long hydrocarbon chain (d)	26.4	19	0
47[c]	Unknown	26.7	8	0
48	Docosanoic acid, methyl ester	27.4	1	0
49[c]	Double unsaturated C_8–C_6 ester	27.7	11	0
50[c]	Unknown	27.8	14	0
51[c]	Wax ester	27.9	16	0
52[c]	Unknown	29.0	20	0
53[c]	Unknown	29.1	9	0
54[c]	Unknown	29.2	10	0
55[c]	Hexadecyl octanat (wax ester)	29.3	8	0
56[c]	Unknown	30.1	11	0
57[c]	Unknown	30.2	1	0
58[c]	Unknown	30.4	15	0
59[c]	Unknown	31.5	16	0
60[c]	Unknown	31.6	0	8
61	Hydrocarbon	31.7	0	1
62[c]	A steroid	31.7	5	0
63[c]	Unsaturated C_{30} hydrocarbon (e)	32.3	0	20
64[c]	Unknown	32.3	0	8
65[c]	A steroid	32.4	5	1
66	Unknown	32.4	3	0
67	Unknown	32.5	1	0
68[c]	Hexamethyl-tetracosatetraene	32.5	5	7
69[c]	A steroid	32.6	2	0
70[c]	C_{26} wax ester	32.8	11	0
71[c]	Wax ester	32.8	6	0
72[c]	Unsaturated C_{30} hydrocarbon	32.9	0	18
73	Unknown	33.0	0	1
74[c]	A steroid	33.0	4	0
75[c]	Unsaturated hydrocarbon	33.2	8	0
76	A steroid	33.2	0	1
77[c]	Unknown	33.3	0	11
78[c]	Saturated C_{14}–C_{14} wax ester with side chain	33.3	5	0
79[c]	Unknown	33.4	0	1
80	Unknown	33.5	0	2
81[c]	Wax ester	33.6	11	0
82[c]	Unknown	33.9	2	0
83[c]	Wax ester	33.9	18	0
84[c]	Unknown	33.9	0	3
85[c]	Unknown	33.9	0	1
86[c]	Unknown	34.0	0	10
87[c]	Unknown	34.3	4	0
88	Unknown	34.4	0	3

TABLE 4. CONTINUED

No.	Tentatively identified compound[b]	Retention time (min)	Samples (*N*)	
			Male	Female
89[c]	Unknown	34.9	15	0
90[c]	A steroid	34.9	3	0
91[c]	Unknown	35.9	11	0
92[c]	Tetradecyl-tetradecanoate (wax ester)	36.1	12	0
93[c]	Vitamine E	36.6	3	0
94[c]	Unknown	36.9	12	0
95[c]	C_{29} wax ester (f)	37.2	20	0
96[c]	Unsaturated C_{30} wax ester	37.5	1	0
97[c]	Cholestan-3-ol-(3-β,5-α)	37.8	5	11
98[c]	A steroid	37.8	9	13
99[c]	Unknown (g)	38.0	11	19
100[c]	Saturated C_{15}–C_{15} wax ester	38.1	19	0
101[c]	Unsaturated C_{31} wax ester	38.4	4	0
102[c]	A steroid	38.5	17	17
103	Dihydrocholesterol	38.6	3	0
104[c]	A steroid	38.8	13	12
105	A steroid (coprostan-3-ol)	39.0	0	1
106[c]	Unsaturated wax ester (h)	39.2	17	0
107[c]	Cholest-7-ene-3-ol (3-β,5-α)	39.2	0	13
108[c]	Unknown	39.3	3	0
109[c]	A steroid	39.3	5	0
110[c]	A steroid (i)	39.5	0	6
111[c]	A steroid	39.8	2	0
112[c]	4-α-Methylcholest-8 (14)-ene-3-β (j)	39.8	13	17
113[c]	Wax ester	39.9	4	0
114[c]	Unknown (k)	40.1	17	0
115	A steroid	40.1	0	3
116[c]	4-Methylcholest-7-ene-3-one	40.1	0	9
117[c]	Unknown	40.3	0	16
118[c]	Saturated C_{31} wax ester	40.3	19	0
119[c]	A steroid	40.4	0	14
120[c]	Cholest-4-ene-3-one	40.5	1	0
121[c]	A steroid	40.9	0	13
122[c]	Unknown	41.0	0	5
123[c]	Dihydrolanosterol	41.2	0	12
124[c]	Heptadecanoic acid, pentadecyl ester	41.2	20	0
125[c]	A steroid	41.4	0	9
126[c]	A steroid	41.4	2	1
127[c]	4-β-Methyl-24(*R*)-methylcholest-8 (14)-ene-3-beta	41.5	0	3
128[c]	Lanosta-8,24-diene-3-ol (3-β)	41.9	0	19
129[c]	Double unsaturated C_{32} wax ester	42.0	20	0
130[c]	A steroid (l)	42.0	0	18
131[c]	Unsaturated C_{32} wax ester (m)	42.2	20	0
132[c]	Hexadecanoic acid, hexadecyl ester	42.3	19	0

TABLE 4. CONTINUED

No.	Tentatively identified compound[b]	Retention time (min)	Samples (*N*)	
			Male	Female
133	9-Octadecenoic acid, hexadecyl ester	42.8	2	0
134[c]	Unsaturated C_{33} wax ester	43.0	19	0
135	Cholest-3-ene (5-α)	43.0	0	2
136[c]	Saturated C_{33} wax ester (n)	43.2	18	0
137[c]	Unknown	43.4	3	0
138[c]	A steroid	43.4	3	0
139	Octadecanoic acid, hexadecylester	43.5	2	0
140[c]	Unknown	44.1	18	0
141[c]	Unknown	44.4	0	5
142[c]	A steroid	44.5	0	8
143[c]	Unknown	44.6	8	0
144[c]	A steroid	44.7	3	9
145	Unknown	44.8	4	0
146[c]	Double unsaturated C_{34} wax ester	44.9	8	0
147[c]	Unsaturated C_{34} wax ester	45.0	6	0
148[c]	A steroid (o)	45.0	12	16
149[c]	A steroid	45.5	5	4
150[c]	Unknown	45.5	9	6
151[c]	A steroid (p)	45.8	17	19
152[c]	A steroid	45.8	1	0
153[c]	Unknown	46.1	11	0
154[c]	A steroid	46.6	12	2
155[c]	Unknown	47.1	9	0
156	A steroid	48.6	2	0
157	Unknown	48.9	5	0
158[c]	Unknown	49.1	14	0
159[c]	Wax ester	49.4	3	0
160[c]	A steroid	49.8	11	0
161[c]	A steroid	50.1	18	0
162	A steroid	55.2	0	1
163	A steroid	57.3	0	1

[a] Letters in parentheses match those in Figure 2.
[b] The identities have not been verified with known samples.
[c] Compounds were found both in the snow control experiment and in the dead animals used in the control experiment.

DISCUSSION

Our results support the prediction that castoreum is most frequently deposited on scent marks (96 of 96) and appears, therefore, to be the main scent signal used in the defense of Eurasian beaver territories during January–March. AGS, however, was deposited only on 4 of 96 scent marks. This suggests that beavers do not specifically deposit AGS on scent mounds, but that the compounds we found

possibly were remnants of AGS from the feet and/or fur after pelage lubrication (Walro and Svendsen, 1982). Beavers may also get AGS on their feet and fur following coprophagy (Wilsson, 1971). AGS may, therefore, have other functions.

Beaver scent marks with castoreum might be a volatile alerting signal for attracting attention (Müller-Schwarze, 1999). Alerting signals contain no information about an individual or even a species (Müller-Schwarze, 1999). Responses to single compounds support the hypothesis that castoreum is used for signaling territorial occupancy, which requires only one bit of information in the signal for making a decision by receivers, i.e., whether the territory is occupied or not (Müller-Schwarze and Houlihan, 1991; Schulte et al., 1994; Sun and Müller-Schwarze, 1999). It may be that the lighter, volatile compounds in the castoreum direct receivers toward the less volatile but potentially more informative chemical components still present at the scent mark. This is supported by the fact that 94% of the compounds had a molecular weight below 300.

Schulte (1998) found that North American beavers discriminated among castor-fluid scents from family, neighbor, and nonneighbor adult males. The Eurasian beaver can also discriminate among scents (castoreum and AGS) from neighbor and non-neighbor individuals (Rosell and Bjørkøyli, unpublished data). However, no significant difference was found in the number of castoreum compounds between dead males and females whose castoreum chromatograms were similar (see also Pedersen, 1999). Likewise, Sun and Müller-Schwarze (1999) failed to find any consistent difference between male and female castoreum profiles in North American beaver and concluded that castoreum is unlikely to be used for sex recognition. This conclusion is in accordance with the evidence that castoreum compounds are mainly dietary derivatives, which do not differ between the two sexes (Müller-Schwarze, 1992). By contrast, the composition of AGS in both the North American and Eurasian beaver exhibits chemical sexual dimorphism (Grønneberg, 1978–1979; Grønneberg and Lie, 1984; Sun and Müller-Schwarze, 1999; this study). Whether the Eurasian beaver uses the sex difference in AGS to distinguish between individuals of different sex, needs further study.

AGS also contains information about individuality, kinship, and family membership (Sun and Müller-Schwarze, 1999). Sun and Müller-Schwarze (1997) have shown that North American beavers use AGS to discriminate between unfamiliar sibling and unfamiliar nonrelatives and that this discrimination was not shown when castoreum samples were tested. Sun and Müller-Schwarze (1998b) showed that beavers' response to AGS from unfamiliar adult males remained at about the same level, but their response to castoreum showed a descending trend. The descending trend in response to the same signal without matching the signaler demonstrates a declining importance of the signal over time, i.e., the scent-matching hypothesis (Gosling, 1982) was supported. The scent-matching hypothesis predicts, among other things, that the territory owner should make itself available for scent matching by the intruder (Gosling, 1982). Sun and Müller-Schwarze (1998a)

recently documented that related individuals shared more features in the chemical AGS profile than did unrelated individuals, and Sun and Müller-Schwarze (1998c) also demonstrated that it is possible to use some AGS compounds to classify different families.

AGS may act as a chemical messenger in the water territory (Grønneberg and Lie, 1984) sensed through close range or contact with the animal. The latter is supported by the fact that only 12.5% and 32.5% of the compounds detected in AGS of females and males, respectively, had a molecular weight below 300. It could be advantageous for a swimming mammal such as the beaver to present chemical signals in the form of lipid substances that would concentrate at the air–water interface (Albone, 1984). By lubricating the fur with AGS, which would be released into the water, beaver could also act as a "living scent mark." As AGS is insoluble in water (Svendsen, 1978), beavers downstream would receive a concentrated flow of chemical scent information in the surface film from upstream territories (Rosell et al., 1998). The recently discovered vomeronasal organ in Eurasian beavers may play a significant role here (Rosell and Pedersen, 1999). Furthermore, anal glands, which are located in the anus (Svendsen, 1978), may add AGS to the feces when beavers defecate in the water. For instance, the large complex of sebaceous and apocrine glands located in and around the anus of many species of antelope may add individual-specific secretion to feces (Barrette, 1977; Mainoya, 1980; Gosling, 1982).

However, Rosell and Bergan (1998) observed on July 21 two adult Eurasian beavers depositing AGS at the border of their territory by everting the "cloaca," protruding the anal gland openings and rubbing them against the surface as the animal walked over the scent mound. Therefore, further analyses need to clarify if beavers use the AGS on scent marks of other times of the year.

Acknowledgements—We thank Ole-Kristian Kristensen for help with the preparation and collection of the samples, Bjørn Steen for help with the chemical analyses, Per Christian Hagen for statistical advice, and Dr. Göran Hartman, Per Martin Holm, Prof. Dietland Müller-Schwarze, Dr. Howard Parker, Dr. Bruce A. Schulte, and Dr. Lixing Sun for comments on an earlier draft. The study was supported financially by Telemark University College, Bø, Norway.

REFERENCES

ALBONE, E. S. 1984. Mammalian Semiochemistry. John Wiley, New York.

BALDWIN, B. A., and MEESE, G. B. 1977. The ability of sheep to distinguish conspecifics by means of olfaction. *Physiol. Behav*. 19:803–808.

BARETTE, C. 1977. Scent-marking in captive muntjacs, *Muntiacus reevesi. Anim. Behav.* 25:536–541.

BRADBURY, J. W., and VEHRENCAMP, S. L. 1998. Principles of Animal Communication. Sinauer Associates, Sunderland, Massachusetts.

BUECH, R. R. 1995. Sex differences in behavior of beavers living in near-boreal lake habitat. *Can. J. Zool*. 73:2133–2143.

EPPLE, G., GOLOB, N. F., and SMITH, A. B. III. 1979. Odor communication in the tamarin *Saguinus fuscicollis* (Callitrichidae): Behavioral and chemical studies, pp. 117–130, *in* F. J. Ritter (ed.). Chemical Ecology: Odour Communication in Animals. Elsevier, Amsterdam, The Netherlands.

GORMAN, M. L. 1984. Scent marking and territoriality. *Acta Zool. Fenn.* 171:49–53.

GORMAN, M. L. and TROWBRIDGE, B. J. 1989. The role of odor in the social lives of carnivores, pp. 57–88, *in* J. L. Gittleman (ed.). Carnivores Behavior, Ecology, and Evolution. Cornell University Press, Ithaca, New York.

GORMAN, M. L., JENKINS, D., and HARPER, R. J. 1978. The anal scent sacs of the otter*(Lutra lutra). J. Zool. London* 186:463–474.

GOSLING, L. M. 1982. A reassessment of the function of scent marking in territories. *Z. Tierpsychol*. 60:89–118.

GRØNNEBERG, T. Ø. 1978–1979. Analysis of a wax ester fraction from anal gland secretion of beaver (*Castor fiber*) by chemical ionization mass spectrometry. *Chemi. Scr.* 13:56–58.

GRØNNEBERG, T. Ø. and LIE, T. 1984. Lipids of the anal gland secretion of beaver (*Castor fiber*). *Chem. Scr.* 24:100–103.

HODGDON, H. E. 1978. Social dynamics and behavior within an unexploited beaver (*Castor canadensis*) population. PhD dissertation. University of Massachusetts, Amherst, Massachusetts.

JOHNSTON, R. E. 1985. Communication, pp. 121–154, *in* H. I. Siegel (ed.). The Hamster: Reproduction and Behavior. Plenum Press, New York.

JOHNSTON, R. E., DERZIE, A., CHIANG, G., JERNIGAN, P., and LEE, H. 1993. Individual scent signatures in golden hamsters: Evidence for specialization of function. *Anim. Behav*. 45:1061–1070.

MAINOYA, J. R. 1980. Observations on the histology of the inguinal glands of the Thomson's gazelle, *Gazella thomsoni. Afri. J. Ecol*. 18:277–280.

MARTIN, I. G. and BEAUCHAMP, G. K. 1982. Olfactory recognition of individuals by male cavies (*Cavia aperea*). *J. Chem. Ecol*. 8:1241–1249.

MÜLLER-SCHWARZE, D. 1983. Scent glands in mammals and their functions, pp. 150–197, *in* J. F. Eisenberg and D. G. Kleiman (eds.). Recent Advances in the Study of Mammalian Behavior. American Society of Mammalogists, Shippensburg.

MÜLLER-SCHWARZE, D. 1992. Castoreum of beaver (*Castor canadensis*): Function, chemistry and biological activity of its components, pp. 457–464, *in* R. L. Doty and D. Müller-Schwarze (eds.). Chemical Signals in Vertebrates 6. Plenum Press, New York.

MÜLLER-SCHWARZE, D. 1999. Chemical signals in the beaver. Signal specialization and evolution in mammals, pp. 1–14, *in* R. E. Johnston, D. Müller-Schwarze, and P. W. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Kluwer Academic/Plenum Publishers, New York.

MÜLLER-SCHWARZE, D. and HOULIHAN, P. W. 1991. Pheromonal activity of single castoreum constituents in beaver (*Castor canadensis*). *J. Chem. Ecol*. 17:715–734.

MÜLLER-SCHWARZE, D., MOREHOUSE, L., CORRADI, R., ZAHO, C., and SILVERSTEIN, R. M. 1986. Odor images: responses of beaver to castoreum fractions, pp. 561–570, *in* D. Duvall, D. Müller-Schwarze, and R. M. Silverstein (eds.). Chemical signals in Vertebrates IV. Plenum Press, New York.

NOLET, B. A. and ROSELL, F. 1994. Territoriality and time budgets in beavers during sequential settlement. *Can. J. Zool*. 72:1227–1237.

OLSTAD, O. 1937. Beverens (*Castor fiber*) utbredelse i Norge. Statens viltundersøkelser. *Nytt mag. Naturvitenskapene* 77:217–273.

OSBORN, D. J. 1955. Techniques of sexing beaver, *Castor canadensis. J. Mammal.* 36:141–142.

PARKER, H. and ROSELL, F. 2001. Parturition dates for Eurasian beaver *Castor fiber*: When should spring hunting cease? *Wildl. Biol.* 7:237–241.

PEDERSEN, B. 1999. Kjemisk analyse av flyktige stoffer i castoreum fra europeisk og amerikansk bever. MSc thesis. Norges Teknisk-Naturvitenskapelige Universitet, Trondheim.

QUAY, W. B. and MÜLLER-SCHWARZE, D. 1971. Relations of age and sex to integumentary glandular regions in black-tailed deer, *Odocoileus hemionus columbianus. J. Mammal*. 51:675–694.

RALLS, K. 1971. Mammalian scent marking. *Science* 171:443–449.

ROEDER, J. J. 1980. Marking behaviour and olfactory recognition in genets (*Genetta genetta* L., Carnivora-Viveridae). *Behaviour* 72:200–210.

ROSELL F. and BERGAN F, 1998. Free-ranging Eurasian beavers,*Castor fiber*, deposit anal gland secretion when scent marking. *Can. Field-Nat*. 112:532–535.

ROSELL, F. and BERGAN, F. 2000. Scent marking in Eurasian beaver *Castor fiber* during winter. *Acta Theriol*. 45:281–287.

ROSELL, F. and NOLET, B. A. 1997. Factors affecting scent-marking behaviour in Eurasian beaver (*Castor fiber*). *J. Chem. Ecol*. 23:673–689.

ROSELL, F. and PEDERSEN, K. V. 1999. Bever. Landbruksforlaget, Oslo.

ROSELL, F. and SUN, L. 1999. Use of anal gland secretion to distinguish the two beaver species *Castor canadensis* and *C. fiber. Wildl. Biol*. 5:119–123.

ROSELL, F., BERGAN, F., and PARKER, H. 1998. Scent-marking in the Eurasian beaver (*Castor fiber*) as a means of territory defense. *J. Chem. Ecol*. 24:207–219.

ROSELL, F., JOHANSEN, G., and PARKER, H. 2000. Eurasian beavers (*Castor fiber*) behavioral response to simulated territorial intruders. *Can. J. Zool*. 78:1–5.

SCHULTE, B. A. 1993. Chemical communication and ecology of the North American beaver (*Castor canadensis*). PhD dissertation. State University of New York, Syracuse, New York.

SCHULTE, B. A. 1998. Scent marking and responses to male castor fluid by beavers. *J. Mammal*. 79:191–203.

SCHULTE, B. A., MÜLLER-SCHWARZE, D., TANG, R., and WEBSTER, F. X. 1994. Beaver (*Castor canadensis*) responses to major phenolic and neutral compounds in castoreum. *J. Chem. Ecol*. 20:3063–3081.

SCHULTE, B. A., MÜLLER-SCHWARZE, D., TANG, R., and WEBSTER, F. X. 1995. Bioactivity of beaver castoreum constituents using principal components analysis. *J. Chem. Ecol.* 21:941–957.

SIEGEL, S. and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral sciences. McGraw-Hill, New York.

SOKAL, R. R. and ROHLF, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. W. H. Freeman and Company, New York.

SUN, L. 1996. Chemical kin recognition in the beaver (*Castor canadensis*): Behavior, relatedness and information coding. PhD dissertation. State University of New York, Syracuse.

SUN, L. and MÜLLER-SCHWARZE, D. 1997. Sibling recognition in the beaver: A field test of phenotype matching. *Anim. Behav*. 54:493–502.

SUN, L. and MÜLLER-SCHWARZE, D. 1998a. Anal gland secretion codes for relatedness in the beaver, *Castor canadensis*. *Ethology* 104:917–927.

SUN, L. and MÜLLER-SCHWARZE, D. 1998b. Beaver response to recurrent alien scent: Scent fence or scent match? *Anim. Behav*. 55:1529–1536.

SUN, L. and MÜLLER-SCHWARZE, D. 1998c. Anal gland secretion codes for family membership in the beaver. *Behav. Ecol. Sociobiol*. 44:199–208.

SUN, L. and MÜLLER-SCHWARZE, D. 1999. Chemical signals in the beaver. One species, two secretions, many functions?, pp. 281–288, *in* Advances in Chemical Signals in Vertebrates. R. E. Johnston, D. Müller-Schwarze and P. W. Sorensen (eds.). Kluwer Academic/Plenum Publishers, New York.

SVENDSEN, G. E. 1978. Castor and anal glands of the beaver (*Castor canadensis*). *J. Mammal*. 59:618–620.

SVENDSEN, G. E. 1980. Patterns of scent-mounding in a population of beaver (*Castor canadensis*). *J. Chem. Ecol*. 6:133–148.

TANG, R., WEBSTER, F. X., and MÜLLER-SCHWARZE, D. 1993. Phenolic compounds from male castoreum of the North American beaver (*Castor canadensis*). *J. Chem. Ecol*. 19:1491–1500.

TANG, R., WEBSTER, F. X., and MÜLLER-SCHWARZE, D. 1995. Neutral compounds from male castoreum of North American beaver, *Castor canadensis. J. Chem. Ecol*. 21:1745–1762.

VALEUR, P. 1988. Beverens territorial-atferd som populasjonsregulerende faktor. *Fauna* 41:20–34.

VAN NOSTRAND, F. C. and STEPHENSON, A. B. 1964. Age determination for beavers by tooth development. *J. Wildl. Manage*. 28:430–434.

WALRO, J. M. and SVENDSEN, G. E. 1982. Castor sacs and anal glands of the North American beaver (*Castor canadensis*): Their histology, development and relationship to scent communication. *J. Chem. Ecol*. 5:809–819.

WELSH, R. G. and MÜLLER-SCHWARZE, D. 1989. Experimental habitat scenting inhibits colonization by beaver, *Castor canadensis. J. Chem. Ecol*. 3:887–893.

WILSON, E. O. 1963. Pheromones. *Sci. Am*. 208:100–111.

WILSSON, L. 1971. Observations and experiments on the ethology of the European beaver (*Castor fiber L.*). *Viltrevy* 8:115–266.

WYNNE-EDWARDS, V. C. 1962. Animal Dispersal in Relation to Social Behavior. Oliver and Boyd, Edinburgh.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

WOLF SPIDERS SHOW GRADED ANTIPREDATOR BEHAVIOR IN THE PRESENCE OF CHEMICAL CUES FROM DIFFERENT SIZED PREDATORS

M. H. PERSONS[1,*] and A. L. RYPSTRA[2]

[1]*Department of Zoology*
Miami University
Oxford, Ohio, 45056

[2]*Department of Zoology*
Miami University
Hamilton, Ohio, 45011

(Received March 26, 2001; accepted July 25, 2001)

Abstract—The wolf spider, *Pardosa milvina*, displays effective antipredator behavior (reduced activity) in the presence of silk and excreta cues from adults of another cooccurring wolf spider, *Hogna helluo*. However, *Pardosa* and *Hogna* engage in size-structured intraguild predation, where *Pardosa* may be either the prey or predator of *Hogna*. We tested the ability of adult female *Pardosa* to vary antipredator responses toward kairomones produced by *Hogna* that vary in size. *Hogna* were maintained on filter paper for 24 hr. We then presented the paper to adult female *Pardosa* simultaneously paired with a blank sheet of paper. One treatment had two sheets of blank paper to serve as a control. The *Hogna* stimulus treatments were as follows ($N = 15$/treatment): (1) 1 *Hogna* half the mass of *Pardosa*; (2) 1 *Hogna* of equal mass of a *Pardosa*; (3) 1 adult *Hogna*, 30 times the mass of *Pardosa*; and (4) 8 *Hogna* each 0.25 the mass of *Pardosa*. *Pardosa* decreased activity in the presence of kairomones from *Hogna* of equal or larger size, but showed no change in activity in the presence of a blank control or from a single *Hogna* smaller than itself. *Pardosa* showed a reduction in activity in the presence of cues from eight small *Hogna*. *Pardosa* avoided substrates with adult *Hogna* cues, but showed no avoidance response to any other treatment. These results suggest that *Pardosa* is showing graded antipredator behavior relative to the quantity of predator kairomones present rather than directly discriminating among the different sizes of the predator.

Key Words—*Pardosa milvina*, Lycosidae, kairomone, wolf spider, predator, size, *Hogna helluo*, antipredator, size discrimination, chemical cue.

*To whom correspondence should be addressed at Department of Biology, Susquehanna University, Selinsgrove, Pennsylvania 17870. E-mail: persons@susqu.edu

INTRODUCTION

Many animals show defensive behavior in the presence of chemical cues produced by predators (see Kats and Dill, 1998, for a review). Antipredator responses include reduced locomotion, increased cover-seeking, or increased vigilance (reviewed in Lima and Dill, 1990; Lima, 1998). These behaviors are generally costly because they interfere with both foraging (Lima and Dill, 1990) and reproduction (Forsgren, 1992; Berglund, 1993; Hedrick and Dill, 1993; Godin, 1995). The "threat-sensitive predator avoidance" hypothesis suggests that prey should show graded responses of defensive behavior proportional to the perceived risk (Dill and Fraser, 1997; Sih, 1997; Puttlitz et al., 1999).

Prey that respond to chemical cues from predators (kairomones) rather than using other sensory cues may overestimate predation risk by exhibiting antipredator behavior even in the absence of the predator itself. This additional cost may be reduced by extracting more information about predation risk from predator cues. Variation in the composition of predator excreta may provide information about the predator's diet (Crowl and Covich, 1990; Chivers et al., 1996; Murray and Jenkins, 1999; Venzon et al., 2000; Persons et al., 2001), which, in turn, may provide information about the proclivity of a predator to feed on particular prey types (Punzo and Kukoyi, 1997; Persons and Rypstra, 2000). Other features such as age or type of chemical cue also may provide information about how recently a predator was in the area (Venzon et al., 2000) as well as the kind of predator present. In some cases, the intensity of kairomone-mediated antipredator responses may be related to the relative size of the prey compared to the predator. For example, dragonfly larvae are effective predators of small tadpoles, but are less efficient preying on tadpoles of larger sizes (Caldwell et al., 1980). Toad tadpoles show a corresponding reduction in antipredator behavior when detecting kairomones from dragonfly larvae as tadpole size increases (Laurila et al., 1997). Collectively, these sources of information may provide animals with a more accurate assessment of predation risk and allow them to modify defensive behavior, mitigating some of the costs of antipredatory behavior.

The wolf spiders *Hogna helluo* and *Pardosa milvina* are among the most abundant ground predators in agricultural fields of the midwestern United States (Marshall and Rypstra, 1999). These two species exhibit size-structured intraguild predation, whereby the role of predator and prey is determined by the relative mass of each spider during an encounter. Adult *Hogna* are typically 20–30 times the body mass of an adult *Pardosa* and are more likely to be the predator during an interspecific encounter, yet we have observed adult *Pardosa* feeding on juvenile *Hogna* as well as adult *Hogna* feeding on adult *Pardosa*. *Pardosa* may benefit from the ability to perceive relative *Hogna* size through kairomone cues since predation risk increases with increasing asymmetry in size (Samu et al., 1999). Adult female *Pardosa* exhibit reduced speed and frequency of movement in the presence of

excreta and silk from adult *Hogna* (Persons et al., 2001). Adult female *Pardosa* also will avoid substrates containing cues from adult *Hogna* when given a choice. These responses are absent when *Pardosa* are presented silk and excreta from conspecifics (Persons et al., 2001). Reduced activity results in longer periods of time in areas with predator cues; however, such behavior is adaptive since slower movement and inactivity increases survival of *Pardosa* in the presence of *Hogna* (Persons et al., 2001).

Pardosa and *Hogna* provide an opportunity to test the threat-sensitive predator avoidance hypothesis. Adults of the smaller *Pardosa* should closely match antipredator response with predation risk. *Pardosa* that interpret chemical cues from a small *Hogna* as a potential predator will incur a number of unnecessary costs. Reduced activity may impair reproductive behavior and may also impair feeding efficiency by causing the spider to avoid a potential prey item. If *Pardosa* can match defensive behavior with relative risk by assessing predator size indirectly, then the spider may minimize the associated costs of defensive behavior. It is unknown if silk and excreta change qualitatively or quantitatively with spider size or if *Pardosa* is able to detect such differences. Here, we test several predictions related to the ability of *Pardosa* to perceive *Hogna* size based on chemical information. First, adult female *Pardosa* will show decreasing activity in the presence of chemical cues from *Hogna* of increasing size, but will show no reduction in activity in the presence of chemical cues from *Hogna* smaller than *Pardosa*. Second, adult female *Pardosa* will not reduce activity in the presence of large quantities of chemical cues from many *Hogna* each smaller than itself. Third, when given a choice, *Pardosa* will avoid silk and excreta produced by *Hogna* larger than itself, but show no avoidance of cues produced by smaller *Hogna*.

METHODS AND MATERIALS

Spider Collection and Maintenance. Spider maintenance, experimental design, and testing protocols are similar to those found in Persons et al. (2001). Seventy-five adult female *Pardosa* were collected from within and along the margins of soybean fields (Oxford, Ohio, Butler County, Ecology Research Center, Miami University) in September 1998. *Pardosa* used as test spiders were fed a meal of two domestic cricket nymphs (*Acheta domesticus*) once a week followed by five additional cricket nymphs one day prior to testing to minimize possible hunger effects on activity. All spiders were maintained in 8-cm-diam. $\times$ 5-cm-high plastic translucent containers lined with moistened peat moss and kept in environmental chambers on a 13L:11D light cycle, 25°C, and 70% relative humidity. Fifty-five adult male and female *Hogna helluo* were collected from the same habitats as *Pardosa* and bred in captivity in the summer of 1998. The offspring of these

field-collected *Hogna* were used as stimuli for all trials and were maintained in a manner similar to that of *Pardosa*.

Stimulus Preparation. Silk and excreta from *Hogna* of four different sizes were collected on filter paper. One treatment consisted of chemical cues from a large *Hogna* ca. 30 times the body mass of a single *Pardosa* (mean $\pm$ SE $=$ 792.9 ± 15.9 mg). *Hogna* of this size can readily consume up to 10 adult female *Pardosa* in a single feeding bout. A second treatment consisted of silk and excreta from a medium-sized *Hogna* that was approximately equal in size to an adult female *Pardosa* (23.3 ± 1.2 mg). Predation among wolf spiders of equal mass has been shown to be infrequent (Samu et al., 1999). A third treatment used cues from a single small *Hogna* about one half the mass of an adult female *Pardosa* (11.2 ± 1.3 mg) and that can readily be consumed by *Pardosa*. We used a fourth treatment in order to uncouple cue quantity and quality. We collected silk and excreta from eight small *Hogna*, each approximately one fourth the body mass of *Pardosa* (collective mean weight $= 54.6 \pm 1.8$ mg). Each chemical stimulus treatment was paired with a blank sheet of filter paper that had never been exposed to a spider of any size or type (same experimental setup as in Persons et al., 2001). This served as a control to monitor behavioral differences. A fifth treatment consisted of only a blank sheet of filter paper.

All *Hogna* that were used as predator cues were maintained on a diet of appropriately sized crickets (*A. domesticus*) for at least two weeks prior to placing them on filter paper substrates. All stimulus *Hogna* were watered *ad libitum* and fed as many prey as they could consume within a 24-hr period. This was done to minimize any differences in silk production or excreta due to differences in amount of food allocated among groups. *Hogna* were then placed onto sheets of filter paper for 24 hr prior to testing *Pardosa* responses. Each container was previously rinsed with 95% ethanol to remove all extraneous odor cues and allowed to dry. Stimulus spiders for each size treatment had access to a small cap of water in the center of the container. The cap was moved periodically to allow silk or excreta to be deposited beneath it. The lid was closed after *Hogna* were introduced. After the 24-hr period, the filter paper was removed from the container and introduced into a test arena immediately prior to testing. For the treatment consisting of eight *Hogna*, 16 spiders were used for each paper stimulus sheet. Eight spiders were introduced onto a single stimulus sheet of paper and maintained under individual vials to prevent cannibalism or agonistic behavior between individuals. Each set of eight spiders was replaced every 6 hr with another set of eight and allowed to receive water in a separate container. These were then reintroduced onto the substrate under vials again after 6 hr.

Pardosa milvina Testing. Seventy-five adult female *Pardosa* were randomly assigned to one of five treatment pairs ($N = 15$/treatment). As described above, these treatments were: (1) large *Hogna*/blank, (2) medium *Hogna*/blank, (3) small *Hogna*/blank, (4) eight small *Hogna*/blank, or (5) blank/blank. Test *Pardosa* for

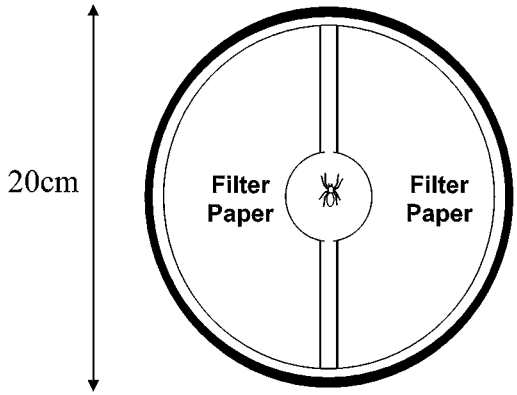


FIG. 1. Experimental apparatus used for testing chemically mediated size discrimination among *Pardosa*. Test spiders are placed in the center of the apparatus and allowed to acclimate for 1 min, after which the spider is allowed to move freely in the container for a period of 1 hr.

each treatment were fed crickets to satiation 24 hr prior to testing and weighed on an analytical balance (27.3 ± 1.0 mg).

We examined the locomotor patterns and behavior of adult female *Pardosa* exposed to each treatment pair and across pairs. Test arenas for *Pardosa* were 20-cm-diam. $\times$ 8-cm-high plastic containers consisting of two half circles of filter paper that collectively covered most of the surface area of the arena except for a 5- to 6-mm band between the two sheets (Figure 1). Additionally, a half circle was cut in the center of each sheet, and this area was used to introduce the spider.

Each spider was introduced into the center of the arena under a clear plastic vial. Each of the 15 spiders per treatment pair was presented a stimulus from a different *Hogna* of the appropriate size class, and no filter paper sheets were used more than once. After a 1-min acclimation period, spiders were allowed to move across the paired treatments for 1 hr each. Spatial positions of filter paper treatments within pairings were randomized among test subjects to eliminate any potential side bias. The test arena was swabbed with 95% ethanol between stimulus presentations to each subject and allowed to dry. Test trials were illuminated equally on all sides with four 15-W fluorescent lights installed in a square configuration above the testing arena. All spiders were tested between 07:00 and 19:00 hr.

Locomotor behavior was measured by monitoring the spiders remotely using an automated digital data collection system (Videomex-V, Columbus Instruments, Columbus, Ohio, USA) integrated into a video camera (JVC high band Saticon GXS 700). All behaviors were recorded automatically. For each test spider,

we recorded the following behaviors separately for each filter paper treatment: (1) time spent moving forward (locomotion), (2) time spent in nonforward movement (any movement of the appendages or turning of the body in place without forward locomotion), (3) time spent immobile (no visible indications of movement), (4) distance traveled (centimeters), (5) residence time (time spent on filter paper), and (6) speed (distance traveled/time spent moving forward). We also recorded the initial choice of sides for each pairing. Changes in spider movement were recorded every second and required a movement of at least one body length in that time period to be measured as forward movement. All locomotor patterns were output onto a computer printer every 5 min, and behaviors for each subject were summed over the 1-hr period.

Statistical Analyses. Because none of the behavior classes showed deviations from a normal distribution (Wilk-Shapiro normality statistic), parametric statistics were used for analyses. We used paired *t* tests to compare behavioral differences between paired filter paper treatments for each class of behavior. We used ANOVA to determine differences for each behavioral category across the three size class treatments and the blank control. A planned series of two-sample *t* tests was used to test for differences for each class of behavior between the eight *Hogna* treatment versus the medium *Hogna* treatment. This was done to determine if *Pardosa* behavior is the result of variation in the quantity or quality of *Hogna* cues.

A second set of analyses was done to compare differences in spider responses across each *Hogna* size treatment. Six one-way ANOVAs were completed with residence time, time in forward locomotion, time in nonforward locomotion, distance moved, time spent immobile, and speed as dependent variables for the blank, large *Hogna*, medium *Hogna*, and small *Hogna* size treatments. Each stimulus treatment paired with a blank was used as the independent variable. Chi-squared goodness of fit tests were used to determine differences in initial choice of sides in each treatment pairing.

RESULTS

In general, there was a decrease in *Pardosa* activity with cues from *Hogna* of increasing size. *Pardosa* showed a significant reduction in activity in the presence of all *Hogna* size treatment groups compared to blank controls except when on substrates from the small *Hogna* treatment (Figure 2). Based on paired *t* tests, mean time spent immobile and mean residence time were different when the cues were from a medium or large *Hogna* (Figure 2A, B). *Pardosa* spent more time in nonforward locomotion in the presence of cues from large *Hogna* than on a blank control sheet of paper (Figure 2A). There was no difference in any class of locomotor behavior among the double blank treatments, indicating no side bias to the testing container (Figure 2D).

TABLE 1. COMPARISON OF MEAN ACTIVITY LEVELS IN SIX CATEGORIES OF *Pardosa* BEHAVIOR FOR EACH *Hogna* SIZE TREATMENT[a]

Behavior	Control	*Hogna*			$F_{3,59}$	P
		Small	Medium	Large		
Residence time (sec)	1608.90 a	2153.30 ab	2431.30 b	2577.7 b	4.06	0.0111
Forward locomotion (sec)	267.46	175.00	242.33	228.87	0.80	0.5024
Nonforward movement (sec)	382.47	394.13	460.87	540.33	0.99	0.4049
Distance moved (cm)	1107.6 a	354.70 b	498.95 b	551.06 ab	4.31	0.0084
Time immobile (sec)	891.33 a	1566.90 ab	1719.60 ab	1828.30 b	3.32	0.0262
Speed (cm/sec)	3.31 a	1.77 b	1.79 b	2.18 b	7.32	0.0003

[a] Different letters indicate significant differences between *Hogna* size treatment groups based on a Tukey post-hoc comparison of means test. F ratios are based on one-way ANOVAs. $N = 15$/treatment.

There was a significant difference in total time spent on each *Hogna* treatment (Table 1). Based on a Tukey *post-hoc* comparison of means, *Pardosa* spent longer periods of time on the medium and large *Hogna* treatments compared to the control. Residence time on the small *Hogna* treatment was intermediate between the control and other *Hogna* size treatments (Table 1). There was no difference in forward locomotion or nonforward locomotion across any size treatment group (Table 1). The total mean distance moved was different, with *Pardosa* traveling shorter distances on the small *Hogna* and medium *Hogna* treatments compared to the control substrate. Distance moved on the large *Hogna* substrate was intermediate between the control and other size treatments (Table 1). Time spent immobile was different on the large *Hogna* treatment compared to the control. Small and medium *Hogna* treatments were intermediate with respect to this behavior (Table 1). Speed of *Pardosa* movement was also measured and compared across treatment groups. Speed was determined by dividing distance moved by time spent in forward locomotion. *Pardosa milvina* moved slower in all *Hogna* size treatments compared to the blank control, but there were no differences in speed across size treatments (Table 1).

To determine if *Pardosa* individuals were responding to quantity of *Hogna* cues, or some qualitative difference in cues based on size, we compared the eight *Hogna* treatment to a blank control and then compared the eight *Hogna* treatment to a treatment of a single medium *Hogna*. *Pardosa milvina* showed a strong and significant reduction in activity in the presence of eight *Hogna* compared to a blank control (Figure 2E). Spiders showed more time immobile and longer residence times on the side with eight *Hogna* compared to the blank control. No other class of behavior differed between pairs. When each behavioral category (nonforward locomotion, forward locomotion, time immobile, residence time, and distance moved) was compared between substrates containing eight small *Hogna* versus a single *Hogna*, there was a significant difference in time spent in nonforward

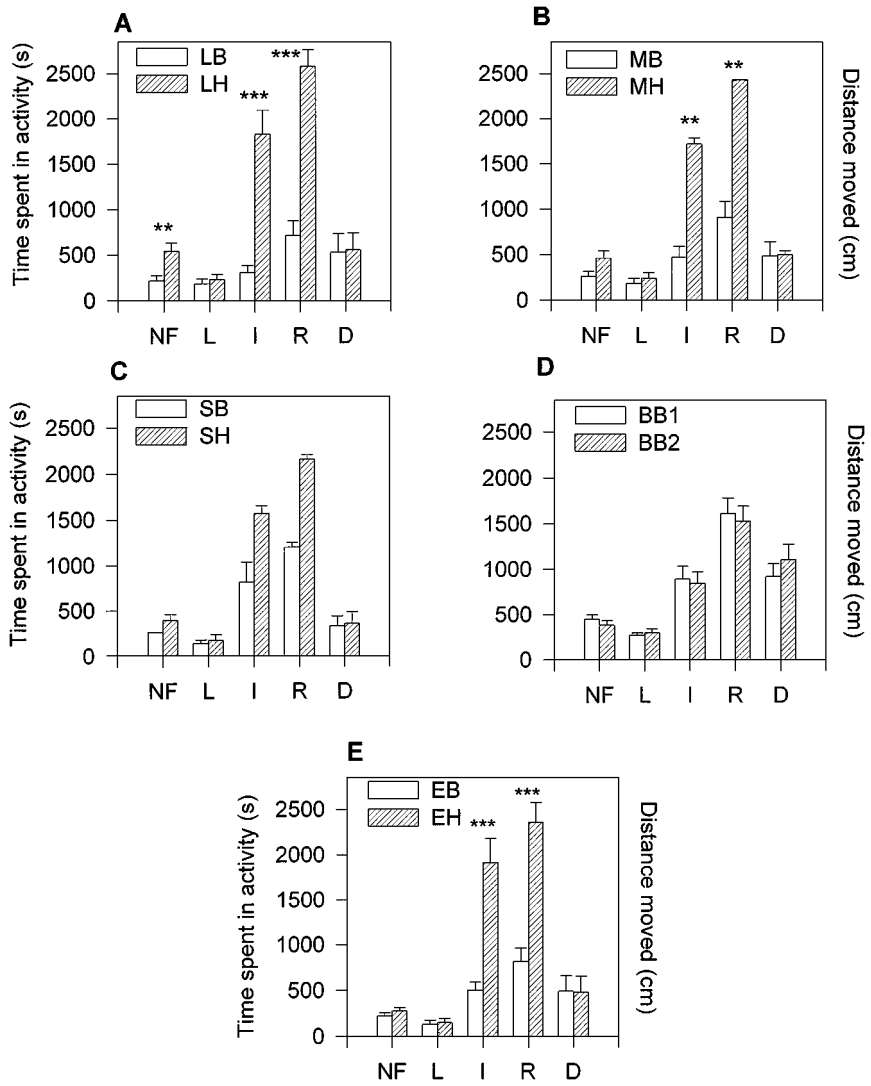


FIG. 2A–E. Comparison of activity level of adult female *Pardosa milvina* exposed to paired treatments of filter paper previously occupied by *Hogna* of different sizes or a blank sheet of paper. Behavioral categories on ordinate are as follows: NF = time spent in nonforward locomotion, L = time spent moving forward (locomote), I = time spent immobile, R = residence time on that substrate, D = distance moved. Distance moved is measured in centimeters and is represented on the same scale as time. All other behaviors are measured in seconds. Results are expressed as means ($N = 15$/treatment) ± SE. Significance levels are based on paired *t* tests for each pair of behaviors denoted as follows: $^{*}<0.05$, $^{**}<0.01$,

TABLE 2. COMPARISON OF MEAN ACTIVITY LEVELS IN SIX CATEGORIES OF *Pardosa* BEHAVIOR FOR EIGHT *Hogna* AND MEDIUM *Hogna* STIMULUS TREATMENTS[a]

Behavior	Eight *Hogna*	Medium *Hogna*	T_{28}	P
Residence time (sec)	2353.9	2431.30	0.24	0.809
Forward locomotion (sec)	151.00	242.33	1.23	0.238
Nonforward movement (sec)	275.27	460.87	2.10	0.045
Distance moved (cm)	478.37	498.95	0.09	0.929
Time immobile (sec)	1910.30	1719.60	0.51	0.615
Speed (cm/sec)	2.32	1.793	1.30	0.203

[a] Results are based on two-sample *t* tests. $N = 15$/treatment.

locomotion (Table 2). Spiders spent more time engaged in nonforward movement in the medium *Hogna* treatment compared to the eight *Hogna* treatment. However, there were no other behavioral differences between the two treatments (Table 2).

Avoidance behavior was found only when *Pardosa* encountered chemical cues from large *Hogna*. When given a choice between a blank sheet of paper and one with cues from an adult female *Hogna*, spiders chose the blank side more often than expected by chance (12 of 15, $\chi^2 = 5.4$, df 14, $P < 0.025$). *Pardosa milvina* showed no initial side preference when given a choice between a blank sheet and *Hogna* cues from medium *Hogna*, small *Hogna*, or eight *Hogna*.

DISCUSSION

In general, *Pardosa* spiders showed reduced activity in the presence of cues from *Hogna* of increasing size. The most commonly modified behavior was time spent immobile. This also explains the counterintuitive response of spiders spending more time on substrates with *Hogna* cues. *Pardosa* tend to become immediately quiescent upon contact with *Hogna* silk and excreta. Spiders also showed a drop in distance moved across *Hogna* size treatments, suggesting it takes much longer for *Pardosa* to leave these substrates than when encountering areas devoid of *Hogna* cues.

$^{***}<0.001$. (A) Chemical cues from large adult female *Hogna* (LH) vs. a blank (LB). (B) Chemical cues from a medium-sized *Hogna* (MH) equal in body mass to the *Pardosa* being tested vs. a blank control (MB). (C) chemical cues from a small *Hogna* (SH) half the body mass of the test spider vs. a blank (SB). (D) a double blank control, B1 and B2. (E) chemical cues from eight small *Hogna* (EH) each ca. one fourth the mass of the *Pardosa* being tested vs. a blank control (EB). Locomotor activity and initial side choice was recorded for each spider.

The overall locomotor behaviors for each *Hogna* size treatment appeared to be qualitatively the same. Although *Pardosa* responses to the small *Hogna* treatment were not significant compared to the control (Figure 2), this appears to be due to a difference in the magnitude of the response. This suggests that variation in the quantity of cue is responsible for variation in *Pardosa* reduction in movement. Moreover, compared to a control sheet, *Pardosa* had a reduction in speed as well as distance moved on the small *Hogna* treatment (Table 1). *Pardosa* exhibited a strong reduction in movement to eight *Hogna*, which appears to be a response equivalent to a medium or large *Hogna*. Since none of the eight small *Hogna* would present a predation threat, these data suggest that it is the quantity of cues that serves as the proximate source of information governing the degree of antipredatory behavior.

Other studies have shown that long periods of immobility greatly increase survival probabilities of *Pardosa* when in the presence of a large *Hogna* (Persons et al., 2001). Reduced locomotion is likely to be adaptive since wolf spider visual systems are strongly biased toward movement rather than shape cues (Rovner, 1996). Wolf spiders are much more likely to lunge at prey that move for longer periods of time (Persons and Uetz, 1997), and reduction in locomotion reduces vibrations generated through the substratum that could also be used by a predatory wolf spider to locate prey (Lizotte and Rovner, 1988).

Pardosa milvina exhibits graded antipredator responses with variation in perceived risk of predation; however, chemical cues alone provide insufficient information for an individual to discriminate between a single large *Hogna* or many smaller individuals. We cannot rule out the possibility that a high density of silk and excreta from large numbers of *Hogna* spiderlings may be an indicator of an adult female *Hogna* nearby. We have found single female *Hogna* capable of producing over 200 offspring (personal observation). The young are carried on the mother's back for a short period of time and then disperse. We believe spiderlings of the same species would rarely occur at densities high enough to produce the amount of kairomone used in this study except immediately after dispersal. It is possible that *Pardosa* could interpret such information as the presence of a large adult female *Hogna* in the area. Without visual, vibratory, or tactile cues to confirm the size of the *Hogna*, *Pardosa* appears to treat any quantity of silk and excreta equal to or greater than that which they produce as a potential predation threat. Nonetheless, *Pardosa* responses to substrates would result in a close match between appropriate antipredator response and the actual risk of predation in most cases. *Pardosa* showed an avoidance response to adult female *Hogna*, the only size class in these treatments that would pose a large potential predation risk. The fact that potential prey apparently can induce significant, albeit lower levels of antipredator behavior, suggests that *Pardosa* weighs the fitness costs of predation more heavily than the loss of a meal. Previous studies have demonstrated that *Pardosa* is unresponsive to silk and excreta from equal-sized conspecifics (Persons et al., 2001), but in this study, *Pardosa* showed

antipredator responses to *Hogna* of equal size. These results suggest that *Pardosa* is capable of discriminating between conspecific and heterospecific silk and excreta.

In general, the results show limited support for two of our three original predictions. Adult female *Pardosa* showed increasing levels of antipredator behavior in the presence of chemical cues from *Hogna* of increasing size. However, *Pardosa* showed significant levels of antipredator behavior in the presence of a *Hogna* equal in size to itself and showed some antipredator behavior, although this was not always statistically significant, in the presence of small *Hogna*. Adult female *Pardosa* appeared to increase antipredator behavior in the presence of increased quantities of chemical cues from *Hogna*, even when those cues were produced from *Hogna* that could be eaten by *Pardosa*. Finally, when given a choice, *Pardosa* tended to avoid substrates previously occupied by a *Hogna* larger than itself, but showed no substrate avoidance if the paper had supported a spider or spiders of equal or smaller size to itself. This indicates that whatever the mechanism, *Pardosa* generally demonstrates antipredator responses appropriate to the degree of predation risk.

Acknowledgments—We thank Doug Meikle for the use of video equipment and lab space for this study. We also thank Leah Winkler, Marie Wills, Mike Brueseke, Tara LaFever, Stephanie Sieg, and Dean Ferrera for their help in collecting and maintaining spiders in the lab. This research was funded by NSF grant DEB 9527710 (for A. Rypstra and S. Marshall), the Department of Zoology, Miami University Research Challenge Grant, and the Hamilton Campus of Miami University.

REFERENCES

BERGLUND, A. 1993. Risky sex: Male pipefishes mate at random in the presence of a predator. *Anim. Behav.* 46:169–175.

CALDWELL, J. P., THORP, J. H., and JERVEY, T. O. 1980. Predator-prey relationships among larval dragonflies, salamanders, and frogs. *Oecologia* 46:285–289.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, J. F. 1996. Damselfly larvae learn to recognize predators from chemical cues in the predator's diet. *Anim. Behav.* 52:315–320.

CROWL, T. A. and COVICH, A. P. 1990. Predator-induced life-history shifts in a freshwater snail. *Science* 247:949–951.

DILL, L. M. and FRASER, A. H. G. 1997. The worm re-turns: Hiding behavior of a tube dwelling marine polychaete, *Serpula vermicularis. Behav. Ecol.* 8:186–193.

FORSGREN, E. 1992. Predation risk affects mate choice in a gobiid fish. *Am. Nat.* 140:1041–1049.

GODIN, J.-G. J. 1995. Predation risk and alternative mating tactics in male Trinidadian guppies (*Poecilia reticulata*). *Oecologia* 103:224–229.

HEDRICK, A. V. and DILL, L. M. 1993. Mate choice by female crickets is influenced by predation risk. *Anim. Behav.* 46:193–196.

KATS, L. B. and DILL, L.M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Ecoscience* 5:361–394.

LAURILA, A., KUJASALO, J., and RANTA, E. 1997. Different antipredator behaviour in two anuran tadpoles: Effects of predator diet. *Behav. Ecol. Sociobiol.* 40:329–336.

LIMA, S. L. 1998. Stress and decision-making under the risk of predation: Recent developments from behavioral, reproductive, and ecological perspectives. *Adv. Study Behav.* 27:215–290.

LIMA, S. L. and DILL, L. M. 1990. Behavioral decisions made under the risk of predation: A review and prospectus. *Can. J. Zool.* 68:619–640.

LIZOTTE, R. S. and ROVNER, J. S. 1988. Nocturnal capture of fireflies by lycosid spiders: Visual versus vibratory stimuli. *Anim. Behav.* 36:1809–1815.

MARSHALL, S. D. and RYPSTRA, A. L. 1999. Patterns in the distribution of two wolf spiders. *Environ. Entomol.* 28:1052–1059.

MURRAY, D. L. and JENKINS, C. L. 1999. Perceived predation risk as a function of predator dietary cues in terrestrial salamanders. *Anim. Behav.* 57:33–39.

PERSONS, M. H. and RYPSTRA, A. L. 2000. Preference for chemical cues associated with recent prey in the wolf spider *Hogna helluo* (Araneae: Lycosidae). *Ethology* 106:27–35.

PERSONS, M. H. and UETZ, G. W. 1997. The effects of prey attack and movement on the patch residence decision rules of *Schizocosa ocreata* (Araneae: Lycosidae). *J. Insect Behav.* 10:737–751.

PERSONS, M. H., WALKER, S. E., RYPSTRA, A. L., and MARSHALL, S. D. 2001. Wolf spider predator avoidance tactics and survival in the presence of diet-associated predator cues. *Anim. Behav.* 61:43–51.

PUNZO, F. and KUKOYI, O. 1997. The effects of prey chemical cues on patch residence time in the wolf spider *Trochosa parthenus* (Chamberlin) (Lycosidae) and the lynx spider *Oxyopes salticus* Hentz (Oxyopidae). *Bull. Br. Arachnol. Soc.* 10:323–326.

PUTTLITZ, M. H., CHIVERS, D. P., KIESECKER, J. M., and BLAUSTEIN, A. R. 1999. Threat-sensitive predator avoidance by larval Pacific treefrogs (Amphibia, Hylidae). *Ethology* 105:449–456.

ROVNER, J. S. 1996. Conspecific interactions in the lycosid spider *Rabidosa rabida*: The roles of different senses. *J. Arachnol.* 24:16–23.

SAMU, F., TOFT, S. and KISS, B. 1999. Factors influencing cannibalism in the wolf spider *Pardosa agrestis* (Araneae, Lycosidae). *Behav. Ecol. Sociobiol.* 45:349–354.

SIH, A., 1997. To hide or not to hide? Refuge use in a fluctuating environment. *Trends Ecol. Evol.* 12:375–376.

VENZON, M., JANSSEN, A., PALLINI, A., and SABELIS, M. W. 2000. Diet of a polyphagous arthropod predator affects refuge seeking of its thrips prey. *Anim. Behav.* 60:369–375.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

SEQUESTRATION OF HOST PLANT GLUCOSINOLATES IN THE DEFENSIVE HEMOLYMPH OF THE SAWFLY *Athalia rosae*

CAROLINE MÜLLER,[1,*] NIELS AGERBIRK,[2] CARL ERIK OLSEN,[2] JEAN-LUC BOEVÉ,[3] URS SCHAFFNER,[4] and PAUL M. BRAKEFIELD[1]

[1]*Institute of Evolutionary and Ecological Sciences, Leiden University P.O. Box 9516, NL-2300 RA Leiden, The Netherlands*

[2]*Chemistry Department, Royal Veterinary and Agricultural University Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark*

[3]*Department of Entomology, Royal Belgian Institute of Natural Sciences Rue Vautier 29, B-1000 Bruxelles, Belgium*

[4]*CABI-Bioscience Switzerland Centre Rue des Grillons 1, CH-2800 Delémont, Switzerland*

(Received June 8, 2001; accepted August 14, 2001)

Abstract—Interactions between insects and glucosinolate-containing plant species have been investigated for a long time. Although the glucosinolate–myrosinase system is believed to act as a defense mechanism against generalist herbivores and fungi, several specialist insects use these secondary metabolites for host plant finding and acceptance and can handle them physiologically. However, sequestration of glucosinolates in specialist herbivores has been less well studied. Larvae of the turnip sawfly *Athalia rosae* feed on several glucosinolate-containing plant species. When larvae are disturbed by antagonists, they release one or more small droplets of hemolymph from their integument. This "reflex bleeding" is used as a defense mechanism. Specific glucosinolate analysis, by conversion to desulfoglucosinolates and analysis of these by high-performance liquid chromatography coupled to diode array UV spectroscopy and mass spectrometry, revealed that larvae incorporate and concentrate the plant's characteristic glucosinolates from their hosts. Extracts of larvae that were reared on *Sinapis alba* contained sinalbin, even when the larvae were first starved for 22 hr and, thus, had empty guts. Hemolymph was analyzed from larvae that were reared on either *S. alba*, *Brassica nigra*, or *Barbarea stricta*. Leaves were analyzed from the same plants the larvae had fed on. Sinalbin (from *S. alba*), sinigrin (*B. nigra*), or glucobarbarin and glucobrassicin (*B. stricta*) were present in leaves in concentrations less than

*To whom correspondence should be addressed. E-mail: roca2911@hotmail.com

0098-0331/01/1200-2505/0 © 2001 Plenum Publishing Corporation

1 μmol/g fresh weight, while the same glucosinolates could be detected in the larvae's hemolymph in concentrations between 10 and 31 μmol/g fresh weight, except that glucobrassicin was present only as a trace. In larval feces, only trace amounts of glucosinolates (sinalbin and sinigrin) could be detected. The glucosinolates were likewise found in freshly emerged adults, showing that the sequestered phytochemicals were transferred through the pupal stage.

Key Words—*Athalia rosae*, sawfly, Hymenoptera, *Sinapis alba*, *Barbarea stricta*, *Brassica nigra*, Brassicaceae, glucosinolates, hemolymph, sequestration.

INTRODUCTION

Defensive secondary metabolites of plants can be utilized by herbivorous insects when they are adapted to them. Such specialists take up certain host plant compounds and concentrate them in particular tissues or organs (Duffey, 1980). Such sequestration of toxic compounds can contribute to making the herbivores themselves unpalatable for members of the third trophic level (Duffey, 1980; Trigo, 2000). Sequestered compounds include alkaloids (Rothschild and Edgar, 1978; Dobler et al., 2000; Abe et al., 2001), cardenolides (Brower and Fink, 1985; Scudder et al., 1986; Carroll et al., 1997; Dobler et al., 1998), and flavonoids (Blum, 1981; Schittko et al., 1999). Most of the insects where sequestration has been demonstrated belong to the Lepidoptera, Coleoptera, or Heteroptera (Duffey, 1980, and references given above).

Glucosinolates are secondary metabolites with a restricted taxonomic distribution (Rodman et al., 1998; Halkier, 1999; Fahey et al., 2001) that form a binary defense system with the glucosinolate-hydrolyzing enzyme myrosinase (Rask et al., 2000). Although the glucosinolate–myrosinase system is believed to be involved in plant resistance to generalist herbivores (Louda and Mole, 1991; Li et al., 2000; Potter et al., 2000) and fungi (e.g., Manici et al., 1997), several specialist insects are not repelled by these phytochemicals (Nielsen et al., 2001, and references therein). To the contrary, glucosinolates and their hydrolysis products, such as volatile isothiocyanates (mustard oils), thiocyanates, nitriles, and others (Chew, 1988a), are involved in host plant finding and acceptance (Nielsen, 1988; Chew, 1988a,b; Louda and Mole, 1991). Even high levels of glucosinolates can apparently be physiologically handled by such specialist insects (e.g., Li et al., 2000; Agerbirk et al., 2001a).

Sequestration of glucosinolates and isothiocyanates has been investigated in two butterfly species; the glucosinolate sinigrin (allylglucosinolate) and allyl isothiocyanate could be detected in whole pupae of *Pieris brassicae* L. and *P. rapae* L. (Lepidoptera: Pieridae) (Aplin et al., 1975). Allyl isothiocyanate was detected in whole-body extracts of larvae and adults of *P. brassicae* (Aplin et al., 1975). However, the glucosinolate detection by Aplin et al. (1975) in *Pieris*

pupae may not be reproducable: none could be detected by the desulfoglucosinolate method in extracts of *P. rapae* pupae whose larvae had fed on cabbage (J. A. A. Renwick, personal communication). Furthermore, it is surprising that Aplin et al. (1975) apparently detected sinigrin as the only glucosinolate in cabbage, while a complex mixture of glucosinolates is normally found in this plant (e.g., Sang et al., 1984; Renwick et al., 1992; Mithen et al., 2000). Since the methods used by Aplin et al. (1975) are now outdated, the report of sequestration of sinigrin (and perhaps also allyl isothiocyanate) in *Pieris* sp. probably needs confirmation with modern analytical methods.

Larvae of the sawfly *Athalia rosae ruficornis* Jakovlev (Hymenoptera: Tenthredinidae, Allantinae) feed on different glucosinolate-containing plant species, such as the Brassicaceae *Armoracia rusticana*, *Barbarea* spp., *Brassica* spp., *Rorippa amphibia, Sinapis alba, Sisymbrium officinale,* and the Tropaeolaceae *Tropaeolum majus* (Liston, 1995). The integument of *A. rosae* larvae is readily disrupted when touched by predators. Droplets of hemolymph are released (Figure 1) that are unpalatable to different antagonists (Ohara et al., 1993; Schaffner et al., 1994). In this study, we investigated whether *A. rosae* takes up glucosinolates from its different host plants, whether these are sequestered in the defensive hemolymph, and whether they are transferred into the adult lifestage.

METHODS AND MATERIALS

Plants. Plants of *Sinapis alba* L. (cultivar: Salvo, seeds from Advanta Seeds B.V., Netherlands), *Brassica nigra* (L.) Koch, and *Barbarea stricta* Andrz. (seeds

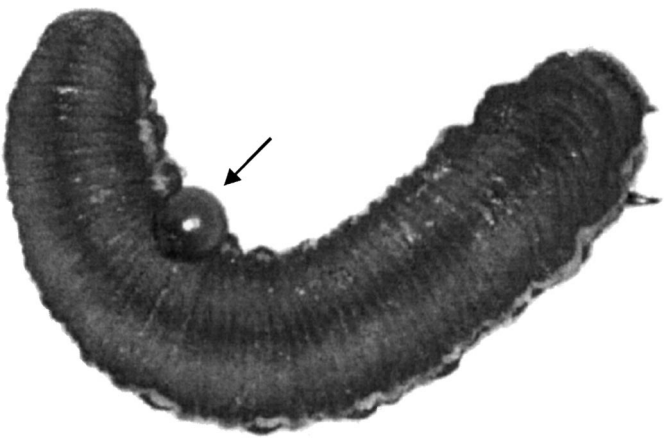


FIG. 1. Larva of *Athalia rosae*, showing a droplet of hemolymph after being touched (length of larva: 1.2 cm).

obtained as described in Agerbirk et al. 2001b) were grown in a greenhouse (20°C, 70% relative humidity, 16L:8D). Four- to 5-week-old, preflowering plants were used.

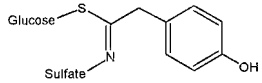
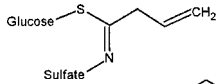
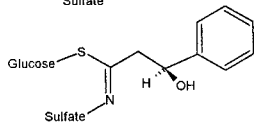
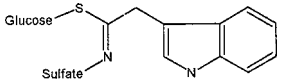
Insects. Cocoons of *Athalia rosae* were obtained from a colony descending from adults collected in the Rhine Valley, Germany, in September 2000. Insects were reared on whole plants of *S. alba* at 20°C, 70% relative humidity, and 16L:8D in the Leiden laboratory. Five fourth-instar larvae (two samples) were taken from the plants and frozen at −80°C. Five additional fourth-instar larvae (two samples) were starved for 22 hr in clean Petri dishes to empty their guts and then frozen. Hemolymph was collected from living fourth- and fifth-instar larvae by piercing the integument carefully with a needle at the middle of the abdomen without damaging the gut. Exuding hemolymph was collected with glass capillaries in 2-ml volume glass vials, weighed, and immediately frozen. Initially, hemolymph was collected from up to 20 larvae, but later samples were collected from smaller numbers. Feces (30–100 mg fresh weight) were collected by placing fourth- and fifth-instar larvae on clean filter paper in Petri dishes without plant material, and then frozen. Five female and five male adults (two samples each) were frozen directly after emergence from pupae. Pupal time lasted 13–16 days.

In addition, second-instar larvae (mean weight: 3.5 mg) that had emerged from leaves of *S. alba* were transferred to leaves of either *B. nigra* or *B. stricta*. When the larvae reached their fourth instar (mean weight: 45 mg), feces and hemolymph were collected as described above. Leaf samples (50–100 mg fresh weight) from all three species were picked from the same plants the larvae had eaten on. All samples were weighed fresh, frozen at −80°C, and freeze dried for 24 hr.

Glucosinolate Extraction and Analysis. Glucosinolates were extracted in boiling aqueous 70% methanol with benzylglucosinolate as internal standard, converted to desulfoglucosinolates, and analyzed by HPLC as described elsewhere (Agerbirk et al., 2001a), except that the detection wavelength was 226 nm (bandwidth 4 nm) because a published relative response factor for sinalbin was available at this wavelength. The relative response factors reported by McGregor (1985), as cited by Buchner (1987), were used for sinalbin (*p*-hydroxybenzylglucosinolate) (0.50), sinigrin (allylglucosinolate) (1.00), glucobrassicin (indolyl-3-methylglucosinolate) (0.26), and benzylglucosinolate (0.86). Glucobarbarin [(*S*)-2-hydroxy-2-phenylethylglucosinolate] was assumed to have the same response factor as benzylglucosinolate since the chromophores in these two analytes are identical. The internal standard and reference compounds were obtained as previously described (Agerbirk et al., 2001a,b; Nielsen et al., 2001).

The identity of the major glucosinolates sinalbin, sinigrin, glucobarbarin, and glucobrassicin was confirmed by comparison of HPLC retention time and diode array UV spectrum with authentic reference compounds and by analysis of hemolymph-derived desulfoglucosinolate samples by HPLC in conjunction with mass spectrometry (LC-MS) (Table 1). LC-MS was done on an HP1100 LC

TABLE 1. STRUCTURES AND ANALYTICAL DATA CONFIRMING IDENTITY OF GLUCOSINOLATES IN LEAVES OF *Sinapis alba*, *Brassica nigra*, AND *Barbarea stricta* AND IN HEMOLYMPH OF *Athalia rosae*[a]

		Desulfoglucosinolates		
Trivial name	Structure	HPLC R_t (min)	UV spectral data (λ_{max}, nm)	Molecular ion $[M+Na]^+$ (a.m.u.)
Sinalbin		17.6	224 276	368
Sinigrin		9.5	225	302
Glucobarbarin		21.5	226 (sh)	382
Glucobrassicin		28.4	219 273 (sh) 280 288 (sh)	(Not determined)

[a]HPLC retention times (R_t) and diode array UV spectral data were identical to values for authentic compounds. The molecular mass of $[M+Na]^+$ was measured by HPLC-MS and confirmed the identifications. sh – shoulder.

coupled to a Bruker Esquire-LC ion trap mass spectrometer. For reversed-phase LC, a C_{18} column (Chrompack Inertsil 3 ODS-3 S15 × 3 COL CP 29126) was used. The mobile phase was water doped with sodium acetate (50 M) (A), and methanol (B), running with a flow rate of 0.25 ml/min in the following gradient: 0–2 min isocratic 100% A; 2–40 min linear gradient 0–60% B; 40–45 min linear gradient 60–100% B, and 45–50 min isocratic 100% B. The mass spectrometer was run in positive ion mode, and $[M+Na]^+$ adduct ions were observed exclusively.

RESULTS

The dominant glucosinolate in leaves of *S. alba* was sinalbin (Table 1), which was found in a concentration of 0.1–0.3 μmol/g fresh weight. Other glucosinolates that are described for seeds and leaves of *S. alba* (Fahey et al., 2001) were not detected. Sinalbin was found in fed larvae of *A. rosae* in about eightfold higher concentration per gram fresh weight compared to leaf material. The glucosinolate was present in starved larvae at a similar concentration, confirming that the high concentration in fed larvae was not largely derived from gut contents. The glucosinolate was sequestered in the larvae's hemolymph, where high concentrations of sinalbin (about 10 μmol/g fresh weight) were found (Table 2). In larval hemolymph and adults reared on *S. alba*, varying amounts (0.1–0.4 μmol/g fresh weight, calculated as sinigrin) of an unidentified peak (possibly 1-methylpropylglucosinolate) with a retention time close to sinigrin were also observed (results not shown). In larval feces, only trace amounts of glucosinolates were detected. Sinalbin was likewise present in freshly emerged adults of *A. rosae*, in females as well as in males (Table 2).

Sinigrin (Table 1) was detected in *B. nigra* leaves, and glucobarbarin (Table 1) in *B. stricta* leaves. Larvae switched at second instar to either of these species, also sequestered sinigrin and glucobarbarin, respectively. While the concentration of glucosinolates was below 1 μmol/g fresh weight in the leaf material, they were found in concentrations between around 12 and 31 μmol/g fresh weight in the hemolymph of larvae fed *B. stricta* or *B. nigra* (Table 2). However, a further prominent glucosinolate in the leaves of *B. stricta*, glucobrassicin, was apparently not sequestered in proportion to its concentration in the leaf tissue (Table 2). Only trace amounts or no glucosinolates were detected in larval feces (Table 2).

Sinalbin could not be detected in hemolymph of larvae that were originally transferred from *S. alba* to *B. nigra* or *B. stricta*, suggesting that any sinalbin sequestered during the short time the larvae fed on *S. alba* was either metabolized or diluted below the detection level by their growth. A sequestration might also happen only in later instars. Neither sinigrin, glucobarbarin, nor glucobrassicin was detected in hemolymph of larvae that had fed exclusively on *S. alba*.

TABLE 2. CONCENTRATIONS OF GLUCOSINOLATES, SINALBIN, SINIGRIN, GLUCOBARBARIN, AND GLUCOBRASSICIN, DETECTED IN SAMPLES OF LEAVES OF *Sinapis alba*, *Brassica nigra*, AND *Barbarea stricta*, AND IN SAWFLY *Athalia rosae* REARED ON DIFFERENT HOST PLANTS[a]

Host plant	Sample	Concentration (mean ± SD, μmol/g fresh weight)				Sample no.
		Sinalbin	Sinigrin	Glucobarbarin	Glucobrassicin	
S. alba	leaf	0.19 ± 0.15	n.d.	n.d.	n.d.	2
	5 larvae, fed	1.61 ± 0.40	n.d.	n.d.	n.d.	2
	5 larvae, starved	1.53 ± 0.03	n.d.	n.d.	n.d.	2
	larval hemolymph	9.54 ± 2.32	n.d.	n.d.	n.d.	6
	larval feces	0.02 (trace)	n.d.	n.d.	n.d.	2
	5 adult females	1.95 ± 0.95	n.d.	n.d.	n.d.	2
	5 adult males	1.61 ± 0.04	n.d.	n.d.	n.d.	2
B. nigra	leaf	n.d.	0.54 ± 0.13	n.d.	n.d.	2
	larval hemolymph	n.d.	30.9 ± 12.8	n.d.	n.d.	2
	larval feces	n.d.	0.03 (trace)	n.d.	n.d.	2
B. stricta	leaf	n.d.	n.d.	0.61	0.29	1
	larval hemolymph	n.d.	n.d.	11.6 ± 3.4	0.05 (trace)	2
	larval feces	n.d.	n.d.	n.d.	n.d.	2

[a] Eggs were laid in *S. alba*, and emerging larvae partly transferred on other host plants as second instars. Starvation of larvae occurred for 22 hr. n.d.—not detected.

DISCUSSION

Larvae of the sawfly species *A. rosae* are able to take up glucosinolates from their host plants and concentrate them effectively in the hemolymph. The glucosinolates are apparently not biosynthesized *de novo* by larvae, since only those particular ones present in the hemolymph were also found in the respective host plant species the larvae had fed on.

Sequestration of plant defensive compounds in different organs has been shown previously in other sawfly species (Codella and Raffa, 1993). Species of the Diprionidae and Pergidae sequester volatile terpenoids from *Pinus* or *Eucalyptus* leaves in larval pouches of the foregut (Prop, 1960; Eisner et al., 1974; Morrow et al., 1976) and regurgitate them when disturbed. The Tenthredinidae *Tenthredo grandis* (Norton) sequesters iridoid glucosides from *Chelone glabra* L. (Bowers et al., 1993); however, the sequestering organ has not been studied. A sequestration in *Salix*-feeding species was suggested by Sevastopulo (1958). In the sawfly *Rhadinoceraea nodicornis* Konow (Blennocampinae), steroid alkaloids from the host plant *Veratrum* spp. (Melanthiaceae) are metabolized and sequestered also in the hemolymph (Schaffner et al., 1994).

Besides *R. nodicornis* and *A. rosae*, several other sawfly species of the subfamilies Blennocampinae and Selandriinae exhibit reflex bleeding when disturbed by predators. Droplets of hemolymph are readily released upon wounding (Heads and Lawton, 1985; Heads, 1986; Schaffner et al., 1994). This phenomenon is often linked to sequestration of toxic plant compounds.

The larval hemolymph of some sawfly species has a high feeding deterrent effect on ants, spiders, and birds (Ohara et al., 1993; Schaffner et al., 1994; Schaffner and Boevé, 1996). Hemolymph of *A. rosae* deterred drinking of the ant *Myrmica rubra* L. (Hymenoptera, Formicidae) even when diluted 10-fold (Schaffner et al., 1994). However, in different sawfly species, different classes of chemicals result in deterrent effects on predators. In *A. rosae*, it remains to be confirmed whether glucosinolates are responsible for the defensive activity of the hemolymph.

Sequestration of plant compounds requires several physiological adaptations (Duffey, 1980; Bowers, 1992). First, the insect must have a specific uptake mechanism. The way glucosinolates pass through the gut wall and are stored in the hemolymph is not known. However, their high concentration in the hemolymph relative to the food source suggests that an active transport mechanism exists. The hypothetical uptake mechanism seems to be nonspecific with respect to the structure of the side chain, since different structural types of glucosinolates were sequestered. Interestingly, the minor amounts of glucobrassicin in hemolymph from *B. nigra*-reared larvae (Table 2) suggests that not all glucosinolates are sequestered with the same efficiency. Second, the insects need to be protected from self-poisoning. Glucosinolates and their hydrolysis products have a deterrent, toxic, or antinutritional effect on most organisms (Louda and Moule, 1991; Potter et al., 2000; Fahey et al., 2001).

Many herbivorous insects excrete toxic plant compounds unchanged with their feces, a physiological process known as the pharmacokinetic strategy (Isman, 1992). Nicotine is, for example, for the most part excreted by the tobacco hornworm *Manduca sexta* (L.) (Lepidoptera: Noctuidae) (Murray et al., 1994). Other toxic compounds are detoxified by specific enzymes in feeding specialists (Witthohn and Naumann, 1987; Kunze et al., 1996) or by mixed function oxidases in generalists (Hodgson and Rose, 1991; Brattsten, 1992). Only trace amounts of glucosinolates were detectable in the feces of *A. rosae* larvae. Either they are almost completely absorbed by the gut wall or a certain amount is hydrolyzed or otherwise degraded while passing through the gut (e.g., Mithen et al., 2000).

With regard to different developmental stages, glucosinolates sequestered by *A. rosae* larvae were apparently passed unchanged, and in similar concentrations, into the adult life stage (Table 2). Since *A. rosae* eggs are laid in the leaf tissue, they were not studied for the presence of glucosinolates.

Female as well as male adults of *A. rosae* are also known to sequester bitter tasting clerodendrines from *Clerodendron trichotomum* (Verbenaceae) in body

tissue (Nishida and Fukami, 1990). These clerodendrines are deterrent to several predators, such as lizards and sparrows (Nishida and Fukami, 1990), and are probably deposited in the insect cuticle (Amano et al., 1999). They also play an important role in the mating behavior of this species (Amano et al., 1999).

In this study, we analyzed only the glucosinolates, but other secondary compounds of the Brassicaceae might also be sequestered, for example, proto- or pseudoalkaloids such as sinapin from *S. alba*, phenolics (e.g., quercetin, rutin), or glucosinolate hydrolysis products. Future studies will show whether the sequestration of glucosinolates is indeed effective against different predators, whether host plant chemistry is important in larval survival, and whether other bioactive compounds are present in the hemolymph.

Acknowledgments—J. K. Nielsen is acknowledged for making the HPLC equipment available for our studies. We thank H. O. Frandsen, I. Mahad, and H. de Vos for technical assistance; A. Barker (Switzerland) for providing us with the first *A. rosae* individuals to start a stock; and E. Schlatmann and coworkers for cultivating the different crucifers. This research was funded by the European Commission and is part of the Research Training Network project INCHECO (Human Potential Program, HPRN-CT-1999-00054).

REFERENCES

ABE, F., YAMAUCHI, T., HONDA, K., OMURA, H., and HAYASHI, N. 2001. Sequestration of phenanthroindolizidine alkaloids by an Asclepiadaceae-feeding danaid butterfly, *Ideopsis similis*. *Phytochemistry* 56:697–701.

AGERBIRK, N., OLSEN, C. E., and NIELSEN, J. K. 2001a. Seasonal variation in leaf glucosinolates and insect resistance in two types of *Barbarea vulgaris* ssp. *arcuata*. *Phytochemistry* 58:91–100.

AGERBIRK, N., PETERSEN, B. L., OLSEN, C. E., HALKIER, B. A., and NIELSEN, J. K. 2001b. 1,4-Dimethoxyglucobrassicin in *Barbarea* and 4-hydroxyglucobrassicin in *Arabidopsis* and *Brassica*. *J. Agric. Food Chem*. 49:1502–1507.

AMANO, T., NISHIDA, R., KUWAHARA, Y., and FUKAMI, H. 1999. Pharmacophagous acquisition of clerodendrins by the turnip sawfly (*Athalia rosae ruficornis*) and their role in the mating behavior. *Chemoecology* 9:145–150.

APLIN, R. T., D'ARCY WARD, R., and ROTHSCHILD, M. 1975. Examination of the large white butterfly and small white butterflies (*Pieris* spp.) for the presence of mustard oils and mustard oil glycosides. *J. Entomol. A* 50:73–78.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

BOWERS, M. D. 1992. The evolution of unpalatability and the cost of chemical defense in insects, pp. 216–244, *in* B. D. Roitberg and M. B. Isman (eds.). Insect Chemical Ecology. An Evolutionary Approach. Chapman and Hall, New York.

BOWERS, M. D., BOOCKVAR, K., and COLLINGE, S. K. 1993. Iridoid glycosides of *Chelone glabra* (Scrophulariaceae) and their sequestration by larvae of the sawfly, *Tenthredo grandis* (Tenthredinidae). *J. Chem. Ecol*. 19:815–823.

BRATTSTEN, L. B. 1992. Metabolic defenses against plant allelochemicals, pp. 175–242, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with

Secondary Plant Metabolites, Vol. II: Evolutionary and Ecological Processes. Academic Press, New York.

BROWER, L. P. and FINK, L. S. 1985. A natural toxic defense system: Cardenolides in butterflies versus birds. *Ann. NY Acad. Sci*. 443:171–186.

BUCHNER, R. 1987. Approach to determination of HPLC response factors for glucosinolates, pp. 50–58, *in* J.-P. Wathelet (ed.). Glucosinolates in Rapeseeds: Analytical Aspects. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

CARROLL, M., HANLON, A., HANLON, T., ZANGERL, A. R., and BERENBAUM, M. R. 1997. Behavioral effects of carotenoid sequestration by the parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol*. 23:2707–2719.

CHEW, F. S. 1988a. Searching for defensive chemistry in the Cruciferae, or, do glucosinolates always control interactions of Cruciferae with their potential herbivores and symbionts? No!, pp. 81–112, *in* K. C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, Toronto, Canada.

CHEW, F. S. 1988b. Biological effects of glucosinolates, pp. 155–181, *in* H. G. Cutler (ed.). Biologically Active Natural Producs—Potential Use in Agriculture. American Chemical Society Symposium, Washington, D.C.

CODELLA, S. G., and RAFFA, K. F. 1993. Defense strategies of folivorous sawflies, pp. 261–294, *in* M. R. Wagner and K. F. Raffa (eds.). Sawfly Life History. Adaptations to Woody Plants. Academic Press, San Diego, California.

DOBLER, S., DALOZE, D., and PASTEELS, J. M. 1998. Sequestration of plant compounds in a leaf beetle's defensive secretion: Cardenolides in *Chrysochus*. *Chemoecology* 8:111–118.

DOBLER, S., HABERER, W., WITTE, L., and HARTMANN, T. 2000. Selective sequestration of pyrrolizidine alkaloids from diverse host plants by *Longitarsus* flea beetles. *J. Chem. Ecol*. 26:1281–1298.

DUFFEY, S. S. 1980. Sequestration of plant natural products by insects. *Annu. Rev. Entomol*. 25:447–477.

EISNER, T., JOHANESSEE, J. S., CARREL, J., HENDRY, L. B., and MEINWALD, J. 1974. Defensive use by an insect of a plant resin. *Science* 184:996–999.

FAHEY, J. W., ZALCMANN, A. T., and TALALAY, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51.

HALKIER, B. A. 1999. Glucosinolates, pp. 193–223, *in* R. Ikan (ed.). Naturally Occuring Glycosides. John Wiley & Sons Ltd., London.

HEADS, P. A. 1986. Bracken, ants and extrafloral nectaries. IV. Do wood ants (*Formica lugubris*) protect the plant against insect herbivores? *J. Anim. Ecol*. 55:795–809.

HEADS, P. A. and LAWTON, J. H. 1985. Bracken, ants and extrafloral nectaries. III. How insect herbivores avoid ant predation. *Ecol. Entomol*. 10:29–42.

HODGSON, E. and ROSE, R. 1991. Insect Cytochrome P 450, pp. 75–91, *in* E. Arinc, J. B. Schenkman, and E. Hodgson (eds.). Molecular Aspects of Monooxygenases and Bioactivation of Toxic Compounds. Plenum Press, New York.

ISMAN, M. B. 1992. A physiological perspective, pp. 156–176, *in* B. D. Roitberg and M. B. Isman (eds.). Insect Chemical Ecology. An Evolutionary Approach. Chapman and Hall, New York.

KUNZE, A., AREGULLIN, M., RODRIGUEZ, E., and PROKSCH, P. 1996. Fate of the chromene encecalin in the interactions of *Encelia farinosa* and its specialized herbivore *Trirhabda geminata*. *J. Chem. Ecol*. 22:491–498.

LI, Q., EIGENBRODE, S. D., STRINGHAM, G. R., and THIAGARAJAH, M. R. 2000. Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. *J. Chem. Ecol*. 26:2401–2419.

LISTON, A. 1995. Compendium of European sawflies. Chalastos Forestry, Gottfrieding, Germany.

LOUDA, S. and MOLE, S. 1991. Glucosinolates: Chemistry and ecology, pp. 123–164, *in* G. A. Rosenthal and M. R. Berenbaum (eds.): Herbivores and Their Interactions with Secondary Plant Metabolites 2E (1): Chemical Participants. Academic Press, New York.

MANICI, L. M., LAZZERI, L., and PALMIERI, S. 1997. In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products towards plant pathogenic fungi. *J. Agric. Food Chem*. 45:2768–2773.

MCGREGOR, D. I. 1985. Determination of glucosinolates in *Brassica* seed. *Eucarpia Cruciferae Newsl*. 10:132–136.

MITHEN, R. F., DEKKER, M., VERVERK, R., RABOT, S., and JOHNSON, I. T. 2000. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. *J. Sci. Food Agric*. 80:967–984.

MORROW, P. A., BELLAS, T. E., and EISNER, T. 1976. Eucalyptus oil in the defensive oral discharge of Australian sawfly larvae (Hymenoptera: Pergidae). *Oecologia* 24:193–206.

MURRAY, C. L., QUAGLIA, M., ARNASON, J. T., and MORRIS, C. E. 1994. A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. *J. Neurobiol*. 25:23–34.

NIELSEN, J. K. 1988. Crucifer-feeding Chrysomelidae: Mechanisms of host plant finding and acceptance, pp. 25–40, *in* P. Jolivet, E. Petitpierre, and T. H. Hsiao (eds.): Biology of Chrysomelidae. Kluwer Academic Publishers, Dordrecht, The Netherlands.

NIELSEN, J. K., HANSEN, M. L., AGERBIRK, N., PETERSEN, B. L., and HALKIER, B. A. 2001. Responses of the flea beetles *Phyllotreta nemorum* and *P. cruciferae* to metabolically engineered *Arabidopsis thaliana* with an altered glucosinolate profile. *Chemoecology* 11:75–83.

NISHIDA, R. and FUKAMI, H. 1990. Sequestration of distasteful compounds by some pharmacophagous insects. *J. Chem. Ecol*. 16:151–164.

OHARA, Y., NAGASAKI, K., and OHSAKI, N. 1993. Warning coloration in sawfly *Athalia rosae* larva and concealing coloration in butterfly *Pieris rapae* larva on similar plants evolved through individual selection. *Res. Popul. Ecol*. 19:223–230.

POTTER, M. J., VANSTONE, V. A., DAVIES, K. A., and RATHJEN, A. J. 2000. Breeding to increase the concentration of 2-phenylethyl glucosinolate in the roots of *Brassica napus*. *J. Chem. Ecol*. 26:1811–1820.

PROP, N. 1960. Protection against birds and parasites in some species of tenthredinid larvae. *Arch. Neerl. Zool*. 13:380–447.

RASK, L., ANDRÉASSON, E., EKBOM, B., ERIKSSON, S., PONTOPPIDAN, B., and MEIJER, J. 2000. Myrosinase: Gene family evolution and herbivore defense in Brassicaceae. *Plant Mol. Biol*. 42:93–113.

RENWICK, J. A. A., RADKE, C. D., SACHDEV-GUPTA, K., and STÄDLER, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3: 33–38.

RODMAN, J. E., SOLTIS, P. S., SOLTIS, D. E., SYTSMA, K. J., and KAROL, K. G. 1998. Parallel evolution of glucosinolate biosynthesis inferred from congruent nuclear and plastid gene phylogenies. *Am. J. Bot*. 85:997–1006.

ROTHSCHILD, M. and EDGAR, J. A. 1978. Pyrrolizidine alkaloids from *Senecio vulgaris* sequestered and stored by *Danaus plexippus*. *J. Zool*. 186:347–349.

SANG, J. P., MINCHINTON, I. R., JOHNSTONE, P. K., and TRUSCOTT, R. J. W. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Can. J. Plant Sci*. 64:77–93.

SCHAFFNER, U. and BOEVÉ, J.-L. 1996. Sequestration of plant alkaloids by the sawfly *Rhadinoceraea nodicornis*: Ecological relevance for different life stages and occurence among related species. *Entomol. Exp. Appl*. 80:283–285.

SCHAFFNER, U., BOEVÉ, J.-L., GFELLER, H., and SCHLUNEGGER, U. P. 1994. Sequestration of *Veratrum* alkaloids by specialist *Rhadinoceraea nodicornis* Konow (Hymenoptera, Tenthredinidae) and its ecoethological implications. *J. Chem. Ecol*. 20:3233–3250.

SCHITTKO, U., BURGHARDT, F., FIEDLER, K., WRAY, V., and PROKSCH, P. 1999. Sequestration and distribution of flavonoids in the common blue butterfly *Polyommatus icarus* reared on *Trifolium repens*. *Phytochemistry* 51:609–614.

SCUDDER, G. G. E., MOORE, L. V., and ISMAN, M. B. 1986. Sequestration of cardenolides in *Oncopeltus fasciatus*: Morphological and physiological adaptations. *J. Chem. Ecol*. 12:1171–1187.

SEVASTOPULO, D. G. 1958. Defensive action of a sawfly larva (Hym., Symphyta). *Entomol. Month. Mag*. 94:275.

TRIGO, J. R. 2000. The chemistry of antipredator defense by secondary compounds in neotropical Lepidoptera: Facts, perspectives and caveats. *J. Braz. Chem*. Soc. 11:551–561.

WITTHOHN, K. and NAUMANN, C. M. 1987. Cyanogenesis—a general phenomenon in the Lepidoptera? *J. Chem. Ecol*. 13:1789–1809.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

ETHYL *m*-DIGALLATE FROM RED MAPLE, *Acer rubrum* L., AS THE MAJOR RESISTANCE FACTOR TO FOREST TENT CATERPILLAR, *Malacosoma disstria* Hbn.

MAMDOUH M. ABOU-ZAID,[1,*] BLAIR V. HELSON,[1] CONSTANCE NOZZOLILLO,[2] and J. THOR ARNASON[2]

[1]*Natural Resources Canada, Canadian Forest Service*
Great Lakes Forestry Centre
1219 Queen St. East, Sault Ste. Marie, Ontario, Canada P6A 2E5

[2]*Ottawa-Carleton Institute of Biology*
University of Ottawa
Ottawa, Ontario, Canada K1N 6N5

(Received March 10, 2001; accepted August 1, 2001)

Abstract—An ethanolic extract of red maple (*Acer rubrum* L.) leaves (RME) applied to trembling aspen (*Populus tremuloides* Michx.) leaves reduced feeding in choice test assays with forest tent caterpillar larvae (*Malacosoma disstria* Hbn.) (FTC), whereas a trembling aspen foliage extract, similarly applied, stimulated feeding. Compounds isolated from the RME were gallic acid, methyl gallate, ethyl gallate, *m*-digallate, ethyl *m*-digallate, 1-*O*-galloyl-β-D-glucose, 1-*O*-galloyl-α-L-rhamnose, kaempferol 3-*O*-β-D-glucoside, kaempferol 3-*O*-β-D-galactoside, kaempferol 3-*O*-β-L-rhamnoside, kaempferol-3-*O*-rhamnoglucoside, quercetin 3-*O*-β-D-glucoside, quercetin 3-*O*-β-L-rhamnoside and quercetin 3-*O*-rhamnoglucoside, (−)-epicatechin, (+)-catechin and ellagic acid. All of the gallates, (−)-epicatechin, and kaempferol 3-*O*-β-L-rhamnoside deterred feeding on trembling aspen leaf disks when applied at 0.28 mg/cm^2. The two digallates deterred feeding by 90% and were the most effective. HPLC analysis indicated that ethyl *m*-digallate is present in amounts 10–100 × higher in RME (∼2.5–250 mg/g) than any other compound. Thus, ethyl *m*-digallate appears to be the major compound protecting red maple from feeding by FTC, with a minor contribution from other gallates.

Key Words—*Acer rubrum* L., red maple, *A. saccharum* L., sugar maple, Aceraceae, *Populus tremuloides* Michx., trembling aspen, Salicaceae, *Malacosoma disstria* Hubner, forest tent caterpillar, Lepidoptera, Lasiocampidae, feeding deterrence, phenolics, gallates, flavonols.

*To whom correspondence should be addressed. E-mail: mabouzai@NRCan.gc.ca

INTRODUCTION

Hardwood forests are endangered by severe defoliation caused by insects. The forest tent caterpillar (FTC) (*Malacosoma disstria* Hubner) is a major pest of Canadian deciduous forests feeding mainly on poplar (*Populus* spp.), particularly trembling aspen (*P. tremuloides* Michx.). It also defoliates other species, including sugar maple (*Acer saccharum* Marsh) (Howse, 1995; Nicol et al., 1997). Red maple (*A. rubrum* L.), another prominent maple occupying a similar range (Farrar, 1995), is seldom attacked by FTC. Beavers (*Castor canadensis*) also have been reported to avoid red maple trees (Müller-Schwarze et al., 1994). Except for a 13% higher content of polyphenols in red maple (Ricklefs and Matthews, 1982), leaves do not exhibit major differences in characteristics, e.g., toughness, sugar content, water content, or nitrogen content, from those of sugar maple. Among polyphenols, tannins are considered as the major defense compounds in tree leaves, but since red and sugar maple leaves contain similar amounts of these complex molecules (Bate-Smith, 1978), some other constituent(s) must provide red maple leaves with a deterrent to feeding by FTC. In the present study, the constituents of an aqueous ethanolic extract of red maple leaves were isolated, identified, and then tested in leaf disk feeding assays in order to determine the phytochemical basis of insect resistance in red maple leaves.

METHODS AND MATERIALS

Plant Material. Red maple leaves were collected in June 1992 from 10 mature trees in Sault Ste. Marie, Ontario, Canada (lat. 46.34N, long. 84.17W). Leaves of trembling aspen were collected locally (Sault Ste. Marie) or grown in the greenhouse as needed for feeding tests. Pressed voucher specimens are deposited in the Canadian Forest Service-Sault Ste. Marie herbarium as *Acer rubrum* L. (CFS-SSM #s 1001–1010), family Aceraceae and trembling aspen (*Populus tremuloides* Michx.) (CFS-SSM #s 1255–1265), family Salicaceae.

Extraction. Fresh red maple leaves (2 kg fresh wt) were extracted at room temperature. First, leaves were steeped for 24 hr in 100% EtOH (1 g fresh wt/10 ml solvent), followed by chopping in a commercial Waring blender, and decanting the solvent. Next, the chopped residue was steeped for an additional 24 hr in 50% aqueous EtOH (10 g fresh wt/100 ml solvent). The combined ethanolic extracts (RME) were evaporated under reduced pressure until most or all of the EtOH had been removed. The residue was freeze-dried to obtain 242 g of crude extract (RME). Thus, from each gram fresh weight of leaves, 121 mg of RME was obtained. Fresh aspen leaf material (2 kg fresh wt) was similarly extracted to yield 204 g residue.

Fractionation. RME (100 g) was adsorbed onto 200 g of polyvinylpolypyrrolidone (PVPP) powder (Sigma Chem. Co., St. Louis, Missouri) preconditioned by

soaking in distilled water for 24 hr and packed in a Buchner funnel (4 liters). Elution was carried out at a slow rate using 5 liters of water followed by 5 liters aliquots of increasing concentrations (20, 50, 70, 100%) of ethanol. All five fractions were tested for antifeedant activity (data not shown) with maximum activity found in the fraction eluting with 50% ethanol, henceforth referred to as the phenolic fraction (RMP). The active fraction was concentrated under vacuum and chromatographed on Whatman No.1 chromatography paper (PC) using either BAW (*n*-butanol–acetic acid–water, 4:1:5, upper phase), water, or acetic acid–water (15:85) to isolate the pure compounds. Further fractionation was carried out on a PVPP column (7 × 120 cm ID.) using the following solvent systems: (i) CH_2Cl_2–EtOH–MeCOEt–Me_2CO (1:1:1:1), (ii) EtOH–MeCOEt–Me_2CO–H_2O (1:1:1:1), and (iii) EtOH–H_2O (1:1). Purification was achieved on a Chemco low-prep pump (model 9 1-M-8R), and fractions were tested for purity by HPLC.

HPLC. A Waters Delta Prep 4000 liquid chromatograph was used equipped with a computer and Millennium 2010 software, an autoscan photodiode array detector (Waters 996), and a Waters Nova-Pak C_{18} reverse-phase analytical column (4 μm, 60 Å, 3.9 × 150 mm ID). A modified gradient chromatographic technique (van Sumere et al., 1993) was used at room temperature: solvent A = MeOH; B = 5% aq. HCOOH; and a flow rate of 0.9 ml/min. Two fixed detection wavelengths were used, 280 nm and 350 nm, and resolved peaks were scanned from 250 to 400 nm by the photodiode array detector.

Identification of Isolated Compounds. UV spectra were recorded on a UV-Vis Beckman DU series 640 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer, at 500 MHz and 125 MHz, respectively; samples were dissolved in DMSO-d_6 with TMS as an internal standard. Structures of purified compounds were determined by comparison with authentic samples where available using standard methods (Harborne, 1994; Markham, 1982): acid hydrolysis in 2 M and 0.1 M HCl (mild hydrolysis) at 100°C for 60 min, enzymatic hydrolysis with β-glucosidase (Sigma) using an acetate buffer (pH 5), hydrogen peroxide oxidation, UV spectroscopy, ^{1}H NMR, ^{13}C NMR, and FAB-mass spectroscopy (positive and negative). The glycosides and aglycones obtained from hydrolysis of isolated compounds were identified by cochromatography with authentic samples (Aapin and Extrasynthese) using PC, TLC, and HPLC. Sugars released by hydrolysis were identified by PC and TLC using standards. Standard gallic acid and gallate derivatives were kindly provided by Profs. G. Gross, Universtät Ulm, Ulm, Germany, and T. Yoshida, Okayama University, Okayama, Japan.

Quantitative and Qualitative Analysis. A dilute solution (10 mg/ml) of RME or RMP was filtered and 10 μl was injected onto an HPLC column (van Sumere et al., 1993) with and without spiking with standards. Peaks were identified on the basis of retention times and UV spectra. Peak heights, measured at 280 nm, were converted to milligrams per milliliter using conversion factors obtained

for standards (Slacanin et al., 1991). Such HPLC analyses were performed in triplicate.

Insect Material. Egg bands of forest tent caterpillar, *Malacosoma disstria* Hubner, Lepidoptera, Lasiocampidae (FTC) were collected from trembling aspen trees from Cochrane and Thunder Bay, Ontario, Canada. Eggs were stored at 2°C for at least 13 weeks. Larvae were reared to the required stage on artificial diet (Addy, 1969; Singh and Moore, 1985) in environmental chambers (21°C, 80% relative humidity, and 16L:8D).

Leaf Disk Assays. Leaf disks (2-cm diam.) were cut with a #13 cork borer from freshly collected leaves and placed into a 9-cm-diam. plastic Petri plate (two disks of each species in alternating positions) to compare feeding on red maple and trembling aspen leaves. The disks were pinned in place through small holes in the bottom of the plate. Five newly molted fourth-instar larvae were placed into each of seven replicate plates. After 24 hr, the leaf disks were photocopied and the percentage of each disk remaining was determined with an Image Analyser (Artek, Image Editor and Video Memory, model 940). The antifeedant index (AI) for each plate was calculated as $\%AI = (C - T)/(C + T) \times 100$ where C is the percentage of two control disks eaten, and T is the percentage of two treated disks eaten (Blaney et al., 1994).

A second choice leaf disk assay method was used to determine the feeding deterrency of red maple and aspen leaf extracts applied to aspen leaf disks in side by side comparisons. Four, 1.5-cm-diam. disks from greenhouse-grown trembling aspen leaves were placed into a 9-cm-diam. plastic Petri plate and pinned in place. The upper surfaces of two opposite disks were each treated with four applications of 12.5 μl of 5% solutions (acetone–water, 80:20) of the extracts for a total of 2.5 mg of each crude extract. The other two disks were treated with solvent only. Five fourth-instar larvae were placed in each of 12 replicate plates for each extract. The percentage of foliage eaten was estimated visually until 35–65% of the disks in a dish were consumed. The AI was then calculated as above. The RMP extract (13 replicate plates) was also tested separately in the same fashion.

Feeding deterrency of individual compounds found in the RME extract, either available commercially or purified from the extract, was determined with the second leaf disk assay method. An equal mass (0.5 mg) of each compound was similarly applied to individual aspen leaf disks. Six to 29 replicate plates in at least duplicate tests were used for each compound. Methyl gallate was tested in 116 replicate plates as a regular standard.

Statistical analysis using appropriate parametric or nonparametric procedures was undertaken with GraphPAD InSTAT software (GraphPad, San Diego, California) or SigmaStat Statistical Software, Version 2 (SPSS Inc., Chicago, Illinois).

TABLE 1. HPLC ANALYSIS OF CRUDE RED MAPLE LEAF EXTRACT (RME) AND PHENOLIC FRACTION FROM RED MAPLE LEAF EXTRACT (RMP) ELUTED FROM PVPP WITH 50% ETHANOL–WATER

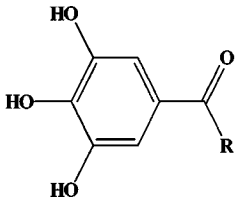
Compound	mg/g dried extract	
	RME	RMP
1-*O*-galloyl-β-D-glucose[a]	10.3	5.4
Gallic acid[a]	24.5	22.9
1-*O*-galloyl-α-L-rhamnose[a]	30.2	16.1
Methyl gallate[a]	2.5	2.5
Ethyl gallate[a]	2.0	3.0
m-Digallate[a]	24.5	58.6
Ethyl *m*-digallate[a]	248.5	302.5
Quercetin 3-*O*-β-D-glucoside[a]	6.1	12.2
Quercetin 3-*O*-rutinoside[a]	5.3	5.3
Kaempferol 3-*O*-β-D-galactoside[a]	6.2	4.1
Quercetin 3-*O*-β-L-rhamnoside[a]	15.0	28.0
Kaempferol 3-*O*-β-D-glucoside[a]	4.1	6.2
Kaempferol-3-*O*-rutinoside[a]	4.1	6.2
Kaempferol 3-*O*-β-L-rhamnoside[a]	4.1	8.3
Kaempferol (galloyl?)rutinoside[b]	28.0	32.0
Kaempferol (galloyl?)rhamnnoside[b]	3.0	3.0

[a] Compounds isolated from red maple leaf extract and used in Table 3.
[b] The tentatively identified acylated kaempferol rutinoside and rhamnoside were most probably hydrolysed to the free glycosides during the prolonged separation and isolation steps.

RESULTS AND DISCUSSION

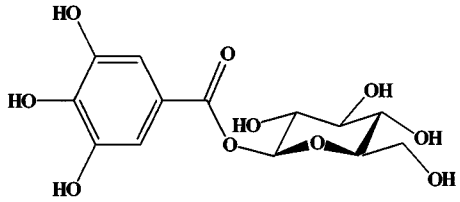
Identification of Isolated Compounds. Seventeen compounds were isolated and identified from *Acer rubrum* L. including the 14 listed in Table 1 and an additional three, (−)-epicatechin, (+)-catechin and ellagic acid (listed in Table 3 below). Structures (Figure 1) were established using standard procedures as described in Methods and Materials (Harborne, 1994; Markham, 1982). UV spectra, ^{1}H NMR, ^{13}C NMR, and MS were identical with standards.

Quantitative and Qualitative Analysis. The depside ethyl *m*-digallate, present at 248.5 mg/g is the predominant compound in both RME and RMP extracts (Table 1). Gallic acid is an important constituent of red maple leaf extracts (24.5 mg/g) as are 1-*O*-galloyl-β-D-glucose (10.3 mg/g), 1-*O*-galloyl-α-L-rhamnose (30.2 mg/g) and *m*-digallate (24.5 mg/g). By contrast, methyl gallate and ethyl gallate are present in RME in relatively low amounts (2.5 mg/g and 2 mg/g; respectively).

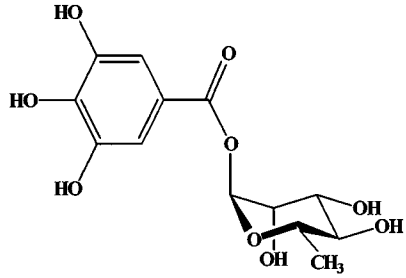


R = OH Gallic acid

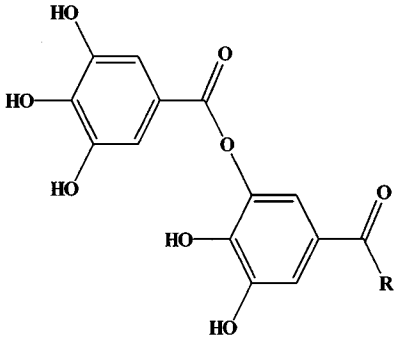
R = OCH_3 Methyl gallate



1-*O*-Galloyl-β-D-glucose

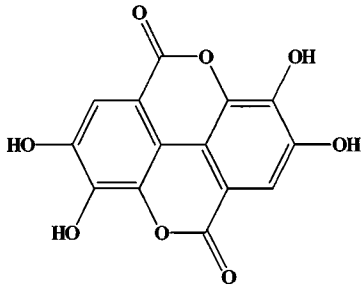


1-*O*-Galloyl-α-L-rhamnose

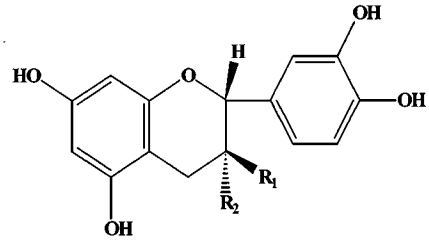


R = OH *m*-Digallate

R = OC_2H_5 Ethyl *m*-digallate

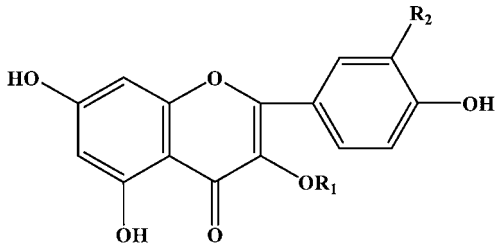


Ellagic acid



R_1=OH, R_2=H (+)-Catechin

R_1=H, R_2=OH (-)-Epicatechin



R_1= glucosyl, R_2=H	kaempferol 3-*O*-β-D-glucoside
R_1= galactosyl, R_2=H	kaempferol 3-*O*-β-D-galactoside
R_1= rhamnosyl, R_2=H	kaempferol 3-*O*-β-L-rhamnoside
R_1= rhamnoglucosyl, R_2=H	kaempferol-3-*O*-rhamnoglucoside
R_1= glucosyl, R_2= OH	quercetin 3-*O*-β-D-glucoside
R_1= rhamnosyl, R_2= OH	quercetin 3-*O*-β-L-rhamnoside
R_1= rhamnoglucosyl, R_2= OH	quercetin 3-*O*-rhamnoglucoside

FIG. 1. Structures of some of the phenolic compounds that have been isolated from ethanolic extracts of red maple (*Acer rubrum* L.) leaves.

The presence in maple leaves of gallic acid, its esters, and glycosides was previously examined by Haslam (1965), but the identification of 1-*O*-galloyl-α-L-rhamnose is a recent discovery (Abou-Zaid and Nozzolillo, 1999). Bailey et al. (1986) isolated methyl gallate from methanolic extracts of silver maple leaves (*A. saccharinum* L.), but this constitutes the first report of its presence in red maple leaves (Table 1). Haddock et al. (1982) pointed out the significance of the depside as a hydrolyzable tannin component of red maple and related species, but this is the first report of its presence as a free compound.

Ellagic acid, (+)-catechin, and (−)-epicatechin were not detectable in the HPLC traces, but small amounts of each were present in red maple extract. Several flavonol peaks also were present, of which seven were isolated and identified. Delendick (1990) identified the three quercetin glycosides, but only one of the four kaempferol glycosides. Only quercetin 3-*O*-β-L-rhamnoside was present in excess of 10 mg/g of extract (Table 1). However, there was an estimated 28 mg/g of the putative kaempferol galloyl rhamnoglucoside in leaf extracts that was most probably hydrolyzed during the lengthy isolation procedure and purified as kaempferol 3-*O*-rhamnoglucoside.

Antifeedant Tests. Red maple leaf disks strongly deterred feeding by FTC larvae. In the choice test employed, 100% of aspen leaf disks were eaten in contrast to only 3.3 ± 6.9% of red maple leaf disks after 24 hr, giving an AI of 94.3% ($N = 7$). This result confirms the field observation by Nicol et al. (1997) that red maple is not a preferred host of FTC.

RME applied to aspen leaf disks at 1.4 mg/cm^2 deterred feeding, yielding an AI of 56.2% (Table 2). In contrast, feeding was stimulated by crude aspen leaf extract (Table 2). These findings suggest that the resistance of red maple to feeding by FTC larvae is due to specific phytochemicals present in the leaves of this species. Since the RMP fraction contains higher levels of phenolic compounds and had an

TABLE 2. CHOICE BIOASSAYS SHOWING FEEDING RESPONSES OF FOREST TENT CATERPILLAR LARVAE TO RED MAPLE LEAF EXTRACT (RME), ASPEN LEAF EXTRACT (AE), OR PHENOLIC FRACTION OF RED MAPLE EXTRACT (RMP)

Test	mg/cm^2 of extract applied	Replicates (N)	Antifeedant index (%)[a]
Experiment 1			
RME	1.4	12	$+56.2 \pm 32.9^{s}$
AE	1.4	12	-54.4 ± 23.5^{s}
Experiment 2			
RMP	1.4	13	$+88.1 \pm 16.3^{s}$

[a] + = antifeedant; − = phagostimulant. Wilcoxon's one-tailed test (H_1; $\mu > 0$), differences from untreated (control) aspen leaf disks indicated as s = significant ($P < 0.05$) or ns = not significant, ($P > 0.05$).

AI of 88.1% (Table 2), we suggest that these compounds might be responsible for the antifeedant activity of this fraction.

Previously reported choice tests with six commercially available phenolic compounds present in red maple also demonstrated that gallic acid and methyl gallate were feeding deterrents at 0.28 mg/cm^2 (Abou-Zaid et al., 2000b). When the full range of 14 phenolic compounds identified in red maple leaf extract was tested at 0.28 mg/cm^2 in the present study, all the gallates (gallic acid, methyl gallate, ethyl gallate, *m*-digallate, ethyl *m*-digallate, 1-*O*-galloyl-β-D-glucose, and 1-*O*-galloyl-α-L-rhamnose) showed antifeedant activity (Table 3). However, ethyl *m*-digallate and *m*-digallate were clearly the most inhibitory to feeding, with AIs of about 90% (Table 3). Only ethyl *m*-digallate was the predominant compound in both RME and RMP. Neither compound is present in sugar maple leaves (Abou-Zaid et al., 2000a). The amount of ethyl *m*-digallate in RME and RMP applied to the leaf disks in Table 2 was 0.36 and 0.42 mg/cm^2, respectively, as compared to 0.28 mg/cm^2 of the pure compound in Table 3. Only RMP inhibited feeding to the same extent as the digallate. Other compounds in RME may act as stimulants to reduce insect feeding deterrence since this extract inhibited feeding by only

TABLE 3. CHOICE BIOASSAYS TO TEST FEEDING RESPONSES OF FOREST TENT CATERPILLAR LARVAE TO ASPEN LEAF DISKS TREATED WITH PHENOLIC COMPOUNDS ISOLATED FROM RED MAPLE (0.28 mg/cm^2 OF LEAF DISK)

Compound	μM	Antifeedant index (mean % $\pm$ SD)[a]	*N*
m-Digallate	0.9	$+90.4 \pm 12.5^{s}$	9
Ethyl *m*-digallate	0.8	$+89.2 \pm 9.1^{s}$	12
Methyl gallate	1.6	$+52.1 \pm 29.5^{s}$	116[b]
1-*O*-galloyl-α-L-rhamnose	0.9	$+43.7 \pm 33.8^{s}$	8
1-*O*-galloyl-β-D-glucose	0.9	$+40.5 \pm 36.5^{s}$	10
(−)-Epicatechin	1.0	$+35.7 \pm 31.8^{s}$	14
Gallic acid	1.7	$+33.7 \pm 33.8^{s}$	20
Ethyl gallate	1.4	$+33.4 \pm 32.9^{s}$	9
Kaempferol 3-*O*-β-L-rhamnoside	0.7	$+29.2 \pm 38.1^{s}$	10
Quercetin 3-*O*-β-L-rhamnoside	0.6	$+13.6 \pm 28.9^{ns}$	11
Ellagic acid	0.9	$+5.2 \pm 23.3^{ns}$	18
Quercetin 3-*O*-rhamnoglucoside	0.5	$+2.8 \pm 23.2^{ns}$	29
Quercetin 3-*O*-β-D-glucoside	0.6	-6.8 ± 32.3^{ns}	18
(+)-Catechin	1.0	-6.9 ± 29.0^{ns}	10
Kaempferol 3-*O*-β-D-glucoside	0.6	-15.8 ± 54.0^{ns}	6
Kaempferol-3-*O*-rhamnoglucoside	0.5	-24.2 ± 29.1^{s}	10

[a] + = antifeedant; − = phagostimulant. Wilcoxon's one-tailed test (H_1; $\mu > 0$), differences from untreated (control) aspen leaf disks indicated as s = significant, ($P < 0.05$) or ns = not significant, ($P > 0.05$).
[b] Used as a standard for all tests.

56%. These include the flavonol glycosides that stimulated feeding in tests with the pure compounds (Table 3). A crude extract such as RME also contains free sugars, amino acids, and other soluble metabolites that are not present in RMP and may stimulate feeding. Further evidence of the potential importance of such metabolites to feeding activity is provided by the test with aspen leaf extract (Table 2), which stimulated feeding in contrast to the inhibitory effect of a similar amount of RME.

Of the two building blocks of proanthocyanidins, (+)-catechin and (−)-epicatechin, only the latter elicited inhibitory activity similar to gallic acid. Only kaempferol 3-*O*-β-L-rhamnoside showed an antifeedant effect of the six flavonols tested (Table 3).

Expression of the concentrations of the compounds in molar amounts (Table 3) makes it possible for direct comparison of biological activity. For gallic acid and its esters, biological activity would be lower than that recorded on a milligram basis. By the same token, kaempferol 3-*O*-β-L-rhamnoside is a more effective antifeedant than the AI value of 29.2 would indicate.

In conclusion, FTC are deterred by gallates. The five most active compounds in red maple all possess the galloyl moiety, suggesting that this structure may constitute the basis for the feeding deterrence of these compounds and that the functional groups attached to this basic structure may influence the level of deterrency. Of all the gallates, ethyl *m*-digallate has both the highest antifeedant activity and concentration in red maple, suggesting it is the major resistance factor.

Acknowledgments—We acknowledge the financial support of the Integrated Forest Pest Management Green Plan Initiative and the Canadian Biotechnology Strategy Fund.

REFERENCES

ABOU-ZAID, M. M. and NOZZOLILLO, C. 1999. 1-*O*-Galloyl-α-L-rhamnose from *Acer rubrum*. *Phytochemistry* 52:1629–1631.

ABOU-ZAID, M. M., NOZZOLILLO, C., HELSON, B. V., and LOMBARDO, D. A. 2000a. Phenolic composition of leaves of six maple species in the Great Lakes Forest region of Canada. *Polyphenol Commun.* 1:95–96.

ABOU-ZAID, M. M., GRANT, G. G., HELSON, B. V., BENINGER, C. W., and DE GROOT, P. 2000b. Phenolics from deciduous leaves and coniferous needles as sources of novel control agents for lepidopteran forest pests, pp. 398–417, *In* Shahidi and Chi-Tang Ho (eds.). Phytochemicals and Phytopharmaceuticals. AOCS Press, Champaign, Illinois.

ADDY, N. D. 1969. Rearing the forest tent caterpillar on an artificial diet. *J. Econ. Entomol.* 62: 270.

BAILEY, A. E., ASPLUND, R. O., and ALI, M. S. 1986. Isolation of methyl gallate as the antitumor principle of *Acer saccharinum*. *J. Nat. Prod.* 49:1149–1150.

BATE-SMITH, E. C. 1978. Systematic aspects of the astringent tannins of *Acer* species. *Phytochemistry* 17:1945–1948.

BLANEY, W. M., SIMMONDS, M. S. J., LEY, S. V., ANDERSON, J. C., SMITH, S. C., and WOOD, A. 1994. Effect of azadirachtin-derived decalin (perhydronaphthalene) and dihydrofuranacetal (furo[2,3-*b*]pyran) fragments on the feeding behavior of *Spodoptera littoralis. Pest. Sci.* 40:169–173.

DELENDICK, T. J. 1990. A survey of foliar flavonoids in the Aceraceae. *Mem. NY Bot. Gard.* 54:1–129.

FARRAR, J. L. 1995. Trees in Canada. Canadian Forest Service & Fitzhenry and Whiteside Ltd., Markham, Ontario, Canada.

HADDOCK, E. A., GUPTA, R. K., AL-SHAFI, S. M. K., LAYDEN, K., HASLAM., E., and MAGNOLATO, D. 1982. The metabolism of gallic acid and hexahydroxydiphenic acid in plants A: Biogenetic and molecular taxonomic considerations. *Phytochemistry* 21:1049–1062.

HARBORNE, J. B. 1994. The Flavonoids: Advances in Research Since 1986. Chapman and Hall, London.

HASLAM, E. 1965. Galloyl esters in the Aceraceae. *Phytochemistry* 4:495–498.

HOWSE, G. M. 1995. Forest Insect Pests in the Ontario Region. *In* J. A. Armstrong and W. G. H. Ives (eds.). Forest Insect Pests in Canada. Natural Resources Canada, Ottawa, Ontario, Canada.

MARKHAM, K. R. 1982. Techniques of Flavonoid Identification, Academic Press, London.

MÜLLER-SCHWARZE, D., SCHULTE, B. A., SUN, L., MÜLLER-SCHWARZE, A., and MÜLLER-SCHWARZE, C. 1994. Red maple (*Acer rubrum*) inhibits feeding by beaver (*Castor canadensis*). *J. Chem. Ecol.* 20:2021–2034.

NICOL, R. W., ARNASON, J. T., HELSON, B. V., and ABOU-ZAID, M. M. 1997. Effect of host and non-host trees on the growth and development of the forest tent caterpillar, *Malacosoma disstria* Hubner (Lepidoptera: Lasiocampidae). *Can. Entomol*. 129:995–1003.

RICKLEFS, R. E. and MATTHEWS, K. 1982. Chemical characteristics of the foliage of some deciduous trees in southeastern Ontario. *Can. J. Bot.* 60:2037–2045.

SINGH, P. and MOORE, R. F. 1985. Handbook of Insect Rearing, Vol II. Elsevier Science Publishers, Amsterdam, The Netherlands, 369 pp.

SLACANIN, I., MARSTON, A., HOSTETTMANN, K., DELABAYS, N., and DARABELLAY, C. 1991. Isolation and determination of flavonol glycosides from *Epilobium* species. *J. Chromatogr.* 557:391–394.

VAN SUMERE, C., FACHE, P., CASTEELE, K. V., DE COOMAN, L., EVERAERT, E., DE LOOSE, R., and HUTSEBAUT, W. 1993. Improved extraction and reversed phase high performance liquid chromatographic separation of flavonoids and identification of *Rosa* cultivars. *Phytochem. Anal*. 4:279–292.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

EFFECT OF METHYL JASMONATE ON HYDROXAMIC ACID CONTENT, PROTEASE ACTIVITY, AND BIRD CHERRY–OAT APHID *Rhopalosiphum padi* (L.) PROBING BEHAVIOR

E. SLESAK,[1,]* M. SLESAK,[2] and B. GABRYS[3]

[1]*Institute of Botany*
University of Wroclaw
Kanonia 6/8 50-328 Wroclaw, Poland

[2]*Department of Plant Protection*
Agricultural University
Cybulskiego 20 50-205 Wroclaw, Poland

[3]*Department of Biology*
Institute of Biotechnology and Environmental Sciences
University of Zielona Gora
Monte Cassino 21B 65-561 Zielona Gora, Poland

(Received October 24, 2000; accepted August 8, 2001)

Abstract—The concentration of hydroxamic acids increased in wheat seedlings exposed to methyl jasmonate. The proteins isolated from such seedlings inhibited trypsin activity. Free-moving aphids avoided plants that had been exposed to methyl jasmonate prior to the experiment. These aphids were able to reject such plants within 15 min, and the inclination to stay on control plants lasted at least 24 hr. Electronic recording (EPG) of aphid stylet activities showed a trend towards reduction in penetration time and total time of phloem sap ingestion on jasmonate–treated plants. However, the duration of the first period of feeding was similar on treated and control plants.

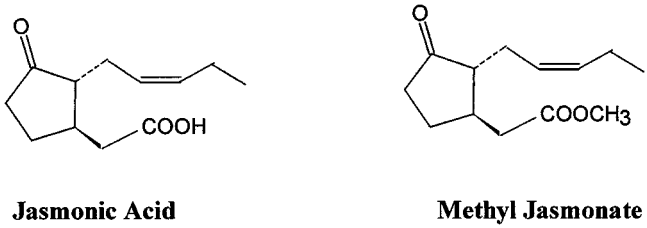
Key Words—Semiochemicals, induced resistance, methyl jasmonate, hydroxamic acids, *Rhopalosiphum padi*, EPG, aphid behavior.

INTRODUCTION

Jasmonic acid (JA) and its methyl ester—methyl jasmonate (JA-ME; Figure 1)—are endogenous regulators of plant growth and development (Loake, 1996). These compounds occur in a large number of higher plant species and their synthesis is stimulated by a variety of processes, including mechanical wounding, herbivore

*To whom correspondence should be addressed.

0098-0331/01/1200-2529$19.50/0

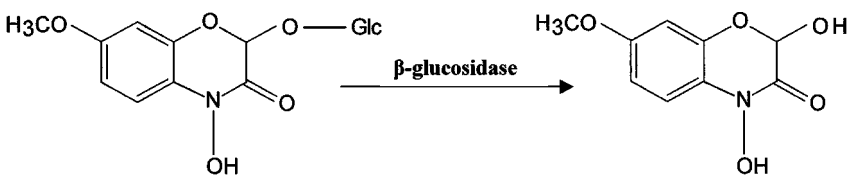


Jasmonic Acid **Methyl Jasmonate**

FIG. 1. Chemical structures of jasmonic acid (JA) and jasmonic acid methyl ester (=methyl jasmonate) (JA-ME).

attack, osmotic stress, and desiccation (Creelman and Mullet, 1997). JA is a key signal molecule carrying information about injury and its concentration in wounded plants increases rapidly (Reinbothe et al., 1994). JA induces expression of plant defense genes responsible for the synthesis of proteinase inhibitors, specific jasmonate-induced proteins, and metabolites directly involved in plant resistance, e.g., glucosinolates (Bennett and Wallsgrove, 1994; Bodnaryk, 1994). Similar plant responses are induced by the volatile JA-ME, which predestines it to a key role in interplant communication and induction of resistance against herbivores (Karban and Baldwin, 1997).

Cyclic hydroxamic acids (Hx) are a group of secondary metabolites characteristic of the family Poaceae (=Graminae). Hx occur in wild grasses, as well as in cultivated crops such as maize, wheat, rye, and triticale, but are absent from barley, oats, and rice (Zuniga et al., 1983; Niemeyer, 1988). In wheat, the most abundant Hx is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) -glycoside. Upon wounding of plant tissues, this molecule is hydrolyzed to benzoxazinone (DIMBOA) (Figure 2) by endogenous β-glucosidase (Niemeyer and Perez, 1995). When consumed by herbivores, DIMBOA interferes with digestive processes within the gut by reducing digestibility and conversion of digested food, probably by inhibition of protease activity (Atkinson et al., 1992). DIMBOA inhibits the activity of many other enzymes including ATPases and enzymes of the



DIMBOA - glycoside **DIMBOA**

FIG. 2. Enzymatic hydrolysis of DIMBOA-glycoside.

oxidative chain (Bravo and Niemeyer, 1986). The level of DIMBOA in plants is associated with insect and disease resistance and allelopathic effects of cereals (Copaja et al., 1991). The highest concentration of Hx in plants is found in vascular tissues (Argandona and Corcuera, 1985; Argandona et al., 1987). Within the vascular bundle, Hx are present in phloem sap (Givovich et al., 1994), but absent from xylem (Argandona et al., 1987). Leszczynski and Dixon (1990) and Givovich et al. (1992) found Hx in the honeydew of aphids feeding on cereals, demonstrating that aphids ingest these compounds with the phloem sap. Hydroxamic acids seem to be natural defensive factors against insects, and increasing their level in plants has been suggested in breeding programs to obtain aphid-resistant cereal varieties (Escobar and Niemeyer, 1993).

The bird cherry–oat aphid, *Rhopalosiphum padi* (L.), is a worldwide pest of all major cereals and is able to transmit many persistent and nonpersistent viruses (Blackman and Eastop, 1985).

The aim of this study was to find whether the level of hydroxamic acids in plants might be affected by external factors such as JA-ME and whether this volatile chemical induces changes in the behavior of the bird cherry–oat aphid during settling, probing, and feeding on wheat. The effect of proteins from plants exposed to JA-ME on protease activity was also studied.

METHODS AND MATERIALS

Plants and Aphids. Winter wheat, *Triticum aestivum* cv. Kobra, was grown under controlled environmental conditions in hydroponic culture with keramsyte as the supporting medium [temperature 26°C (day)/21°C (night); 16L:8DL photoperiod; 65–70% relative humidity; light intensity 200 μM/m^2/sec FAR]. Hoagland's solution [$Ca(NO_3)_2$ 3.25 mmol/liter; KNO_3, 1 mmol/liter; KH_2PO_4, 1 mmol/liter; $MgSO_4$, 1 mmol/liter; Fe EDTA, 0.05 mmol/liter; H_3BO_4, 1 μmol/liter; $MnSO_4$, 0.05 μmol/liter; $CuSO_4$, 0.5 μmol/liter; $ZnSO_4$, 0.01 μmol/liter; $(NH_4)_6Mo_6O_{24}$, 0.01 μmol/liter; the acidity of the medium was set at pH 6.5 using 0.1 N H_2SO_4 or 1 N KOH] provided nutrients for the growing plants.

Aphids, *Rhopalosiphum padi*, were reared on *T. aestivum* cv. Kobra under similar controlled conditions as plants. Young apterous virginoparous females were used for experiments.

Application of JA-ME. Methyl jasmonate (JA-ME) was obtained from Serva, Germany. Six-day-old wheat seedlings were exposed to 50 μl of JA-ME that was applied to strips of filter paper attached to the top of a glass chamber (16 cm high $\times$ 9 cm diam.) for 24 or 48 hr. After exposure, seedlings were used for EPG study, direct observation of aphid behavior, and chemical analysis.

Airborne Concentration of Methyl Jasmonate. The concentration of JA-ME in the glass chamber was measured by GC-MS (GC model HP 5890II, MD model

HP 5972, column HPS) and found to be 217 nmol/liter. Only the (+)-epi form of methyl jasmonate is biologically active and rapidly isomerizes to the more stable, but biologically inactive, *trans* configured isomer. This process takes place under physiological conditions and results in a 1:9 ratio of *cis*-(3*R*,7*S*) epimer to *trans*-(3*R*,7*R*) epimer (Mueller, 1997). Therefore, we were able to determine that the concentration of total JA-ME in our glass chamber was 20 nmol/liter. According to data provided by Serva, the JA-ME preparation consists of three isomers, which means that the physiologically active concentration of JA-ME in our study was several nanomoles per liter. This fits within the range of physiological concentrations reported by other authors (Falkenstein et al., 1991; Farmer and Ryan, 1992).

Quantification of Hydroxamic Acids. Hx content was analyzed according to the procedure described by Leszczynski (1996). Plant material was homogenized in distilled water and left at room temperature for 30 min. The homogenate was adjusted to pH 3 and centrifuged at 6000 *g* for 15 min. The supernatant was extracted into ethyl ether (1:1, v/v $\times$ 3); the combined ether fractions were evaporated to dryness, dissolved in 80% methanol, and the $FeCl_3$ reagent was added. Absorbance of the complex was measured at 590 nm by using a Beckman DU 640 spectrophotometer. The concentration of Hx was determined by comparing the absorbance of the extract with a standard curve made with 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) because standards of hydroxamic acids are not commercially available. Free Hx aglucones were isolated according to the method of Woodward et al. (1978) and Leszczynski (1996). The preparation was crystallized in hexane (2$\times$) and purity determined by the method of Woodward et al. (1978). This was used as the Hx standard based on its main component—DIMBOA. Additional measurements of DIMBOA using TLC-UV (Zuniga et al., 1983) confirmed the results obtained with the ferric chloride method.

Extraction of Protease Inhibitors. Protease inhibitors were isolated by using a modification of the Jongsma et al. (1994) method. Plant tissue was immersed in liquid nitrogen and homogenized in buffer (0.1 M Tris HCl, pH 7.6) containing 5% polyvinylpolypyrrolidone (Sigma), 2 mg/ml phenylthiourea, 5 mg/ml DIECA, and 0.05 M EDTA. The homogenate was centrifuged (MPW 375) at 12,000 *g* for 20 min at 4°C. Proteins in the supernatant were precipitated with chilled $(NH_4)_2SO_4$ and centrifuged at 4°C for 10 min. The precipitate was resuspended in buffer (0.1 M Tris HCl, pH 7.6) and added to the reaction mixture for determination of enzyme activity.

Enzyme Assay. Trypsin activity was evaluated using ox trypsin type III (Sigma) and on the basis of the azocasein methods of Brock et al. (1982) and Harsulkar et al. (1999). The reaction mixture consisted of trypsin, azocasein [0.1% azocasein (Sigma) in 0.1 M glycine NaOH)], and the isolated plant protease inhibitor. Incubation was carried out at 37°C for 30 min and the reaction terminated with 5% TCA. The mixture was centrifuged at 10,000 *g* for 10 min after incubation at room temperature. An equivalent amount of 1 N NaOH was added to the

supernatant, and the absorbance at 450 nm was measured. One protease activity unit was defined as an increase in absorption of one extinction unit per minute. The unit of protease inhibition was defined as the inhibition of one protease unit under described conditions.

Olfactometry. Experiments were carried out in a Pettersson olfactometer (Pettersson, 1995). JA-ME (4 μM) was applied to one of the four arms of the olfactometer, the remaining arms served as controls (water). Five apterous, virginoparae were placed in the central arena of the olfactometer. The number of aphids in the individual arms was counted every minute for 15 min. The airflow was 4 ml/sec. Experiments were replicated eight times and the results analyzed using the *t* test.

Direct Observation of Aphid Settling: Choice Test. Two wheat leaves (one untreated control and the other from plants treated with JA-ME for 24 or 48 hr) were placed in a Petri dish containing wet filter paper. Twenty apterous, virginoparae were placed in the center of the dish, and the number on each of the leaves was counted after 15, 30, 60, and 120 min, and 24 hr. The experiment was replicated eight times and the results were analyzed by ANOVA.

EPG. The Electrical Penetration Graph technique (Tjallingii, 1995) provides information on aphid stylet activities in plant tissues and is helpful in the localization of plant resistance factors, if there are any. This technique was used to follow aphid behavior on plants exposed to JA-ME. Probing behavior of apterous, virginoparae *R. padi* was recorded continuously for 8 hr on untreated and JA-ME-treated plants prepared as described earlier. Each experiment was replicated 15 times using fresh aphids. The results were analyzed using the Kruskal-Wallis test.

RESULTS

There was an increase in Hx concentration in wheat seedlings exposed to methyl jasmonate. Hx content increased by 49% and 129% in seedlings exposed for 24 and 48 hr, respectively (Table 1). The proteins isolated from wheat seedlings exposed to JA-ME inhibited trypsin activity with the strongest inhibition found for seedlings exposed for 48 hr (Table 2).

TABLE 1. HYDROXAMIC ACID CONTENT IN WHEAT SEEDLINGS EXPOSED TO METHYL JASMONATE (JA-ME)

	Hydroxamic acid content (mmol/kg fresh wt)		
Treatment	0 hr	24 hr	48 hr
Control	0.81 ± 0.11^a	0.79 ± 0.12a^b	0.72 ± 0.09a
JA-ME		1.21 ± 0.14b	1.86 ± 0.10b

[a] Mean $\pm$ SD ($N = 6$).
[b] Different letters in columns represent a difference ($P \geq 0.05$) by *t* test.

TABLE 2. INHIBITION OF TRYPSIN ACTIVITY BY PROTEINS ISOLATED FROM WHEAT SEEDLINGS EXPOSED TO METHYL JASMONATE (JA-ME)

	Trypsin inhibition (% of control ± SD)	
Treatment	24 hr	48 hr
Control	100[a]	100
JA-ME	81 ± 3	55 ± 4

[a] Activity expressed as percent of control ± SD.

In the olfactometer, fewer aphids (8.8 on average) visited the arm containing methyl jasmonate than the control arms (11.2 on average), but the difference was not significant.

Aphids did not settle on plants that had been exposed to methyl jasmonate, regardless of exposure time. Such behavior was observed during the entire 24-hr experiment (Table 3).

Electronic recording of aphid stylet activities showed a trend towards the reduction of total penetration time, and, among all penetration activities, a decrease in phloem sap ingestion from plants exposed to JA-ME (Figure 3). During the first hour of recording, probing time was 25% and 50% shorter on plants exposed to JA-ME for 24 and 48 hr, respectively, than on control plants. On the control plants, the typical aphid behavior might be observed. During the second hour after aphids had access to plants, stylet probing constituted 90% of all aphid activity (40% pathway and 50% phloem sap ingestion). Aphids showed only penetration activity from the sixth hour until the end of the recording; 90% of it was the ingestion of

TABLE 3. RESPONSE OF APTEROUS *Rhopalosiphum padi* TO WHEAT SEEDLINGS EXPOSED TO METHYL JASMONATE (JA-ME)

Time from start of experiment	Mean number of aphids on leaves			
	24 hr exposure		48 hr exposure	
	Control	JA-ME	Control	JA-ME
15 min	8.6a[a]	4.0b	14.4a	2.8b
30 min	10.8a	2.9b	15.4a	1.9b
60 min	12.6a	1.5b	17.6a	0.4b
120 min	13.1a	2.0b	15.0a	2.5b
24 hr	19.1a	0.5b	19.1a	0.3b

[a] Different letters in rows show significant differences ($P \geq 0.05$) in choice tests analyzed by ANOVA.

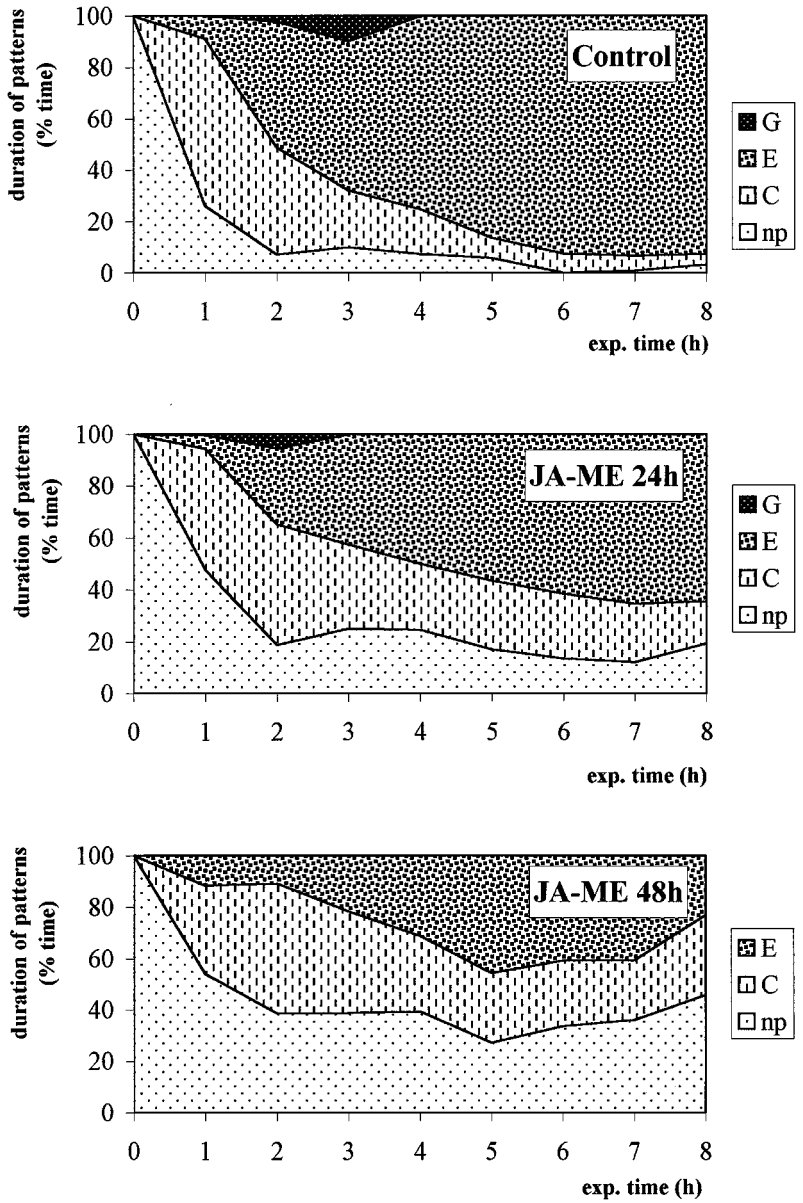


FIG. 3. *Rhopalosiphum padi* stylet penetration activity on wheat exposed to methyl jasmonate (8-hr EPG recording). np, non penetration; C, pathway activity; E, phloem sap ingestion; G, xylem sap ingestion.

phloem sap. Stylet penetration comprised 80% of aphid activity (60% pathway, and 20% phloem sap ingestion) after 2 hr spent on plants exposed to JA-ME for 24 hr. From the sixth through the eighth hours, total probing time did not change and comprised approximately 80% of all activities (pathway—20%, phloem sap ingestion—60%). Nonprobing activities took around 40% from the second hour until the end of the experiment on plants exposed for 48 hr. During that time, pathway activities decreased from 50% in the second hour to 30% in the eighth hour, and phloem sap ingestion increased from 10% to 30%, respectively. All aphids found phloem vessels and started feeding by the fourth hour of the experiment on control plants. On plants exposed to JA-ME for 24 hr, all tested aphids showed phloem sap ingestion waveforms, but much later than on control plants, i.e., after the sixth hour of the experiment. On plants exposed to JA-ME for 48 hr, 20% of tested aphids failed to locate their food source: no waveforms indicating phloem contact occurred during probing by these aphids (Figure 4).

The most important differences between aphid probing on control and JA-ME-treated plants was in total penetration time (20–30% reduction on JA-ME-treated plants), total phloem sap ingestion time (50% reduction on JA-ME-treated plants), pathway activities during the first hour (25–50% reduction on JA-ME-treated plants), and number of probes (twofold increase on JA-ME-treated plants). The duration of the first period of feeding was similar on control and treated plants (Table 4).

The time needed by *R. padi* to locate phloem sieve elements after having access to the plant was similar on control and JA-ME-treated plants. However, within a probe the duration of pathway before the beginning of sap ingestion was

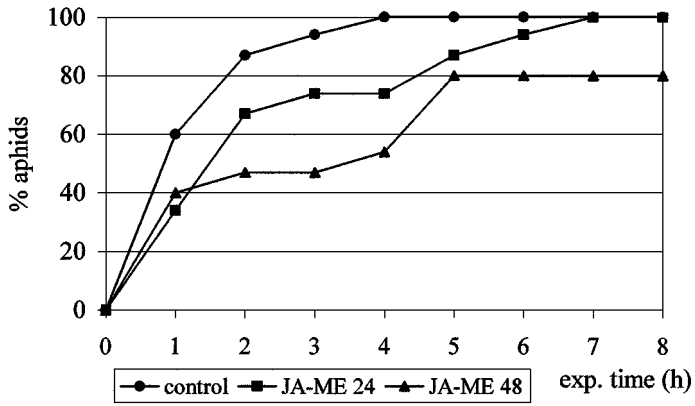


FIG. 4. Cumulative percent of *Rhopalosiphum padi* reaching phloem vessels on wheat exposed to methyl jasmonate (8-hr EPG recording).

TABLE 4. EPG PARAMETERS OF *Rhopalosiphum padi* STYLET PENETRATION ACTIVITIES ON WHEAT SEEDLINGS EXPOSED TO JA-ME

EPG Parameter	Control	JA-ME Exposure	
		24 hr	48 hr
Total penetration time (hr)	6.7 ± 1.9a[a]	5.8 ± 2.2a	4.6 ± 2.5b
Total pathway time (hr)	1.6 ± 2.1a	3.4 ± 4.0a	2.2 ± 2.6a
Total duration of pathway during the 1st hour of experiment (min)	41.5 ± 15.1a	31.1 ±15.7b	19.4 ± 13.6c
Total duration of E1 and E2 phases[b] (hr)	5.1 ± 2.1a	4.7 ± 4.1a	2.3 ± 2.6b
Total duration of E2 phases (hr)	4.8 ± 2.1a	3.5 ± 4.1ab	2.2 ± 2.6b
Number of probes	6.7 ± 4.4a	12.4 ± 0.5b	12.1 ± 7.6b
Number of probes before 1st E phase	3.0 ± 2.6a	7.5 ± 7.8a	4.6 ± 4.6a
Number of E phases	4.6 ± 4.1a	5.2 ± 5.3a	3.2 ± 3.4a
Number of E2 phases	3.6 ± 3.6a	3.1 ± 3.3a	2.9 ± 3.2a
Time from 1st probe to 1st E phase (hr)	1.0 ± 0.8a	1.7 ± 1.4a	1.5 ± 1.7a
Pathway to E phase within a probe (min)	17.3 ± 10.8a	29.8 ± 30.9a	11.6 ± 0.2b
Duration of 1st E phase (hr)	2.1 ± 3.1a	3.4 ± 3.0a	1.0 ± 1.8a
Duration of 1st E2 phase (hr)	2.1 ± 3.1a	1.7 ± 2.6a	1.0 + 1.8a

[a] Mean ± SD of 15 aphids continuously recorded for 8 hr. Different letters in rows indicate significant differences ($P \geq 0.05$) by the Kruskal-Wallis test.
[b] E1: phloem salivation; E2: phloem sap ingestion activities.

two times shorter on plants exposed to JA-ME for 48 hr than on the control ones, and three times shorter than on plants exposed for 24 hr (Table 4).

DISCUSSION

EPG studies supported by direct observation of aphid settling showed that the behavior of *Rhopalosiphum padi* changed on plants that had been exposed to vapors of methyl jasmonate. Host-plant acceptance was delayed in the case of the tethered aphids, suggesting that these individuals might have left the plant given freedom of movement. Indeed, freely moving aphids in another experiment rejected such plants within the first 15 min of the experiment, and the inclination to stay on control plants occurred throughout 24 hr of observation. The reduction of pathway and phloem ingestion activities in aphids on JA-ME-treated plants showed that stylet penetration was deterred by factors present in the mesophyll as well as vascular tissues.

Our results correspond well with data reported by other authors. Several authors have described the negative correlation between DIMBOA content in cereals and the behavior and performance of various species of cereal aphids, including Corcuera et al. (1982), Leszczynski et al. (1989), Leszczynski and Dixon (1990),

and Thackray et al. (1990). Addition of DIMBOA to artificial diets showed its antibiotic effect on aphids (Argandona et al., 1983). DIMBOA-aglucone inhibited ATPase, trypsin, chymotrypsin, and cholinesterase activity (Atkinson et al., 1992; Cuevas and Niemeyer, 1993). According to Gonzales and Rojas (1999), the toxic effect of Hx may be related to reactive forms of oxygen that occur in the presence of Hx and may destroy cell membranes. It seems likely that the reduced ability to feed on jasmonate-treated plants may be attributed to some disorders in the aphid nervous system.

The role of JA as a stress hormone in aphid–plant interactions has not been studied. The results of the present work refer to many aspects of this relationship. The schematic drawing (Figure 5) that combines our findings with literature data shows a hypothetical model of aphid–plant interactions in light of the JA/JA-ME key role in the induction of plant resistance mechanisms against herbivores in wheat.

Exogenous methyl jasmonate (Step I) or jasmonic acid synthesized in response to the wounding of plants by aphid stylets (II) induces expression of genes (III) responsible either for the synthesis of enzymes of the shikimic acid pathway, leading to the accumulation of hydroxamic acids (IV), or the synthesis of specific proteins, the protease inhibitors (VIII).

Synthesis of Hx induced by JA-ME is followed by glycosylation (IV). This is the basic transport form of this compound (Zuniga and Massardo, 1991). In a similar way, glycosylation during synthesis of glucosinolates in Brassicaceae increases their mobility and enables their transport in the phloem (Brudenell et al., 1999). Corcuera et al. (1982) using artificial diets demonstrated that the DIMBOA-aglucone is more toxic to aphids than its glycosylated form (DIMBOA-Glc). DIMBOA-Glc may be transformed into the aglucone by endogenous plant β-glucosidase (V) (Escobar and Niemeyer, 1993) following damage to plant tissue by feeding insects. A recent report shows that wheat contains an unknown β-glucosidase (EC 3.2.1.21) characterized by a specific affinity to DIMBOA-Glc (*Sue* et al., 2000). The risk of plant damage by aphids is minimized. Their stylets penetrate plant tissues within the cell walls rather than through the inside of the cells (Tjallingii and Hogen Esch, 1993). However, sometimes plant cells may deteriorate during pathway activities of aphid stylets. In such cases, plant β-glucosidases would be activated and the concentration of DIMBOA-aglucones would increase. Niemeyer et al. (1989) and Leszczynski and Dixon (1990) showed that the content of DIMBOA-aglucones increased in plants infested by aphids. There is also evidence that salivary factors may elicit specific plant responses (Karban and Baldwin, 1997). Givovich et al. (1994) found that *Rhopalosiphum padi* was more sensitive to Hx than *R. maidis*. If stylet penetration activities of cereal aphids induce synthesis of Hx, then reduction of sampling frequency by *R. maidis* will be the strategy for reducing the possibility of intoxication, which has already been suggested by Givovich et al. (1994). It is still an open question

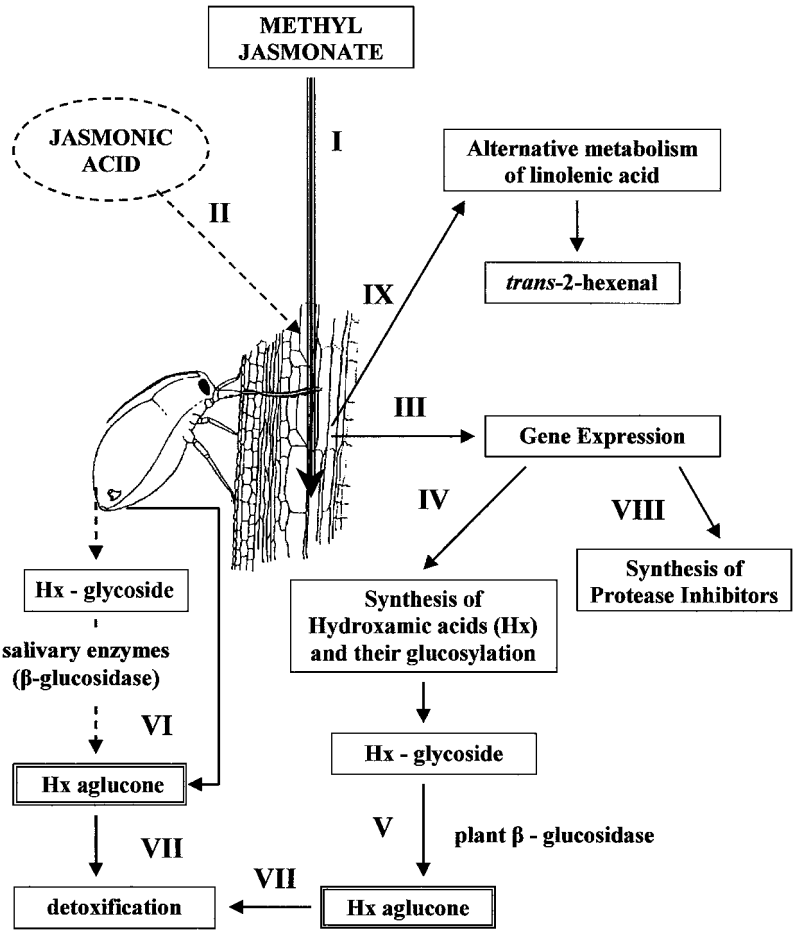


FIG. 5. Schematic representation of jasmonic acid and methyl jasmonate roles in aphid-plant relationships in cereals. **I**—exogenous methyl jasmonate; **II**—jasmonic acid synthesized in response to insect feeding; **III**—expression plant resistance genes; **IV**—synthesis, glucosylation, and accumulation of hydroxamic acids; **V**—hydrolysis of hydroxamic acid glucosides by endogenous plant β-glucosidases; **VI**—hydrolysis of hydroxamic acid glucosides by aphid salivary β-glucosidases; **VII**—detoxification of hydroxamic acids; **VIII**—synthesis of proteinase inhibitors; **IX**—synthesis of *trans*-2-hexenal.

whether β-glucosidases from aphid saliva (VI) are DIMBOA-glycoside-specific and whether they contribute to the increase in DIMBOA-aglucone concentration in aphid-infested plants (Leszczynski et al., 1993). The DIMBOA ingested by aphids with the phloem sap may be detoxified (VII) by glutathione S-transferase

(Leszczynski et al., 1993), or UDP-glucosyltransferase (Leszczynski and Dixon, 1990). UDP-glucosyltransferase may transform the toxic aglucone form of DIMBOA into the glycoside one that may be excreted in honeydew (Leszczynski and Dixon, 1990).

Aphid nutrition is based on easily available free amino acids from the phloem sap (Srivastava, 1987). However, phloem sap also contains a small amount of proteins, including chitin-binding lectins, that are considered a plant resistance factor against aphid feeding (Cole, 1994). A number of other proteins at concentrations within the range occurring in phloem sap increased mortality and inhibited growth of the pea aphid *Acyrthosiphon pisum* (Rahbe and Febvay, 1992). These ingested polypeptides are not processed by aphids (Rahbe et al., 1995), although proteases were found in the salivary glands and midgut of several aphid species including *R. padi* (Srivastava, 1987). Furthermore, aphids have nondigestive proteases sensitive to trypsin and chymotrypsin inhibitors; the oral application of these inhibitors affected the survival and development of the pea aphid (Rahbe et al., 1995). Jasmonate-induced resistance gene expression may lead to the synthesis of protease inhibitors (VIII). We found significant inhibition of trypsin by proteins from wheat exposed to the volatile methyl jasmonate and analogous results were obtained by other authors (Farmer and Ryan, 1992; Jongsma et al., 1994). Casaretto and Corcuera (1998) showed that the protease inhibitors isolated from barley leaves had no effect on the survival of *Schisaphis graminum* on artificial diets, whereas they reduced the survival of *Rhopalosiphum padi* under similar conditions. Therefore, the accumulation of protease inhibitors induced by JA-ME in wheat leaves found in the present study might be the additional factor responsible for decreased feeding activity. If the effect of PIs described for *A. pisum* by Rahbe et al. (1995) is a common phenomenon in aphids, then the jasmonate-induced plant PIs may be an important plant resistance factor applicable in aphid control on cereals. Certainly, this aspect of aphid physiology requires further study.

Pettersson et al. (1996) found that volatiles released by aphid-infected cereals could induce antixenotic effects in neighboring plants, which cause nonpreference in aphids. Hildebrand et al. (1993) observed that volatiles released by damaged potato leaves induced plant responses that reduced aphid fecundity. The volatile blend produced by plants attacked by insects may contain additional compounds than the stress-induced jasmonates that have no effect on aphid behavior (see this work). The main metabolic pathway of linolenic acid leads to jasmonates, but an alternative pathway also exists leading to the synthesis of various volatile compounds, for example *trans*-2-hexenal (IX). *trans*-2-Hexenal, one of the general green-leaf volatiles, is toxic to insects and mites (Lyr and Basamian, 1983; Pare and Tumlinson, 1999). Aphids have olfactory receptors sensitive to these compounds (Visser et al., 1996). In our experiments, free *R. padi* avoided leaves exposed to JA-ME as soon as they had contact with them. It is likely that this response was the

reaction to volatiles produced by these leaves; however, this hypothesis remains to be tested.

In this paper we report evidence for only two jasmonate-induced processes that may act as resistance mechanisms of wheat against insect herbivores. If we accept the idea that methyl jasmonate acts as an airborne signal carrying information about wounding, our results give support to the idea of a multi-way cascade response in plants, activated by stress situations.

Acknowledgments—We would like to thank M. Hojniak, MSc, Institute of Chemistry, Wroclaw University, for his help in GC-MS measurements of airborne JA-ME concentration.

REFERENCES

ARGANDONA, V. H. and CORCUERA, L. J. 1985. Distribution of hydroxamic acids in *Zea mays* tissue. *Phytochemistry* 24:177–178.

ARGANDONA, V. H., CORCUERA, L. J., NIEMEYER, H. M., and CAMPBELL, B. C. 1983. Toxicity and feeding deterrency of hydroxamic acids from Graminae in synthetic diets against the greenbug, *Schizaphis graminum. Entomol. Exp. Appl.* 34:134–138.

ARGANDONA, V. H., ZUNIGA, G. E., and CORCUERA, L. 1987. Distribution of gramine and hydroxamic acids in barley and wheat leaves. *Phytochemistry* 26:1917–1918.

ATKINSON, J., ARNASON, J., CAMPOS, F., NIEMEYER, H. M., and BRAVO, H. R. 1992. Synthesis and reactivity of cyclic hydroxamic acids, pp. 349–360, *in* D. R. Baker, J. G. Fenyes, and J. J. Steffens (eds.). Synthesis and Reactivity of Cyclic Hydroxamic Acids. ACS Symposium Series No.504. American Chemical Society, Washington, D.C.

BENNETT, R. N. and WALLSGROVE, R. M. 1994. Secondary metabolites in plant defense mechanisms. *New Phytol.* 127:617–633.

BLACKMAN R. L. and EASTOP, V. F. 1985. Aphids on the worlds crops: An identification guide. John Wiley & Sons, New York, pp. 413.

BODNARYK, R. P. 1994. Potent effect of jasmonates on indole glucosinolates in oilseed rape and mustard. *Phytochemistry* 35:301–305.

BRAVO, H. R. and NIEMEYER, H. M. 1986. A new product from the decomposition of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid from cereals. *Heterocycles* 24:335–337.

BROCK, R. M., FOSBERG, C. W., and BUCHANAN-SMITH, J. G. 1982. Proteolytic activity of rumen microorganisms and effect of proteinase inhibitors. *Appl. Environ. Microbiol*. 44:561–569.

BRUDENELL, A. J. P., GRIFFITS, H., ROSSITIER, J. T., and BAKER, D. A. 1999. The phloem mobility of glucosinolates. *J. Exp. Bot.* 50:745–756.

CASARETTO, I. A. and CORCUERA, L. J. 1998. Proteinase inhibitor accumulation in aphid-infested barley leaves. *Phytochemistry* 49:2279–2286.

COLE, R. A. 1994. Isolation of a chitin-binding lectin, with insecticidal activity in chemically-defined synthetic diets, from two wild brassica species with resistance to cabbage aphid Brevicoryne brassicae. *Entomol. Exp. Appl.* 72:181–187.

COPAJA, S., BARRIA, B. N., and NIEMEYER, H. M. 1991. Hydroxamic acid content of perennial triticale. *Phytochemistry* 30:1531–1534.

CORCUERA, L. J., ARGANDONA, V. H., PENA, G. F., PEREZ, F. J., and NIEMEYER, H. M. 1982. Effect of a benzoxazinone from wheat on aphids. Proceedings, 5th Symposium on Insect–Plant Relationships, Wageningen, 1982. Pudoc, Wageningen, pp. 33–39.

CREELMAN, R. A. and MULLET, I. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:355–381.

CUEVAS, L. and NIEMEYER, H. M. 1993. Effect of hydroxamic acids from cereals on aphid cholinesterases. *Phytochemistry* 34:983–985.

ESCOBAR, C. A. and NIEMEYER, H. M. 1993. Potential of hydroxamic acids in breeding for aphid resistance in wheat. *Acta Agric. Scand. Sect. B. Soil Plant Sci*. 43:163–167.

FALKENSTEIN, E., GROTH, B., MITHOFER, A., and WEILER, E. W. 1991. Methyl jasmonate and α-linolenic acid are potent inducers of tendril coiling. *Planta* 185:316–322.

FARMER, E. F. and RYAN, C. A. 1992. Interplant communication: air-borne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci. USA* 87:7713–7716.

GIVOVICH, A., MORSE, S., CERDA, H., NIEMEYER, H. M., WRATTEN, S. D., and EDWARDS, P. J. 1992. Hydroxamic acids glucosides in honeydew of aphids feeding on wheat. *J. Chem. Ecol.* 18:841–846.

GIVOVICH, A., SANDSTROM, J., NIEMEYER, H. M., and PETTERSSON, J. 1994. Presence of hydroxamic acid glycoside in wheat phloem sap, and its consequences for performance of *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). *J. Chem. Ecol.* 20:1923–1930.

GONZALES, L. F. and ROJAS, M. C. 1999. Role of wall peroxidases in oat growth inhibition by DIMBOA. *Phytochemistry* 50:931–937.

HARSULKAR, A. M., GIRI, A. P., PATANKAR, A. G., GUPTA, V. S., SAINANI, M. N., RANJEKAR, P. K., and DESHPANDE, V. V. 1999. Successive use of non host plant proteinase inhibitors required for effective inhibition of *Helicoverpa armigera* gut proteinase and larval growth. *Plant Physiol.* 121:497–506.

HILDEBRAND, D. F., BROWN, G. C., JACKSON, D. M., and HAMILTON-KEMP, T. R. 1993. Effects of some leaf-emitted volatile compounds on aphid population increase. *J. Chem. Ecol.* 19:1875–1887.

JONGSMA, M. A., BAKKER, P. L., VISSER, B., and STIEKAMA, W. J. 1994. Trypsin inhibitor activity in mature tobacco and tomato plants is mainly induced locally in response to insect attack, wounding and virus infection. *Planta* 195:29–35.

KARBAN, R. and BALDWIN, I. T. 1997. Induced Responses to Herbivory. The University of Chicago Press, Chicago, pp. 319.

LESZCZYNSKI, B. 1996. Kurs praktyczny w zakresie chemicznych interakcji owady-rosliny na przykladzie mszyc (Aphidoidea). Wyd. Uczel. WSRP w Siedlcach, Siedlce, pp. 390.

LESZCZYNSKI, B. and DIXON, A. F. G. 1990. Resistance of cereals to aphids: Interaction between hydroxamic acids and the aphid *Sitobion avenae* (Homoptera: Aphidiidae). *Ann. Appl. Biol.* 117:21–30.

LESZCZYNSKI, B., WRIGHT, L. C., and BAKOWSKI, T. 1989. Effect of secondary plant substances on winter wheat resistance to grain aphid. *Entomol. Exp. Appl.* 52:135–139.

LESZCZYNSKI, B., TJALLINGII, W. F., and DIXON, A. F. G. 1993. Some remarks on the interaction between hydroxamic acids and grain aphid. *Aphid Resistance Newsl.* 7:13014.

LOAKE, G. J. 1996. Jasmonates: Global regulators of plant gene expression, pp. 215–230, *in* M. Smallwood, J. P. Knox, and D. J. Bowles (eds.) Membranes: Specialized Function in Plants. BIOS Oxford Scientific Publishers, Oxford.

LYR, H. and BASAMIAN, L. 1983. Alkenals, volatile defense substance in plants, their properties and activities. *Acta Phytopathol. Acad. Sci. Hung.* 18:3–12.

MUELLER, J. M. 1997. Enzymes involved in jasmonic acid biosynthesis. *Physiol. Plant.* 100:653–663.

NIEMEYER, H. M. 1988. Hydroxamic acids (4-hydroxy-11,4-benzoxazin-3-ones), defense chemmicals in the Graminae. *Phytochemistry* 27:3349–3358.

NIEMEYER, H. M. and PEREZ, F. J. 1995. Potential of hydroxamic acids in the control of cereal pests, diseases, and weeds. pp. 260–270, *in* Inderjit, K. M. M. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Application. ACS Symposium Series No. 582. Americam Chemical Society, Washington, D.C.

NIEMEYER, H. M., PESEL, E., COPAJA, S. V., BRAVO, H. R., FRANKE, S., and FRANCKE, W. 1989. Changes in hydroxamic acid levels of wheat plants induced by aphid feeding. *Phytochemistry* 28:447–449.

PARE, P. W. and TUMLINSON, I. H. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121:325–331.

PETTERSSON, J. 1995. Aphids, odour, and behavioural ecology, pp. 1–10, *in* H. M. Niemeyer (ed.). Techniques in Plant-Insect Interactions and Biopesticides. Proceedings, IFS Workshop on Chemical Ecology. Santiago, Chile.

PETTERSSON, J., QUIROZ, A., and FAHAD, A. E. 1996. Aphid antixenosis mediated by volatiles in cereals. *Acta Agric. Scand. Sect. B, Soil Plant Sci.* 46:135–140.

RAHBE, Y. and FEBVAY, G. 1993. Protein toxicity to aphids: An in vitro test on *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 67:149–160.

RAHBE, Y., SAUVION, N., FEBVAY, G., PEUMANS, W. J., ANGHARAD, M., and GATEHOUSE, R. 1995. Toxicity of lectins and processing of ingested proteins in the pea aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 76:143–155.

REINBOTHE, S., MOLLENHAUER, B., and REINBOTHE, C. 1994. JIPs and RIPs: The regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *Plant Cell* 1994:1197–1209.

SRIVASTAVA, P. N. 1987. Nutritional Physiology, pp. 99–121, *in* A. K. Minks and P. Harrewijn (eds.). Aphids, Their Biology, Natural Enemies and Control. Elsevier Science Publishers, Amsterdam, The Netherlands

SUE, M., ISHIHARA, A., and IWAMURA, H. 2000. Purification and characterization of a hydroxamic acid glycoside β-glucosidase from wheat (*Triticum aestivum*) seedlings. *Planta* 210:432–438.

THACKRAY, D. J., WRATTEN, S. D., EDWARDS, P. J., and NIEMEYER, H. M. 1990. Resistance to the aphids *Sitobion avenae* and *Rhopalosiphum padi* in Gramineae in relation to hydroxamic acid levels. *Ann. Appl. Biol.* 116:573–582.

TJALLINGII, W. F. 1995. Stylet penetration by Homoptera: Electrical signals from the depth of plant tissues, pp. 49–58 *in* H. M. Niemeyer (ed.). Techniques in Plant–Insect Interactions and Biopesticides. Proceedings, IFS Workshop on Chemical Ecology. Santiago, Chile.

TJALLINGII, W. F. and HOGEN ESCH, TH. 1993. Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiol. Entomol.* 18:317–328.

VISSER, J. H., PIRON, P. G. M., and HARDIE, J. 1996. The aphids' peripheral perception of plant volatiles. *Entomol. Exp. Appl.* 80:35–38.

WOODWARD, M. D., CORCUERA, L. J., HEGELSON, J. P., and UPPER, CH. D. 1978. Decomposition of 2,4-dihydroxy-7-methoxy-2H1,4-benzoxazin-(4H)-one in aqueous solutions. *Plant Physiol.* 61:796–802.

ZUNIGA, G. E. and MASSARDO, F. 1991. Hydroxamic acid content in undifferentiated and differentiated tissue of wheat. *Phytochemistry* 30:3281–3283.

ZUNIGA, G. E., ARGANDONA, V. H., NIEMEYER, H. M., and CORCUERA, L. J. 1983. Hydroxamic acid content in wild and cultivated Gramineae. *Phytochemistry* 22:2665–2668.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

ANALYZING VARIABILITY IN NECTAR AMINO ACIDS: COMPOSITION IS LESS VARIABLE THAN CONCENTRATION

MARK C. GARDENER* and MICHAEL P. GILLMAN

Ecology and Conservation Research Group
Department of Biological Sciences
The Open University
Walton Hall, Milton Keynes, MK7 6AA, UK

(Received May 19, 2001; accepted July 22, 2001)

Abstract—Thirty species of flowering plants were analyzed for floral nectar amino acid composition. High-performance liquid chromatography (HPLC) was used in conjunction with AccQtag derivatization to produce accurate and precise data. For any one species, the total concentration of amino acids varies greatly (average coefficient of variation 0.65), but composition is much less variable (average correlation among samples from a single species 0.85). Absolute concentration of individual amino acids is much more variable than the relative abundance (coefficients of variation 0.98 and 0.77, respectively; $N = 544$, $t = 16.98$, $P < 0.001$). When amino acids that occur in only small relative abundance (<1%) are removed from the analysis, the difference is even more marked (0.78 and 0.51, respectively; $N = 344$, $t = 15.13$, $P < 0.001$). The results highlight the need for large sample sizes when making observations concerning the absolute amounts of amino acids in nectar and for sensitive analyses of the composition, as even small changes may be biologically significant.

Key Words—Nectar, amino acid, correlation, HPLC, flowering plant, composition, variation.

INTRODUCTION

Although early studies showed that the nectar of flowering plants contained substances other than sugars (Ziegler, 1956; Luttge, 1961), it was not until Baker and Baker (1973) showed that amino acids were largely ubiquitous in nectar that studies began to examine variation in composition both between and within species. Early studies used simple ninhydrin staining techniques to quantify total amino

*To whom correspondence should be addressed. E-mail: m.c.gardener@open.ac.uk

0098-0331/01/1200-2545/0 © 2001 Plenum Publishing Corporation

acid concentration (Baker and Baker, 1975). Despite limitations, the method is still used (Bernardello et al., 1991; Forcone et al., 1997). Other studies use thin-layer chromatography (TLC) to separate and identify component amino acids (Baker and Baker, 1976, 1977, 1979; Baker et al., 1978), with many studies electing to use a relative scale of concentration for the various component amino acids. More recent investigations employ high-performance liquid chromatography (Gottsberger et al., 1989; Lanza et al., 1995; Rusterholz and Erhardt, 1998), which provides a more precise and accurate determination of amino acid complement and concentration in a mixed sample (Cohen and Micheaud, 1993).

Based on TLC analysis, Baker and Baker (1977) stated that the amino acid complement of nectar was "remarkably constant" within a plant species. The relative concentrations of the amino acids present in each species were given, which, upon inspection, appeared to show impressive constancy of amino acid complement. However, the study by Lanza et al. (1995) determined that variation existed among individual plants within a single population of *Impatiens capensis* and between separate populations. Nectar from different flowers of a single individual showed no significant variability. More recently, Gardener and Gillman (2001) showed that the amino acid complement could be altered by soil fertilizer treatment, highlighting the fact that the use of HPLC allows greater sensitivity in detecting small changes that might remain undetected using previous techniques.

We present here the first quantitative assessment of the variability of nectar amino acid composition for a wide range of plant species. We also examine the variability of total amino acid concentration in those species. This is important, as previous studies have made assertions concerning the role of amino acids in nectar based upon measurements of the total concentration (e.g., Baker and Baker, 1973, 1977; Gottsberger et al., 1984). It is necessary for future studies to appreciate the variability in composition and concentration if we are to fully understand the ecological role of amino acids in nectar.

METHODS AND MATERIALS

Nectar Collection. Nectar was collected from plants growing wild in the vicinity of the Open University campus in Milton Keynes, Buckinghamshire, United Kingdom. In all cases, samples were taken from a number of individuals in the same neighborhood to minimize variability among populations or caused by soil conditions. Samples were taken at the same time of day (14:00–16:00 hr) and from flowers of approximately the same age (first day of dehiscence). This was to minimize effects of flower aging that have been shown to affect amino acid concentrations in nectar samples (Gottsberger et al., 1990; Petanidou et al., 1996). Predehiscent flowers were covered with a fine net (dress net, 1-mm mesh size) to prevent visitation by insects and so possible contamination or nectar removal. The following day the nectar of those flowers was withdrawn using 5-μl glass

graduated micropipet tubes, capillary action being enough to draw in the nectar. Precautions were taken to minimize possible contamination with pollen, which can release free amino acids in solution (Erhardt and Baker, 1990). In some species, the inflorescence was cut with sharp scissors, allowing the anthers to fall away, revealing the ovary. In other species (e.g., *Epilobium hirsutum*), this was not necessary, as the nectaries were easily accessible. In such species, care was taken to avoid touching the anthers with the pipet. The volume of each sample was determined by measuring the fluid column in the pipet. This measurement was a source of variability. Most samples were in the range of 1–2 μl. With 15 mm representing 1 μl and a measurement accuracy of 0.5 mm, this represents a random error in the range of 1–3.5%. Each sample was aspirated into a glass chromatography vial (Chromacol 02-CTVG) and frozen (at -40°C) shortly afterwards until analysis by HPLC. In total, samples from 30 plant species were collected.

Analysis of Nectar. Samples were thawed and amino acids derivatized using the AccQtag protocol (Waters Corp.) (Cohen and Micheaud, 1993) in a 0.02 M borate buffer (pH 8.6). HPLC was performed, with standards every four samples, using the following equipment: Waters 712 WISP autosampler, Waters 600 pump controller, Waters 600 HPLC pump with 510 pump-heads. Separation was achieved using a Novapak C_{18} (15 cm $\times$ 4.6 mm) cartridge with guard column. The binary solvent system was a 6:4 acetonitrile–water mix and a TEA–phosphate (pH 5.0) buffer. Detection was via a Waters 474 scanning fluorescent detector (excitation at 295 nm and detection at 350 nm). The system was monitored and data collected using the Waters Millenium^{32} software. Chromatograms were analyzed and compared to standards for identification of individual amino acids. Standard amino acids were made up to a concentration of 100 pmol/μl. In addition to all the protein-building amino acids, standards of hydroxyproline, ornithine, taurine, AABA, and GABA were used. Peak areas were compared to standards to determine the concentration of individual amino acids. From these data, the total concentration of all amino acids was determined and the proportion that each made to the total was calculated. A summary of the amino acid composition for each species is given in Appendix 1.

RESULTS

Total Concentration of Amino Acids. For each species the variability in total amino acid concentration among samples was determined by using the coefficient of variation, i.e., the standard deviation divided by the mean. Table 1 shows the coefficients for all the species analyzed. The mean coefficient was 0.67. Given this variability, the error arising from measurement of nectar in the collecting capillary is negligible.

Comparison of Composition. Each nectar sample produced a range of amino acids in varying proportions. In general, a sample contained a few, abundant amino

TABLE 1. SPECIES EXAMINED FOR NECTAR AMINO ACIDS BY HPLC[a]

Species	*N*	Corr	Vc
Agrostemma githago	42	0.91	0.6
Ajuga reptans	8	0.85	1.3
Calystegia sylvatica	7	0.83	1.2
Cardamine pratensis	7	0.94	0.9
Centaurea nigra	6	0.81	0.9
Centranthus ruber	18	0.92	0.2
Chamaenerion angustifolium	7	0.75	0.7
Cirsium vulgare	6	0.82	0.6
Convolvulus arvensis	6	0.93	0.3
Corydalis lutea	7	0.89	0.4
Epilobium hirsutum	7	0.74	1.0
Epilobium montanum	5	0.88	0.3
Lamium purpureum	6	0.99	1.0
Lamium album	7	0.93	0.5
Lavatera arborea	5	0.96	0.5
Lonicera hecrotii, Goldflame	4	0.83	0.3
Lotus corniculatus	10	0.75	1.0
Lunaria annua	5	0.99	0.8
Lychnis flos-cuculi	9	0.84	0.5
Lythrum salicaria	7	0.88	0.9
Primula veris	6	0.92	0.9
Primula vulgaris	6	0.82	0.8
Prunella vulgaris	7	0.96	0.3
Pulmonaria officinalis	3	0.91	0.4
Scrophularia scorodonia	5	0.91	0.6
Silene dioica	36	0.92	1.1
Stachys sylvatica	6	0.86	0.3
Trifolium pratense	7	0.94	0.4
Vicia sativa	6	0.84	1.0
Vinca major	6	0.96	0.4
Average		0.88	0.67

[a] Data columns are: number of samples analyzed (N), average intraspecific correlation (Corr), and coefficient of variation for total concentration of nectar amino acids (Vc). All correlations are statistically significant ($P < 0.001$).

acids that each contributed greater than 10% towards the total concentration, a number of smaller components with fractions in the range of 5–10%, and a larger number of amino acids each contributing <5% towards the total. These latter amino acids, many contributing <1% towards the total concentration, would undoubtedly have remained undetected if less sensitive techniques had been used.

The extent of the similarity of composition between two nectar samples can be determined by correlation of the amino acid concentrations (e.g., Figure 1). In this case, Pearson correlation coefficients were determined for all comparisons of compositions within each species. The mean correlation coefficient could be used

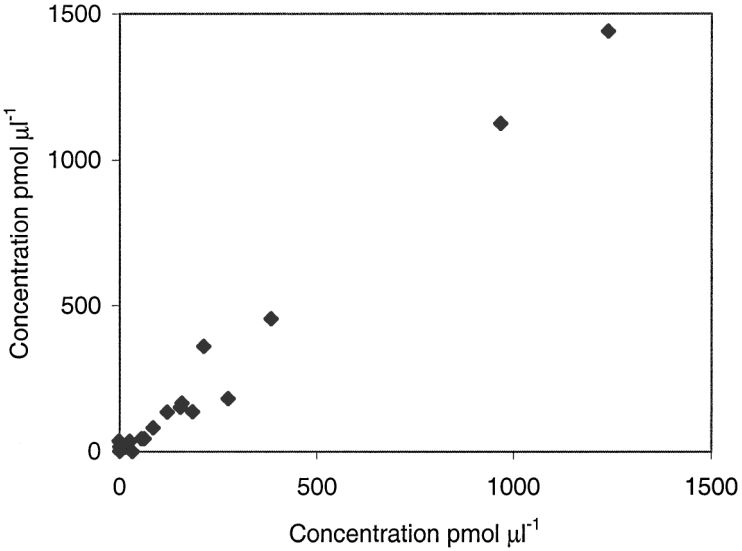


FIG. 1. Example of positive correlation of a pair of nectar samples (*Corydalis lutea*, $R = 0.993$, $N = 19$, $P < 0.001$).

to represent the variation in composition. This is a conservative (and potentially biased) measure, as one errant sample would have a greater effect upon the final mean by virtue of multiple paired comparisons. Table 1 shows the mean coefficient for each species analyzed. The mean of all the coefficients of variation was 0.88.

Correlation provides a useful tool to examine the similarity of samples. It would be expected that nectar samples from conspecifics would be more highly correlated to one another than to heterospecifics, and this could form the basis for an interesting analysis of nectar evolution, perhaps by comparing a cluster analysis of nectar samples to a molecular phylogeny.

Comparing Variability of Composition and Concentration. Although the correlation coefficient is useful as a guide to the constancy of composition, it is not directly comparable to the coefficient of variation of total concentration. In order to improve the comparison, we considered the set of samples from each plant species in turn. For each amino acid in the set of samples, a coefficient of variation for its absolute concentration was calculated. A similar coefficient was calculated for the fraction that amino acid contributed to the total (i.e., the relative abundance of the amino acid). The process was repeated for all 30 of the plant species, producing a database of 544 comparisons.

For each amino acid of each species, the coefficients of variation in absolute amount and relative amount were compared. The data were analyzed by using a

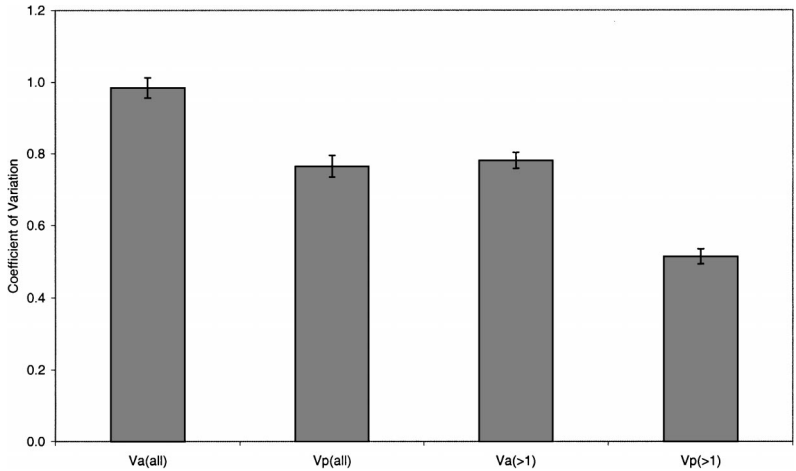


FIG. 2. Coefficients of variation for absolute concentration of amino acid and proportion (Va and Vp, respectively) for amino acids in nectar samples. First group represents all data while the second group is for amino acids contributing >1% to the sample. Error bars are standard error.

t test for paired samples, showing that the variability in absolute concentration was significantly higher than the variability in relative abundance (0.98 and 0.77, respectively, $N = 544$, $t = 16.98$, $P < 0.001$, Figure 2). It might be expected that the more abundant amino acids (>10%) would be less variable than the less abundant ones. To test this, the amino acids were categorized into abundance classes. Removing the last category (0–1%) of amino acids from the analysis, the difference in variability between absolute and relative concentration is more pronounced (0.78 and 0.51, respectively; $N = 344$, $t = 15.13$, $P < 0.001$, Figure 2). Variability of absolute concentration, in the range 0–1% was higher than all other frequency ranges (one-way ANOVA, $F_{6,537} = 18.205$, $P < 0.001$. post-hoc LSD test $P < 0.001$; Figure 3). Variability of relative abundance was also higher for this 0–1% group (one-way ANOVA, $F_{6,537} = 26.791$, $P < 0.001$, post-hoc LSD test $P < 0.001$; Figure 4).

The data in Figures 3 and 4 can be combined to produce a simple index of amino acid variability expressed as the ratio of variability in absolute concentration to variability in relative abundance (Va/V%). The ratio of variability of absolute to relative abundance changed as the component amino acids increase their share of the total (Figure 5). There appear to be three groups, the first being amino acids, contributing >20% to the total. Post-hoc LSD tests showed this group to have significantly higher Va/V% than all other groups ($P < 0.001$, one-way ANOVA, $F_{6,537} = 12.185$, $P < 0.001$). The second group was amino acids occurring in the range of 1–20%. Post-hoc tests showed no significant differences for any of

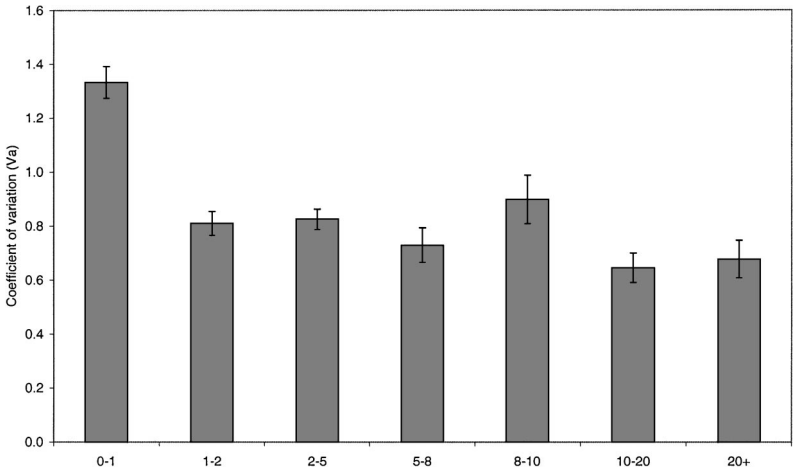


FIG. 3. Variability of absolute concentration of amino acids in nectar for various relative abundance ranges e.g., A0–1 = amino acids that contribute 0–1% to the total in the sample (one-way ANOVA, $F_{6,537} = 18.205$, $P < 0.001$).

the abundance classes within this range. A final category covers amino acids that contribute <1% to the total of the sample in which they occur. Post-hoc LSD tests show this group to have a lower Va/V% ratio than all the others ($P < 0.01$) except for the 8–10% group ($P = 0.08$) and the 10–20% group ($P = 0.13$).

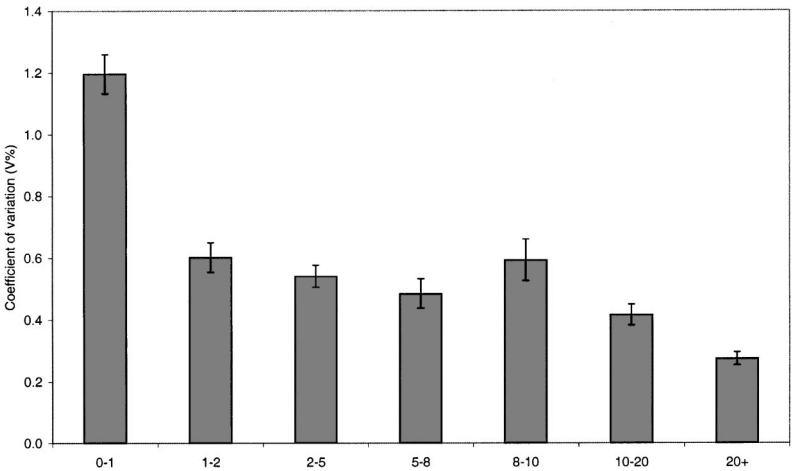


FIG. 4. Variability of relative abundance of amino acids in nectar for various relative abundance ranges (one-way ANOVA, $F_{6,537} = 26.791$, $P < 0.001$).

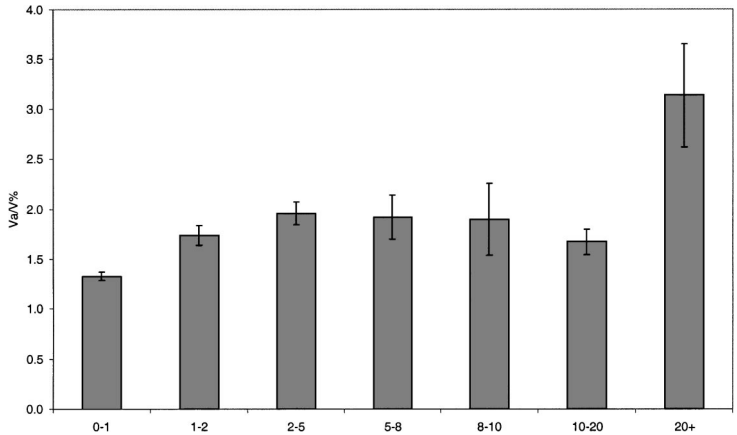


FIG. 5. The ratio of variability in absolute concentration (Va) to variability in relative abundance (V%) for amino acids occurring in nectar samples in various relative abundance ranges (one-way ANOVA, $F_{6,537} = 12.185$, $P < 0.001$).

DISCUSSION

These analyses show that the total concentration of amino acids in the nectar of any one species may vary widely. When making comparisons between species, therefore, it is important to ensure adequate replication and to use appropriately sensitive techniques. The composition, on the other hand, is much less variable. This is not entirely unexpected. The structure of the plant tissues that contribute to nectar production—the nectaries, phloem, and surrounding cells—are fixed by genetic processes and produce nectar of a certain species-specific, composition (Baker and Baker, 1977), although there can be variability among populations of a species (Lanza et al., 1995). The production of nectar is an active, energy-requiring process, which is curbed by respiratory inhibitors (Findlay and Mercer, 1971). Day-to-day environmental variations, in temperature and sunlight for example, are factors that will influence the metabolic processes of nectar production and may lead to changes in overall concentration of the nectar components. Physiological processes such as water relations may influence nectar concentration at the production stage, and evaporation may influence concentration afterwards. Longer-term environmental variables operating within a growing season, such as soil nutrients (Gardener and Gillman, 2001) and CO_2 (Rusterholz and Erhardt, 1998), are more likely to alter nectar composition by a variety of mechanisms e.g., altered metabolite availability and concentration, altered growth of plant tissues.

Acknowledgments—Thanks to Graham Jeffs from the Chemistry Department at the Open University for help and support with all aspects of chromatography. This study forms part of a PhD project funded by the Open University Department of Biological Sciences (grant SB20 5861 0).

APPENDIX 1. NECTAR AMINO ACID COMPOSITION DATA[a]

	Agrostemma githago ($N = 42$)		*Ajuga reptans* ($N = 8$)		*Calystegia sylvatica* ($N = 7$)		*Cardamine pratensis* ($N = 7$)		*Centaurea nigra* ($N = 6$)		*Centranthus ruber* ($N = 18$)	
N	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE
Hyp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Asp	510	64.0	115	37.6	303	158.5	138	54.3	422	269.4	216	11.9
Ser	766	106.3	240	67.4	0	0.0	251	101.8	734	471.8	497	44.3
Asn	33	16.3	49	25.3	4565	2301.6	17	18.5	2085	805.2	0	0.0
Glu	327	32.2	97	28.2	19	19.1	180	52.4	456	329.3	153	18.3
Gly	312	45.1	122	35.6	60	24.1	142	72.0	347	227.1	1118	61.5
Gln	6,193	700.4	1421	970.8	1321	718.9	1583	523.3	3955	1380.7	352	48.0
His	194	21.7	7	3.2	47	15.5	49	15.0	106	36.1	21	5.0
Tau	5	2.9	11	3.8	15	10.5	1	1.1	4	3.8	0	0.0
Arg	287	25.9	125	33.8	406	180.4	152	53.7	412	152.3	105	10.5
Thr	55	5.3	27	5.2	76	19.0	21	8.1	185	44.5	124	9.3
Ala	459	64.1	56	13.3	184	95.0	62	39.0	668	299.1	179	26.6
Pro	1,243	178.6	38	18.3	801	277.4	235	55.5	1697	743.4	31	14.3
Gaba	876	152.3	0	0.0	120	62.3	113	87.8	105	83.8	93	26.9
Aaba	2	1.9	0	0.0	0	0.0	0	0.0	3	3.1	0	0.0
Cys	13	5.3	0	0.0	220	100.3	8	8.3	0	0.0	0	0.0
Tyr	54	5.3	13	4.0	36	11.2	8	6.3	42	19.5	53	3.4
Val	305	26.8	32	8.1	368	185.3	84	28.2	243	80.0	497	18.4
Met	38	6.1	2	1.5	40	8.8	1	1.5	0	0.0	43	2.6
Orn	104	31.6	81	21.4	17	8.3	41	37.1	261	206.8	85	10.0
Lys	118	14.4	12	4.9	29	12.4	22	12.7	99	42.5	28	3.8
Ile	160	11.7	22	5.1	206	102.4	47	17.0	101	35.4	33	2.7
Leu	171	22.9	21	5.3	162	79.0	45	15.2	70	25.9	42	3.4
Phe	496	22.5	15	3.4	732	381.1	22	7.9	76	28.0	23	2.5
Trp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total	12,730	1268.2	2507	1139.6	9726	4486.2	3226	1104.3	12070	4438.2	3692	195.4

APPENDIX 1. CONTINUED

	Chamerion angustifolium ($N = 7$)		*Cirsium vulgare* ($N = 6$)		*Convolvulus arvensis* ($N = 6$)		*Corydalis lutea* ($N = 7$)		*Epilobium hirsutum* ($N = 7$)		*Epilobium montanum* ($N = 5$)	
N	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE
Hyp	0	0.0	3	3.2	0	0.0	0	0.0	0	0.0	0	0.0
Asp	6	3.0	76	34.3	398	74.9	139	9.6	16	9.4	212	91.6
Ser	22	7.3	296	77.9	473	158.1	361	49.1	0	0.0	186	26.2
Asn	0	0.0	357	77.9	2332	373.8	109	41.3	39	7.4	0	0.0
Glu	14	3.1	140	37.7	266	74.9	137	14.7	3	2.3	95	23.5
Gly	2	1.7	141	36.6	264	74.9	140	25.6	5	3.6	81	12.5
Gln	66	18.9	976	293.3	1089	185.6	2231	474.6	22	8.7	200	43.5
His	12	6.4	49	9.0	60	23.8	37	3.1	19	5.5	63	21.1
Tau	0	0.0	4	2.3	92	24.2	1	1.5	0	0.3	0	0.0
Arg	30	2.8	209	51.2	447	97.4	297	24.6	17	3.4	46	4.8
Thr	10	4.4	29	9.3	39	11.0	12	7.8	1	0.6	4	3.9
Ala	3	2.1	176	53.3	266	62.3	85	7.6	47	38.4	0	0.0
Pro	12	11.7	465	57.3	64	42.1	53	27.5	212	93.3	1140	214.5
Gaba	0	0.0	34	15.2	68	36.4	7	4.8	1	1.5	131	56.4
Aaba	0	0.0	0	0.0	0	0.0	0	0.0	1	0.8	0	0.0
Cys	0	0.0	12	11.5	426	92.0	0	0.0	0	0.0	0	0.0
Tyr	1	0.8	38	7.1	55	15.5	21	3.0	1	0.7	0	0.0
Val	1	0.6	79	21.9	452	86.3	33	6.5	3	1.3	31	5.4
Met	0	0.5	0	0.0	29	16.9	56	9.2	0	0.0	25	4.1
Orn	0	0.0	77	23.1	124	37.3	105	20.8	0	0.0	33	13.4
Lys	1	0.7	19	7.5	47	9.4	43	5.5	1	0.7	6	6.4
Ile	2	0.7	49	11.5	320	63.2	14	1.5	2	0.8	18	2.9
Leu	3	0.5	33	11.5	194	45.9	1104	191.0	1	0.9	25	5.1
Phe	6	1.5	58	11.5	3074	305.9	228	33.9	5	2.0	14	2.0
Trp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	81	81.1
Total	190	51.1	3318	792.8	10580	1441.2	5198	695.3	397	157.2	2392	369.4

APPENDIX 1. CONTINUED

	Lamium album ($N = 7$)		*Lamium purpureum* ($N = 6$)		*Lavatera arborea* ($N = 5$)		*Lonicera hecrotii Goldflame* ($N = 4$)		*Lotus corniculatus* ($N = 10$)		*Lunaria annua* ($N = 5$)	
N	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE
Hyp	8	4.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Asp	10	5.6	124	35.4	178	38.8	53	8.4	551	156.6	264	75.4
Ser	55	17.5	155	31.0	0	0.0	130	45.9	385	136.1	200	68.2
Asn	32	8.7	138	50.1	1805	482.7	0	0.0	1876	952.0	288	97.2
Glu	0	0.0	89	24.4	0	0.0	48	5.0	347	115.5	181	83.8
Gly	14	6.1	83	16.7	9	4.3	55	20.2	182	43.5	17	14.7
Gln	142	61.2	3668	1686.4	246	86.3	257	106.4	1066	703.7	5606	2194.4
His	9	2.4	3	3.0	23	3.3	14	5.1	151	72.8	15	9.8
Tau	1	0.7	0	0.0	0	0.0	3	1.5	7	7.4	0	0.0
Arg	54	21.5	51	9.8	85	11.9	83	8.5	335	87.5	61	18.0
Thr	19	4.7	24	9.3	11	2.6	13	1.2	106	31.8	0	0.0
Ala	83	28.8	36	14.2	289	114.1	29	10.3	316	88.1	26	20.0
Pro	625	118.3	69	21.2	1005	153.5	418	30.1	1382	241.5	49	20.3
Gaba	0	0.0	46	16.9	0	0.0	0	0.0	52	34.9	0	0.0
Aaba	0	0.0	0	0.0	0	0.0	48	28.1	14	14.3	0	0.0
Cys	0	0.0	0	0.0	0	0.0	3	2.9	115	70.0	0	0.0
Tyr	243	42.3	3	3.0	7	0.7	8	3.3	1924	547.3	6	4.0
Val	14	3.8	46	9.3	19	6.5	28	6.5	167	51.0	87	29.4
Met	3	1.1	20	3.5	0	0.0	3	0.7	40	11.0	0	0.0
Orn	8	4.1	35	12.7	0	0.0	25	9.6	94	22.7	0	0.0
Lys	3	1.6	3	3.3	0	0.0	8	1.9	70	32.1	5	5.3
Ile	18	4.6	26	4.7	9	2.1	18	4.6	76	20.6	41	9.4
Leu	8	2.2	31	6.1	20	3.1	20	4.9	90	26.9	14	4.4
Phe	627	130.0	16	2.8	5	0.7	8	3.1	1879	519.3	16	4.3
Trp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total	1976	386.9	4668	1922.3	3712	843.1	1271	202.2	11228	3472.4	6878	2580.9

APPENDIX 1. CONTINUED

	Lychnis flos-cuculi ($N = 9$)		*Lythrum salicaria* ($N = 7$)		*Primula veris* ($N = 6$)		*Primula vulgaris* ($N = 6$)		*Prunella vulgaris* ($N = 7$)		*Pulmonaria officinalis* ($N = 3$)	
N	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE
Hyp	0	0.0	4	2.9	0	0.0	0	0.0	0	0.0	0	0.0
Asp	195	47.7	230	93.3	86	37.1	81	12.2	67	12.7	150	61.6
Ser	402	91.5	266	130.4	0	0.0	112	14.6	356	30.4	264	118.3
Asn	150	24.4	244	91.3	278	122.0	210	102.4	0	0.0	16	16.0
Glu	248	49.9	201	119.6	169	54.3	164	31.7	49	15.5	162	61.1
Gly	44	9.6	95	36.3	61	26.3	63	9.3	182	14.6	125	64.8
Gln	3691	818.6	590	242.2	162	63.7	714	208.8	130	21.3	603	47.7
His	62	12.5	32	12.5	12	4.8	3	2.8	28	6.7	0	0.0
Tau	0	0.0	6	4.4	0	0.0	5	5.1	1	1.2	0	0.0
Arg	358	60.6	354	69.5	52	18.8	17	7.7	168	26.7	242	33.2
Thr	138	38.9	69	16.1	14	3.9	19	13.9	25	4.7	11	11.3
Ala	297	86.3	169	58.9	371	137.9	113	38.1	80	11.8	57	24.5
Pro	478	56.4	992	367.2	1022	372.7	726	317.9	955	118.7	141	30.1
Gaba	211	77.5	157	104.4	2	1.6	0	0.0	0	0.0	0	0.0
Aaba	0	0.0	0	0.0	0	0.0	0	0.0	40	40.1	0	0.0
Cys	0	0.0	8	7.9	0	0.0	0	0.0	2	2.4	0	0.0
Tyr	140	30.0	16	7.6	7	3.2	0	0.0	27	4.8	0	0.0
Val	277	60.3	143	43.1	30	10.7	32	6.1	38	5.3	38	11.3
Met	17	3.1	26	10.3	10	3.5	20	1.7	16	5.9	7	6.8
Orn	43	7.5	45	20.4	42	17.9	18	11.2	136	23.8	64	25.7
Lys	131	29.3	37	15.6	25	9.8	8	5.1	29	7.6	0	0.0
Ile	225	47.5	93	37.6	17	6.2	20	2.3	25	3.4	22	8.3
Leu	232	48.7	83	27.5	20	7.2	18	2.9	27	3.6	25	8.2
Phe	1437	244.4	2365	784.2	8	3.1	15	3.1	15	2.3	14	5.5
Trp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total	8777	1561.2	6224	2049.8	2387	867.7	2358	744.6	2396	252.5	1941	484.4

APPENDIX 1. CONTINUED

	Scrophularia scorodonia ($N = 5$)		*Silene dioica* ($N = 36$)		*Stachys sylvatica* ($N = 6$)		*Trifolium pratense* ($N = 7$)		*Vicia sativa* ($N = 6$)		*Vinca major* ($N = 6$)	
N	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE	Av C	SE
Hyp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Asp	29	11.1	78	14.0	26	6.2	277	36.4	193	66.6	135	17.1
Ser	194	72.9	118	23.1	64	15.0	0	0.0	1057	504.8	0	0.0
Asn	0	0.0	5	4.7	0	0.0	1820	332.9	299	138.3	310	102.1
Glu	28	12.0	47	12.0	18	2.3	0	0.0	257	100.7	96	18.9
Gly	411	54.8	59	13.7	30	9.3	88	21.0	258	99.8	4	4.4
Gln	2190	779.2	1261	283.7	129	15.9	472	89.8	601	224.8	77	25.6
His	40	16.1	13	2.1	9	3.2	38	6.8	98	51.3	17	3.0
Tau	0	0.0	4	1.0	2	1.2	0	0.0	0	0.0	0	0.0
Arg	21	20.7	56	9.6	52	6.5	152	29.9	569	151.7	238	33.6
Thr	386	117.6	16	2.2	7	3.5	17	4.7	97	21.7	49	7.3
Ala	20	7.9	80	13.9	15	3.4	211	26.6	515	309.2	158	60.1
Pro	0	0.0	32	10.0	0	0.0	563	175.4	0	0.0	1281	217.6
Gaba	0	0.0	12	5.3	0	0.0	156	38.8	65	65.0	0	0.0
Aaba	0	0.0	0	0.0	0	0.0	49	30.2	0	0.0	0	0.0
Cys	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Tyr	19	4.9	8	1.4	6	2.3	18	4.0	40	22.2	0	0.0
Val	75	8.5	28	5.3	11	2.6	83	15.7	140	65.3	12	2.6
Met	10	3.5	2	1.2	4	1.3	19	4.0	8	5.6	9	1.4
Orn	37	8.7	31	8.8	15	4.4	55	15.8	82	28.6	2	2.3
Lys	814	132.2	6	1.8	6	4.1	16	4.6	28	13.3	3	1.8
Ile	9	4.4	13	2.5	8	2.2	49	9.8	89	46.8	7	1.5
Leu	11	5.1	24	5.6	10	4.1	40	8.7	104	49.3	20	2.6
Phe	10	7.4	11	1.4	22	7.1	28	5.3	80	37.4	3	1.6
Trp	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total	4304	1124.9	1904	350.8	435	55.8	4151	626.7	4581	1928.1	2422	397.5

[a] Each amino acid and totals are given as picomoles of amino acid per microliter of nectar (equivalent to millimolar).

REFERENCES

BAKER, H. G. and BAKER, I. 1973. Amino acids in nectar and their evolutionary significance. *Nature* 241:543–545.

BAKER, H. G. and BAKER, I. 1975. Studies of nectar constitution and pollinator-plant coevolution, pp. 100–140, *In* L. E. Gilbert and P. H. Raven (eds.). Co-evolution of Animal and Plants. University of Texas Press, Austin, Texas.

BAKER, H. G. and BAKER, I. 1977. Intraspecific constancy of floral nectar amino acid complements. *Bot. Gaz.* 138:183–191.

BAKER, H. G., OPLER, P. A., and BAKER, I. 1978. A comparison of the amino acid complements of floral and extrafloral nectars. *Bot. Gaz.* 139:322–332.

BAKER, I. and BAKER, H. G. 1976. Analyses of amino acids in flower nectars of hybrids and their parents, with phylogenetic implications. *New Phytol.* 76:87–98.

BAKER, I. and BAKER, H. G. 1979. Chemical constituents of the nectars of two *Erythrina* species and their hybrid. *Ann. Mio. Bot. Gard.* 66:446–450.

BERNARDELLO, L. M., GALETTO, L., and JULIANI, H. R. 1991. Floral nectar, nectary structure and pollinators in some Argentinean Bromeliaceae. *Ann. Bot.* 67:401–412.

COHEN, S. A. and MICHEAUD, D. P. 1993. Synthesis of a fluorescent derivitization reagent, 6-aminoquionyl-*N*-hydroxysuccinimidyl carbamate and its application for the analysis of hydrolysate amino acids via high performance liquid chromatography. *Anal. Biochem.* 211:279–287.

ERHARDT, A. and BAKER, I. 1990. Pollen amino acids—an additional diet for a nectar feeding butterfly. *Plant Syst. Evol.* 169:111–121.

FINDLAY, N. and MERCER, F. V. 1971. Nectar production in *Abutilon*. III. Sugar secretion. *Aust. J. Biol. Sci.* 24:665–675.

FORCONE, A., GALETTO, L., and BERNARDELLO, L. 1997. Floral nectar chemical composition of some species from Patagonia. *Biochemi. Syst. Ecol.* 25:395–402.

GARDENER, M. C. and GILLMAN, M. P. 2001. The effects of soil fertilizer on amino acids in the floral nectar of corncockle, *Agrostemma githago* L. (Caryophyllaceae). *Oikos* 92:101–106.

GOTTSBERGER, G., SCHRAUWEN, J., and LINSKENS, H. F. 1984. Amino acids and sugars in nectar, and their putative evolutionary significance. *Plant Syst. Evol.* 145:55–77.

GOTTSBERGER, G., ARNOLD, T., and LINSKENS, H. F. 1989. Intraspecific variation in the amino acid content of floral nectar. *Bot. Acta* 102:141–144.

GOTTSBERGER, G., ARNOLD, T., and LINSKENS, H. F. 1990. Variation in floral nectar amino acids with aging of flowers, pollen contamination, and flower damage. *Isr. J. Bot.* 39:167–176.

LANZA, J., SMITH, G. C., SACK, S., and CASH, A. 1995. Variation in nectar volume and composition of *Impatiens capensis* at the individual, plant and population levels. *Oecologia* 102:113–119.

LUTTGE, U. 1961. Uber die zusammensetzung des nektars und den mechanismus seiner sekretion. *I. Planta* 56:189–212.

PETANIDOU, T., VANLAERE, A. J., and SMETS, E. 1996. Change in floral nectar components from fresh to senescent flowers of *Capparis spinosa* (Capparidaceae), a nocturnally flowering Mediterranean shrub. *Plant Syst. Evol.* 199:79–92.

RUSTERHOLZ, H. P. and ERHARDT, A. 1998. Effects of elevated CO_2 on flowering phenology and nectar production of nectar plants important for butterflies of calcareous grasslands. *Oecologia* 113:341–349.

ZIEGLER, H. 1956. Untersuchungen uber die Leitung und Sekretion der Assimilate. *Planta* 47:447–500.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

DETOXIFICATION IN RELATION TO TOXIN TOLERANCE IN DESERT WOODRATS EATING CREOSOTE BUSH

ANTONIO M. MANGIONE,[1,3,]* DENISE DEARING,[2] and WILLIAM KARASOV[3]

[1]*Departamento de Bioquímica y Ciencias Biológicas*
Universidad Nacional de San Luis, Argentina
5700 San Luis, Argentina

[2]*Department of Biology*
University of Utah
Salt Lake City, Utah 84112

[3]*Department of Wildlife Ecology*
University of Wisconsin
Madison, Wisconsin 53706

(Received September 27, 2000; accepted August 8, 2001)

Abstract—We studied the relationship between the use of three detoxification pathways and urine pH and the tolerance of desert woodrats from two populations to a mixture of naturally occurring plant secondary metabolites (mostly phenolics) in resin from creosote bush (*Larrea tridentata*). The two populations of desert woodrats came from the Mojave desert (Mojave woodrats), where woodrats consume creosote bush, and from the Great Basin desert (Great Basin woodrats), where the plant species is absent. We fed woodrats alfalfa pellets containing increasing levels of the phenolic resin and measured three detoxification pathways and urine pH that are related to detoxification of allelochemicals. We found that the excretion rate of two phase II detoxification conjugates, glucuronides and sulfides, increased with increasing resin intake, whereas excretion of hippuric acid was independent of resin intake, although it differed between populations. Urine pH declined with increasing resin ingestion. The molar proportion of glucuronides in urine was three times that of the other conjugates combined. Based on an evaluation of variation in the three detoxification pathways and urine pH in relation to resin intake, we rejected the hypotheses that woodrats' tolerance to resin intake is related to capacity for amination, sulfation, or pH regulation. However, Mojave woodrats had higher maximum glucuronide excretion rates, and we accepted the hypothesis that within and between populations woodrats tolerate more resin because they have a greater capacity for glucuronide excretion.

*To whom correspondence should be addressed. E-mail: amangion@unsl.edu.ar

0098-0331/01/1200-2559/0

Key Words—Desert woodrats, *Neotoma lepida*, creosote bush, *Larrea tridentata*, tolerance, glucuronic acid, hippuric acid, sulfates, urine, pH, detoxification, conjugates.

INTRODUCTION

Herbivores that feed on plants containing plant secondary metabolites (PSMs) can manage owing to enzymes that metabolize the ingested toxins (Brattsten, 1992). Freeland and Jansen (1974) proposed that the amount of PSMs that an individual can ingest is related to its capacity to detoxify and eliminate PSMs. This capacity is ecologically important because it constitutes a constraint on how much of a food containing the PSM might be ingested, and so it can be used to predict diets (Belovsky and Schmitz, 1991).

The ability of herbivores to cope with PSMs can result from: (1) the capacity to habituate to aversive sensory properties of the PSM, (2) differential rates of PSM absorption, (3) variation in target site sensitivity, and (4) the capacity to detoxify PSMs, either by using different detoxification pathways or because of differences in the specific kinetics of detoxification (Lindroth, 1988). This study relates primarily to the fourth factor. Although there is a large body of literature on detoxification pathways used by mammals, and their relative affinities and capacities as enzymatic systems (Caldwell, 1986; Sipes and Gandolfi, 1991), there are three points that deserve more attention. First, almost all detoxification pathways have been studied with humans and laboratory and domestic mammals (Parke, 1968; Caldwell, 1986; Sipes and Gandolfi, 1991) and very little in wild mammal–allelochemical systems (McArthur and Sanson, 1993; Harju-Anu, 1996). Second, one of the major detoxification systems for the conjugation of phenolic compounds, glucuronidation, has been one of the more extensively studied in laboratory mammal–drug systems. A direct consequence of this is that glucuronidation usually is the only conjugation route studied in wild mammal–allelochemical systems (Lindroth and Batzli, 1983; MacCoubrey et al., 1997), although a few studies in birds measured other conjugation pathways along with glucuronidation (Jakubas et al., 1993; Guglielmo et al., 1996; Hewitt and Kirkpatrick, 1997). Third, researchers rarely determine the maximum capacities of conjugation pathways or which pathways are induced with toxin exposure and which are fixed at some constitutive level. Consequently, there are few tests of the idea that the amount of PSMs that an individual can ingest is related to its capacity to detoxify and eliminate them.

The general objective of this study was to establish the relationship between the use of three detoxification mechanisms and urine pH and the tolerance of desert woodrats (*Neotoma lepida*) from two populations to a mixture of naturally occurring PSMs (mostly phenolics) from creosote bush (*Larrea tridentata*). The detoxification mechanisms are phase II detoxification pathways that involve conjugation of endogenous substrates to toxin metabolites: glucuronidation, sulfation,

and amino acid (glycine) conjugation to form hippuric acid. These were chosen because: (1) they are the most common conjugation reactions in mammals; (2) all three can be detected in urine; and (3) their respective endogenous moieties (glucuronic acid, sulfate and glycine) can be limiting factors under nutritional stress and hence detoxification could be compromised, or, if the need for moieties exceeds their supply, the nutritional status of the animals may be compromised (Sipes and Gandolfi, 1991; Guglielmo et al., 1996).

Urine pH and the three detoxification pathways we studied relate to acid–base homoeostasis that may be affected by organic acids produced as a result of the biotransformation and metabolism of PSMs (Foley et al., 1995). These organic acids are ionized at physiological pH (Robinson et al., 1953). The resulting hydrogen ion can be excreted in the urine or buffered. However, if the animal's buffering capacity is saturated, the pH of the urine will decrease.

The two populations of desert woodrats subjected to study came from the Mojave desert (hereafter called Mojave woodrats) and from the Great Basin desert (hereafter called Great Basin woodrats). While the natural diet of Great Basin woodrats is mostly composed by Utah juniper (personal observation), the diet of Mojave woodrats is composed of at least 22% of creosote bush in some areas in the Mojave desert (Mangione et al., 2000). The leaves of creosote bush are covered with a resin (Mabry et al., 1977) that makes up 10–25% leaf dry mass. The resin is composed of 40% dry mass of nordihydroguaiaretic acid (NDGA) and the remainder of the resin is a complex mixture of partially *O*-methylated flavones and flavonols (Rhoades and Cates, 1976; Mabry et al., 1977). The resin is known to deter feeding by arthropods and to complex with protein in vitro. NDGA fed to laboratory rats at 0.5, 1, or 3% produced cysts in the kidney and vacuolation of kidney tubular epithelium (Grice et al., 1968; Goodman et al., 1970). In a previous study (Mangione et al., 2000), we found that there were differences in tolerance to creosote bush resin between these two populations; Mojave woodrats ingested more resin, and maintained body mass constant at higher concentrations of resin in the diet. Possibly, the difference in tolerance relates to differences in detoxification pathways used by woodrats from these two populations.

The specific goals of the study were: (1) to test whether desert woodrats rely more on glucuronidation compared to sulfation and glycine conjugation (hippuric acid), as occurs for other mammals; (2) to find the maximum capacities of each detoxification route and its relationship with the maximum tolerance of resin in the diet; (3) to establish whether the detoxification of the resin affects acid–base balance of woodrats; and (4) to test whether the differential use of detoxification routes explains the tolerance difference between the two populations of desert woodrats studied earlier (Mangione et al., 2000).

We investigated the overall hypothesis that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate the PSMs by testing a number of specific predictions, which are illustrated in Figure 1

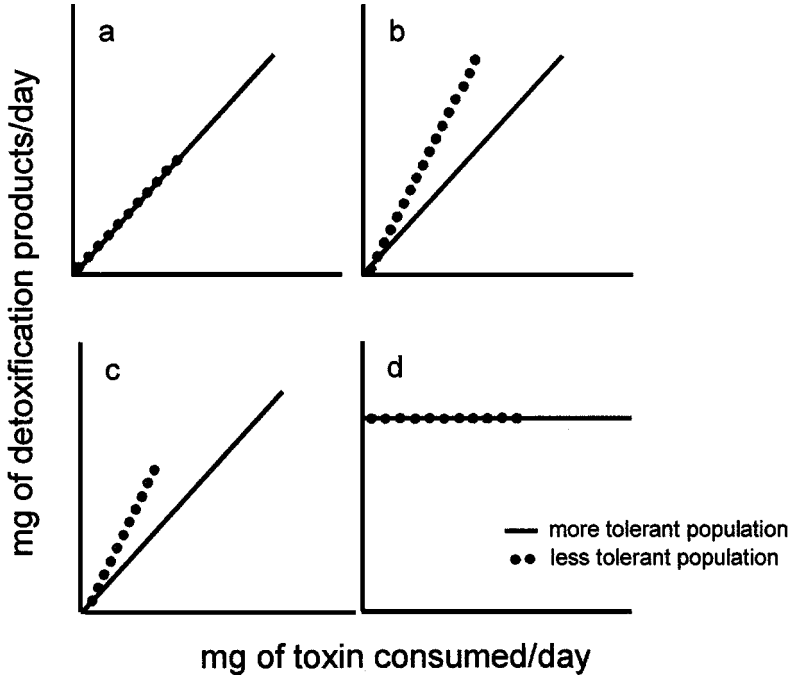


FIG. 1. Possible patterns between detoxification process(es) and toxin intake in two populations with different average maximum toxin tolerance. Plots a–c are based on the hypothesis that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate the toxin. Plot d is based on the assumption that the detoxification process plays no limiting role in determining toxin tolerance. (a) Animals from both populations absorb the same proportion of toxin in food (same regression line), but animals from the more tolerant population can ingest more resin and exhibit higher capacity for the detoxification process. (b) Animals from the more tolerant population absorb a smaller proportion of toxin in food (lower slope), excrete less detoxification products per unit resin ingested, and, therefore, the maximum excretion rate of detoxification products may not be markedly different even though tolerance to resin is different. (c) Animals from the more tolerant population absorb less toxin per unit resin ingested, but also excrete more detoxification products. (d) Detoxification process is unrelated to toxin ingestion in animals from both more and less tolerant populations because the process plays no limiting role in the maintenance of tolerance to the toxin.

for the case of glucuronidation. First, there should be a correlation between glucuronides excreted and resin ingested per day. If animals from both populations absorb the same proportion of toxin in food, then they will fall along the same regression line, animals from the more tolerant population will tolerate more resin (higher maximum tolerable resin intake) and exhibit higher maximum glucuronide

excretion rate (Figure 1a). Alternatively, tolerance to the resin in animals from the more tolerant population occurs because they absorb a smaller proportion of toxin in food, excrete less detoxification products per unit resin ingested, and therefore maximum glucuronide excretion rate may not be markedly different even though tolerance to resin is different (Figure 1b). If differences in both absorption and detoxification are involved, then animals from the more tolerant population not only absorb fewer toxins per unit ingested resin but also excrete more glucuronides (Figure 1c). In either case (Figure 1b and c), in addition to salivary or intestinal binding, a reduction in absorption rate of a toxin could be related to the presence of an intestinal multidrug transporter system that may retard the absorption of the toxin (Epel, 1998). If there were no correlation between resin ingestion rate and reliance on a particular elimination process (Figure 1d), then we would reject the hypothesis that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate toxin(s) by that process.

METHODS AND MATERIALS

Field Site and Sample Collection. Woodrats from the Mojave Desert were trapped at three different sites near Beaver Dam in Grand County, Utah (37°06′N, 113°58′W). Woodrats from the Great Basin were trapped in two locations 70 km apart at Jericho, Juab County, Utah (39°57′N 112°22′W) and Dugway, Tooele County, Utah (40°19′N 112°57′W) (see Mangione et al., 2000 for details on trapping methods). All woodrats were transported to the animal facility at the University of Utah and kept in quarantine for two to five months until they were tested for Sin Nombre hantavirus (Dearing et al., 1998), after which they were transported to the Department of Wildlife Ecology, University of Wisconsin, Madison, Wisconsin. Because detoxification enzymes require very short times for induction, hours to at most three to five days (Sipes and Gandolfi, 1991), and there was no induction of such enzymes, there was no concern for a reduction or alteration of the woodrats' capacity to detoxify creosote bush resin.

Animal Husbandry and Diet Preparation. Woodrats were housed in metal cages (47 × 30 × 21 cm) with screened bottoms. The animals were provided with cotton bedding and a ceramic bowl in which to nest. The room was kept at 21°C and 65% relative humidity. When not involved in experiments, woodrats were fed rabbit chow ad libitum [Harlan Teklad 8630; 17.5% crude protein, 22.0% acid detergent fiber (ADF), 2.5% fat] and 7.5% water. A slice of apple was given to the animals once a week.

Creosote bush collection and resin extraction were performed as indicated in Mangione et al. (2000) and resin was stored at 25°C for up to seven months. Resin-treated diets were prepared as follows: a known amount of resin (depending on the desired concentration of resin in the diet) was dissolved in a volume of

ethanol (95%) equal to 25% of the mass of the ground rabbit chow used in the treatment. Control diet was prepared in the same manner without addition of resin. The resin–ethanol solution and chow were thoroughly mixed, dried overnight at room temperature, and pelleted. To prevent alteration of the properties of the resin diet or palatability by the effects of heat and water (Price et al., 1980; Lindroth et al., 1984; Dietz et al., 1994), the pellet machine was refrigerated using a plastic bag filled with crushed ice and the amount of water added to make the pellets was reduced by using a 60% ethanol solution. The final pellets were dried at room temperature and stored in the freezer.

Feeding Trials. Two feeding trials were performed in this study. All experimental protocols were approved by the Research Animal Resources Center (RARC), University of Wisconsin-Madison.

Experiment 1. Nine woodrats from the Mojave Desert, six males and three females, and 8 woodrats from the Great Basin, three males and five females (five from Dugway, Tooele County, Utah and three from Jericho, Juab County, Utah), were exposed to increasing levels of resin following the procedure described by Mangione et al. (2000). At the beginning of the experiment, woodrats were offered rabbit chow with 0% resin and then switched sequentially to 1, 2, 3, 5, 7, and 9% resin. The animals were exposed to each concentration for six days. This sequence allowed the animals to habituate to the resin and permitted us to evaluate differences between populations at low and high concentrations of resin. Fresh food and water were offered daily. Body mass was measured daily, and animals were removed from the experiment if they lost 15% of their initial body mass or showed signs of sickness (e.g., swaying, not responding to sound or tactile stimuli, etc.). Water was supplied in bottles, and water intake was measured daily by weighing bottles and correcting for spillage and evaporation. Food intake was calculated daily by subtracting the dried orts (uneaten food) from the amount of food offered. Daily values of resin intake were calculated as the product of the amount of dry matter ingested per day multiplied by the proportion of resin in the diet at that particular level.

Urine was collected during days 2, 4, and six of each six-day treatment. On these days, woodrats were restricted to a portion of their cage ($16 \times 19 \times 20$ cm) with a funnel that allowed the collection of uncontaminated urine. Urine drained into an iced plastic vial to minimize bacterial growth and evaporation. The temperature of the vial remained between 0 and 5°C during a 24-hr period. Pilot experiments showed that there were no differences in food intake when woodrats were in either section of the cage (data not shown).

Experiment 2. Because some woodrats reduced their feeding rate and lost mass when fed diets with resin (Mangione et al., 2000), we performed a second, pair-fed control experiment to test whether variation in the excretion of glucuronides in urine of desert woodrats was due to variation in resin intake only and not the

reduction in food intake and mass loss. Four Mojave and six Great Basin woodrats were fed resin-free food daily (pair-fed control woodrats) at the same rate they were eating in experiment 1. Urine was collected for measurement of detoxification products.

Determination of Detoxification Products. Urine samples from the sixth day of each treatment (or from the last day a urine sample was taken if the animals had to be taken out of the experiment) were collected and used for analyses of conjugates. Urine samples were frozen at −25°C until they were used. Glucuronides were determined following the colorimetric assay described by Jakubas et al. (1993) and Blumenkrantz and Asboe-Hensen (1973). Briefly, urine samples were analyzed in duplicate. A urine sample (0.5 ml) was pipetted into a culture tube placed in an iced water bath along with 3 ml of sodium tetraborate–sulfuric acid solution (Blumenkrantz and Asboe-Hensen, 1973). The mix was shaken by Vortex and returned to the iced water bath. Tubes were then heated in a water bath at 100°C for 10 min. After cooling, 50 μl of the reagent *m*-hydroxydiphenyl (Sigma, St. Louis, Missouri) was added to one set of samples (Blumenkrantz and Asboe-Hensen, 1973). A urine sample (blank) was prepared, but the reagent was replaced by 50 μl of 0.5% NaOH. A standard curve was made with known concentrations of glucuronic acid (Sigma) between 25 and 250 μmol. The absorbance was measured at 520 nm in a Beckman DU-64 spectrophotometer. All samples were diluted between 200- and 1000-fold, depending on the treatment.

Hippuric acid concentration was determined by high-pressure liquid chromatography (HPLC) (Chen et al., 1996) on a System Gold Nouveau (Beckman), equipped with a controller (Detector Module 168) and a pump (126 Solvent Module). The analytical column used was an Ultrasphere C_{18} (5 μm) 250 × 4.6 mm ID (Beckman). Two solvents—ethanol (15%) in 20 mmol acetic acid (A) and methanol (B)—were used (see Chen et al., 1996). The total run time was 29.5 min. The gradient used was (%B): 0% at 0 min, 50% at 7 min, and 100% at 14.5 min. Between 14.5 and 19.5 min, B was maintained at 100% and then changed back to 0% at 24.5 min. A 20-μl injection loop was filled with 50 μl to ensure the delivery of a constant volume on every injection. Flow rate was 0.8 ml/min, and the separation was performed at room temperature. The HPLC settings, elution time, and solvent gradient were adapted from Chen et al. (1996) to ensure a good separation of hippuric acid in our samples. Urine samples were diluted with a 1 M sodium acetate buffer (pH 5.0 adjusted with glacial acetic acid) and filtered through 0.2-μm Acrodisc filters (HPLC certified; Fisher Scientific, Chicago, Illinois). The dilution factor varied with the level of resin in the diet. A standard curve was made with known concentrations of hippuric acid (Sigma) ranging from 25 to 250 mg/liter. Two standards were run with every batch of samples. To check for possible carryover during the runs, at least two blanks (mobile phase) were run every day, one

following three samples (six runs), and the other at the end of the day. Duplicate samples were monitored at 210 nm.

Sulfate esters were measured with a turbidimetric assay from Jakubas et al. (1993) and Lundquist et al. (1980). Briefly, 1.5 ml of diluted urine (dilution varied between 10 and 90 times the volume of urine sample available) was mixed with 300 μl of an acidic barium chloride solution to precipitate inorganic sulfate. After 5 min, the solution was centrifuged at 3000 rpm for 10 min. The supernatant was decanted and 300 μl of a 5% (mass per volume) solution of sodium carbonate was added to remove excess barium ions. The solution was centrifuged at 3000 rpm for 10 min, and 1.8 ml of the supernatant was pipetted into a culture tube with 675 μl of 10% (v/v) hydrochloric acid. The tube was topped with nitrogen gas, sealed, and heated in an oil bath at 100°C for 30 min. The hydrolysate was cooled to room temperature, and three aliquots (750 μl each) from each animal were pipetted into test tubes. Two of the aliquots were mixed with Ba-PEG-reagent (Lundquist et al., 1980), while the third was mixed with a PEG-8000 solution (150 g PEG 8000/liter of deionized water) and served as a sample blank. After mixing, samples were allowed to sit for 5 min before determining their absorbance at 600 nm. The 1%, 5%, and 7% treatments had to be discarded because of inconsistent sulfate values when different dilutions were made on the same sample. This could be an indicator that other factors may be influencing sulfate precipitation or hydrolysis. The molar proportion of total conjugates represented by glucuronides, sulfate, and hippuric acid was calculated for 0%, 2%, and 3% resin treatments, because we measured all three conjugates. Molecular weights used were 194.1 for glucuronides (glucuronic acid), 179.2 for hippuric acid, and 96 for sulfate conjugates.

An aliquot of the urine was used to measured pH (Acumet pH meter, model 15). All measurements were taken at 24°C with a pH pencil probe. Two Mojave and one Great Basin woodrat had to be excluded from pH analysis due to the low volume of urine collected.

Statistical Analysis. Relationships between detoxification product excretion rate and either resin intake rate (experiment 1, as in Figure 2) or feeding rate (experiment 2) were compared among the populations by analysis of covariance (ANCOVA, factor = population; covariate = resin or food intake). Residuals were normally distributed when values of excretion rate (milligrams per day) of glucuronides, hippuric acid and sulfates were natural log transformed; this transformation was not necessary in the case of urine pH. Interactions between covariate and factor (i.e., differences in slope) were not significant ($P > 0.05$) and are not reported.

Although regressions describe the relation between resin intake and conjugate excretion, each woodrat is considered three or more times [equal to the number of treatments (resin level)], and so the assumption of independency of data

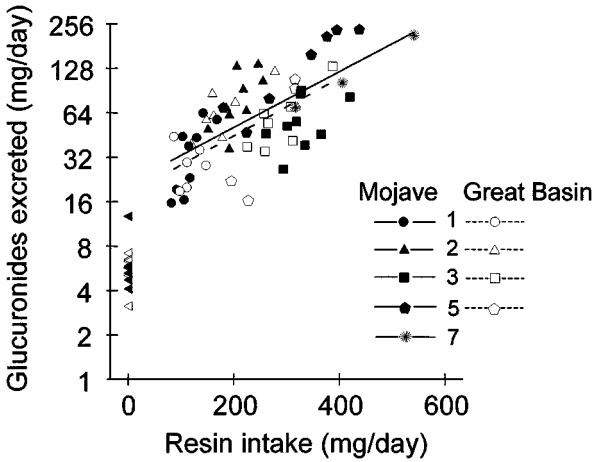


FIG. 2. Excretion of glucuronides as a function of resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed.

points is violated. Therefore, we followed each ANCOVA with a repeated measures one-factor analysis of variance (ANOVA; factor = population, repeated measure = treatment resin level). This test was more conservative because it avoided pseudoreplication and inflation of the degrees of freedom. Treatment resin levels were compared by post-hoc contrasts (Wilkinson, 1992).

Statistical operations were performed according to Wilkinson (1992). In all cases, values are expressed as mean $\pm$ 1 standard error (SE) (N = number of woodrats). Comparisons of means between populations were made by using the t test. For all statistical comparisons, a value of $P < 0.05$ was considered significant, and $0.05 < P < 0.1$ was taken to indicate a trend.

RESULTS

Experiment 1: Effect of Resin Intake on Detoxification Pathways. Woodrats from both populations maintained a relatively constant food intake and body mass at a dietary level of 0% and 1% resin. Some Great Basin woodrats began losing body mass on 2% resin (see Mangione et al., 2000 for detailed mass records). Woodrats were removed from the experiment when eating diets with resin concentrations ranging from 3% to 9% (Table 1). Sixty percent of woodrats were removed from

TABLE 1. STATUS OF WOODRATS WHEN REMOVED FROM EXPERIMENT 1

Woodrat[a]	Treatment[b]	Body mass lost (% from initial)	Observations[c]
60 M	7	−5.1	died[d]
53 M	3	−5.5	non responsive[e]
59 M	9	−5.7	non responsive
45 M	7	−10.6	non responsive
48 M	7	−12.1	swaying[f]
61 M	7	−12.4	swaying
89 G	3	−13.7	moved slowly[g]
118 G	3	−15.0	non responsive
85 G	5	−15.0	non responsive
87 G	3	−15.6	moved slowly
34 G	5	−15.8	moved slowly
88 G	5	−16.8	moved slowly
49 M	7	−17.1	died
51 M	3	−17.4	moved slowly
33 G	5	−17.5	swaying
42 M	5	−20.1	moved slowly
94 G	5	−20.7	moved slowly

[a] M, Mojave woodrat; G, Great Basin woodrats.
[b] Diet resin level (percent) at which woodrats were removed from the experiment.
[c] Status of each woodrat was found at the time it was removed from the experiment.
[d] Woodrat found dead in the cage.
[e] Woodrat did not respond to tactile or sound stimuli and did not move.
[f] Woodrat would sway when touched or when trying to move.
[g] Woodrat moved considerably slower than when it was in treatment 0% (control).

the experiment because they lost 15% or more of body mass, whereas 40% were removed because they exhibited signs of illness before they lost 15% of their body mass (Table 1). Some woodrats that lost only 5% of body mass were removed because they became nonresponsive to tactile or sound stimuli, a sign of possible postabsorptive toxicity.

The excretion of glucuronides was positively correlated with resin intake whether controls (i.e., zero resin intake) were excluded (ANCOVA $F_{1,59} = 50.22$, $P < 0.001$) or included ($F_{1,72} = 169$, $P < 0.001$) (Figure 2). Individual woodrats from both populations exhibited similar patterns of excretion in relation to intake because there was no significant difference by population in either elevation ($F_{1,59} = 0.98$, $P = 0.32$) or slope. The influence of resin ingestion on glucuronide excretion was also apparent in the repeated-measures ANOVA

($F_{3,36} = 43.7$, $P < 0.001$) where population as a factor had no effect ($F_{1,12,} = 1.4$, $P = 0.26$).

Excretion of hippuric acid was independent of resin intake whether controls were excluded (ANCOVA, $F_{1,59} = 0.44$, $P = 0.50$) or included (ANCOVA, $F_{1,72} = 0.6$, $P = 0.44$), but was nearly three times higher in Great Basin woodrats ($F_{1,59} = 18.05$, $P < 0.001$) (Figure 3).

Sulfate excretion rate was independent of resin intake over the range measured ($F_{1,20} = 2.75$, $P = 0.11$), and there was no difference between populations ($F_{1,20} = 0.48$, $P = 0.49$) (Figure 4). However, sulfate excretion increased with resin intake when controls (zero resin intake) were included (ANCOVA $F_{1,40} = 32.6$, $P < 0.001$) (Figure 4). The repeated measure analysis on the 0%, 2%, and 3% treatments suggested that this increment reflects a resin ingestion-dependent sulfate excretion up to a plateau value. The repeated measures analysis confirmed that there was a diet-dependent increase in sulfate excretion rate ($F_{2,20} = 18.2$, $P < 0.001$) with no difference by population ($F_{1,10} = 0.016$, $P = 0.9$). The significant diet effect was between controls and the 2% resin treatment ($F_{2,10} = 7.5$, $P = 0.01$), but not between the 2% and 3% resin treatments ($F_{2,10} = 0.75$, $P = 0.5$).

Urine pH declined with resin intake rate whether controls (zero resin intake) were excluded ($F_{1,63} = 15.9$, $P < 0.001$) or included ($F_{1,78} = 14.1$, $P < 0.001$)

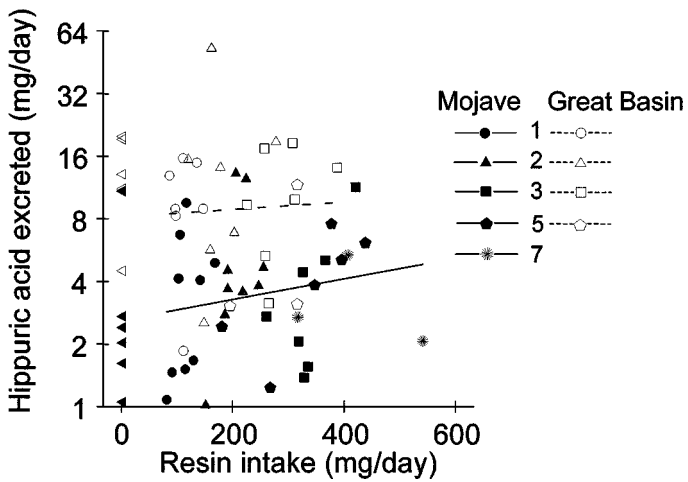


FIG. 3. Excretion of hippuric acid as a function of resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed.

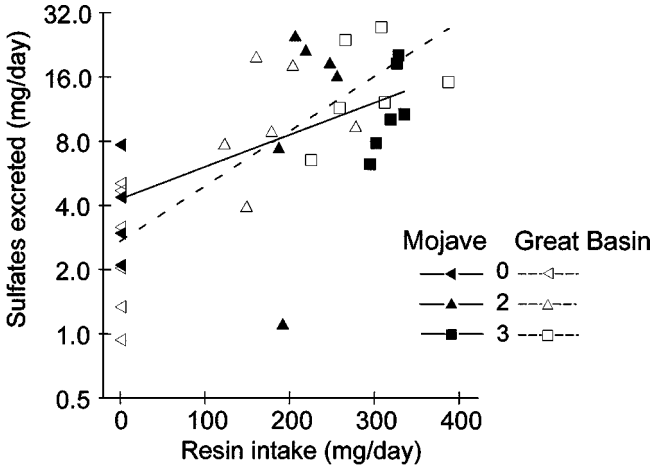


FIG. 4. Excretion of sulfates as a function of resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed. The very lowest value at around 200 mg of resin per day (filled triangle) was identified as an outlier, but the ANCOVA is not substantially different whether it is included or excluded. The datum was included in the final ANCOVA.

(Figure 5). Individual woodrats from both populations apparently exhibited the same urine pH pattern in relation to resin intake because there was no difference by population in either elevation ($F_{1,63} = 1.8$, $P = 0.19$) or slope. The influence of resin ingestion on urine pH was also apparent in the repeated-measures ANOVA ($F_{3,36} = 13.6$, $P < 0.001$), where population was not a significant factor ($F_{1,12} = 3.53$, $P = 0.085$).

Experiment 2: Effect of Food Intake on Detoxification Pathways. Woodrats fed diets with increasing resin concentration decreased their feeding rates by as much as 50% (Mangione et al., 2000). We tested whether the variation in detoxification pathways and urine pH with resin intake (e.g., Figures 2, 4, and 5) might be explained by decreases in feeding rate. Were this the case, glucuronide excretion would increase with decreasing feeding rate on resin-free diet, whereas the opposite pattern was found in woodrats from both populations (Figure 6, top), i.e., increasing glucuronide excretion with increasing feeding rate ($F_{1,34} = 16.5$, $P < 0.001$). Analogously, were this the case for urine pH, then it would decline with decreasing feeding on resin-free diet, whereas it did not change with intake rate in either population ($F_{1,34} = 0.05$, $P = 0.83$) (Figure 6, bottom). Overall, as feeding rate changed, the extent of variation in detoxification or urine pH was nil

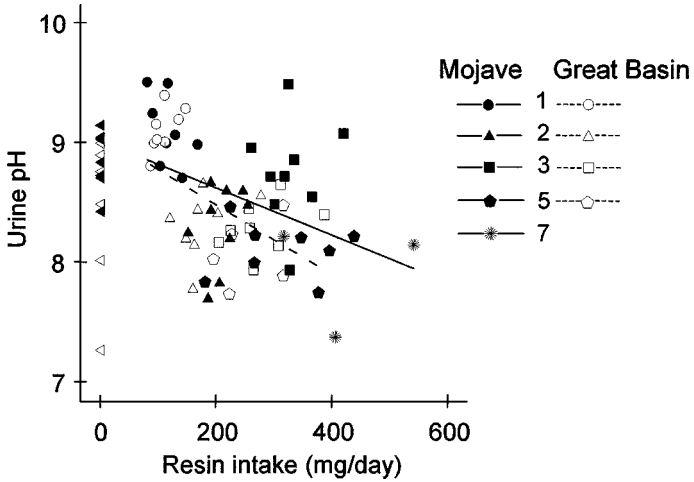


FIG. 5. Relationship between urine pH and resin intake. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats. Each point represents one woodrat exposed to one resin treatment. The numbers between the symbols in the legend represent the treatments (i.e., percentage of resin in the diet) to which the woodrats were exposed.

or in the wrong direction and was very small compared to the much larger changes in woodrats consuming increasing amounts of resin.

DISCUSSION

Detoxification Routes Used by Woodrats. Variation in detoxification can be due to qualitative and quantitative differences (Hansen and Shane, 1994). Qualitative differences correspond to the use of different metabolic routes and the activation or deactivation of metabolic pathways. Quantitative differences are due to variations in enzyme levels. Without simultaneous study of several possible detoxification pathways and the determination of their relative capacities, little progress can be made in understanding the role of detoxification in setting the limits of toxin ingestion (MacCoubrey et al., 1997).

In this study, we considerably advanced knowledge in these regards for one natural herbivore–PSM system: desert woodrats consuming resin from creosote bush. There were no major qualitative differences in detoxification pathways among individuals or populations; all three phase II detoxification routes (glucuronidation, sulfation, and amination) were used to some degree by both woodrat populations exposed to the resin. On resin-free diet, the molar excretion

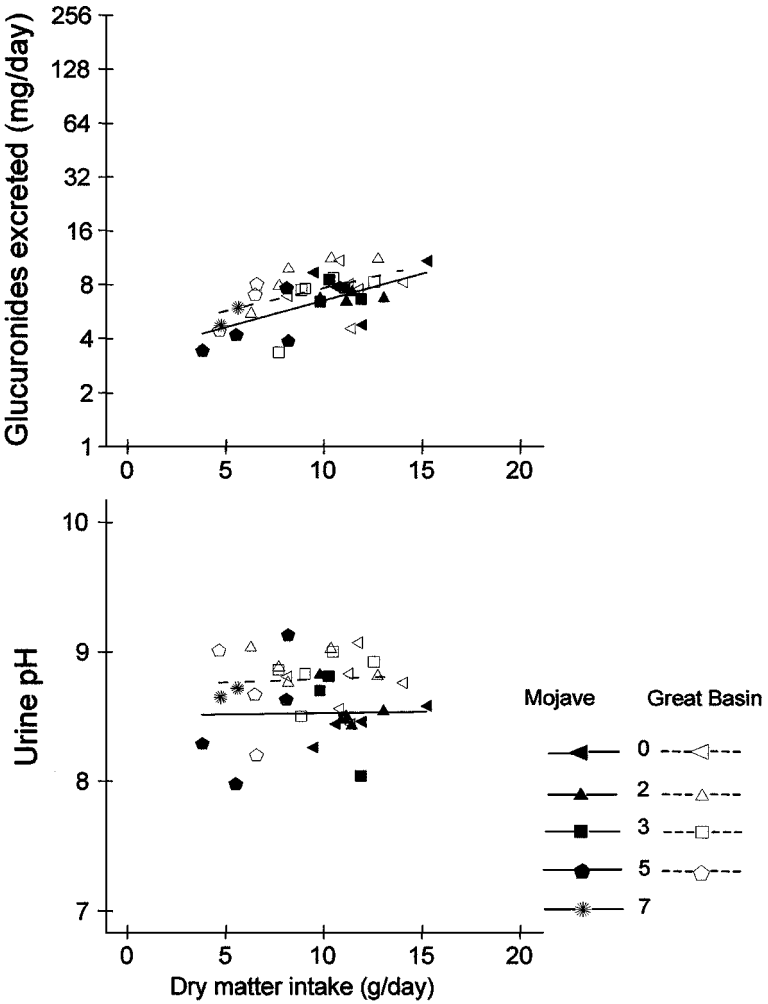


FIG. 6. Variation of glucuronides excreted (top) and urine pH (bottom) as a function of dry matter intake in pair-fed control woodrats (see Methods and Materials). The solid line represents the least-squares regression for Mojave woodrats, the dashed line is the least-squares regression for Great Basin woodrats. Each point represents one woodrat restricted to the same feeding rate it had during experiment 1 at a particular resin treatment. The numbers between the symbols in the legend represent the corresponding treatments (i.e., percentage of resin in the diet), although in this experiment there was no resin in the diets. The *y* axes of the figures match those in Figures 2 and 5 to permit comparison of these excretion rates in woodrats on resin-free diet with those of woodrats eating resin-containing diets.

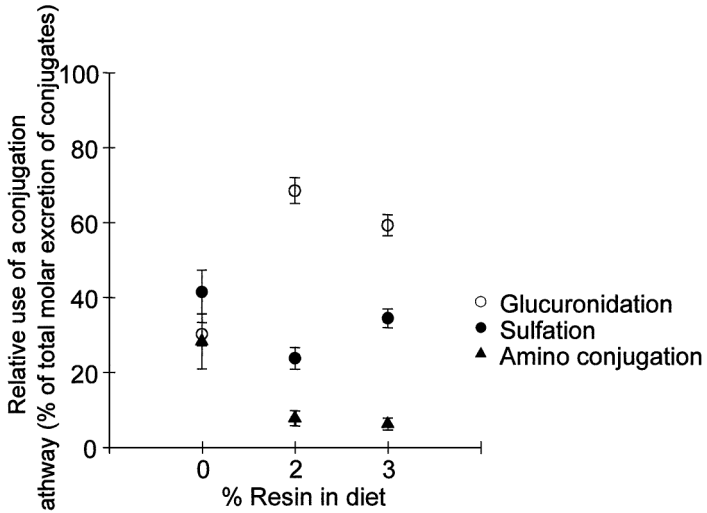


FIG. 7. Variation in the relative use of detoxification pathways versus percentage of resin in the diet. Percentages on the *y* axes were calculated as the ratio of the molar excretion rate of each conjugate to the sum of the molar excretion rates of the three conjugates times 100. Each value is the average of a pool of 12 individuals from both populations.

rates of the three conjugates (glucuronides, glycine, sulfate) were similar (Figure 7). Because glucuronides were the only conjugates that showed consistent increases in excretion with increasing resin intake (c.f. Figures 2–4), glucuronidation became proportionally more important than other conjugation pathways following resin ingestion, coming to represent about 75% of total moles of conjugates excreted (Figure 7). However, we do not know whether this is because of higher levels of specific constitutive enzymes or induction of the specific enzymes involved. Alternatively, reliance on glucuronidation may differ for woodrats reared on other diets or physiological conditions (Price and Jollow, 1988, 1989; Nanbo, 1993), or for woodrats possessing different gut microbial communities (Phipps et al., 1998).

Does Detoxification Capacity Set the Limit on Resin Intake Rate? We found no qualitative difference among woodrats to explain their differential tolerance to creosote bush resin. We also, can reject a number of hypothetical quantitative explanations.

We reject the hypothesis that the amount of resin that indiviuals can ingest is related to their capacity to detoxify and eliminate toxin(s), because hippuric acid excretion rates were independent of resin ingestion rates (Figure 3). Although the level of hippuric acid in urine of Great Basin woodrats was significantly higher than that in Mojave woodrats, this does not explain the latter's greater tolerance to resin.

Hippuric acid is a rather specific conjugate of benzoic acid. If benzoic acid is not a metabolic product of a toxic compound(s), then there would be little relationship between tolerance and hippuric acid excretion. It would be interesting to measure excretion of other benzoic acid metabolites, e.g., flavanoids with different patterns of hydroxylation such as *m*-hydroxybenzoic acid or *m*-hydroxphenyl propionic acid (Harborne, 1991). In addition, there might be other phase II conjugations that involve amino acids other than glycine, e.g., taurine (Caldwell, 1986). A good approach to test this might be to assay urine for glycine and taurine (Konishi et al., 1998; Phipps et al., 1998).

We reject the hypothesis that tolerance to resin is related to woodrat sulfation capacity because sulfate excretion increased with resin intake up to a plateau at 200 mg resin/day (Figure 4), while woodrats continued to consume resin at two to three times this level (Mangione et al., 2000) (Figure 2). The apparent saturation of sulfation in woodrats seems consistent with the observation in other mammals that conjugation with sulfate is a high affinity–medium capacity enzymatic system (Sipes and Galndolfi, 1991).

Two physiological features related to detoxification, glucuronide excretion and urine pH, were correlated with resin intake and consistent with our a priori predictions that the amount of resin that individuals can ingest is related to their capacity to detoxify and eliminate the PSMs (Figure 1). These correlations were not artifactually related to variation in feeding rate (Figure 6). While Figures 2 and 5 illustrate how individual woodrats from the two populations respond to variation in resin intake with regard to glucuronide excretion and urine pH, they are not effective for testing whether a particular detoxification mechanism or buffer capacity might be limiting, and, thereby, explain observed differences in resin tolerance among individuals or between the two woodrat populations. To test this, we regressed each individual's maximum value for glucuronide elimination or minimum value for urine pH against its maximum tolerable resin intake rate—the latter values were taken from Mangione et al. (2000), although they correspond closely to maxima from Figures 2 and 5.

The maximum excretion of glucuronides was positively correlated with maximum resin intake (ANCOVA $F_{1,13} = 8.57$, $P < 0.012$) (Figure 8, top). Individual woodrats from both populations exhibited the same pattern of maximum glucuronide excretion in relation to maximum resin intake because there was no difference by population in either elevation ($F_{1,13} = 0.63$, $P = 0.44$) or slope. This corresponds with the prediction in Figure 1a. Furthermore, the mean maximum glucuronide excretion rate of Mojave desert woodrats, 143.8 ± 23.3 mg/day ($N = 9$), exceeds that of the Great Basin desert woodrats, 67.0 ± 10.8 mg/day ($N = 8$) ($t_{15} = -2.86$, $P < 0.012$). Therefore, in accord with Figure 1a, we cannot reject the hypothesis that animals from both populations absorb the same proportion of toxin in food and that woodrats within and between populations that

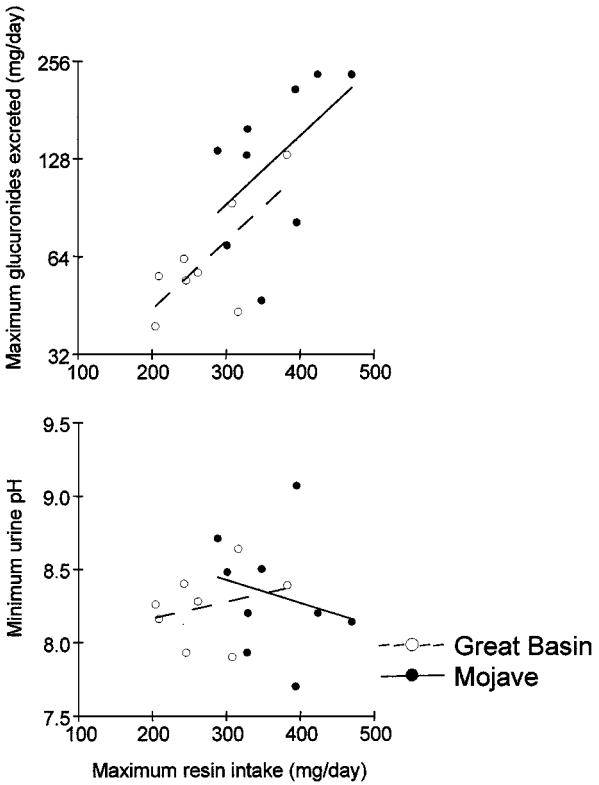


FIG. 8. Maximum glucuronides excreted (top) and minimum urine pH (bottom) as a function of resin intake. Each point represents an individual woodrat's maximum resin intake rate and corresponding maximum glucuronide excretion rate or minimum pH. The solid line represents the least-squares regression for Mojave woodrats, the dashed line represents the least-squares regression for Great Basin woodrats.

tolerate more resin do so because they have a greater capacity for glucuronide excretion.

Instead urine pH can be considered an indicator of an animal's acid–base status and its capacity to detoxify and eliminate metabolites. These metabolites are strong acids that may challenge the animal's acid–base homeostasis (McLean et al., 1993; Foley et al., 1995). However, the tolerance for resin ingestion does not relate as well to urine pH as it does to glucuronide excretion. First, the lowest urine pH of each individual was not correlated with the individual's maximum tolerable resin intake rate (ANCOVA $F_{1,14} = 0.04$, $P = 0.84$) (Figure 8, bottom). Second, the reduction in urine pH to 8 observed in desert woodrats eating creosote resin

seems modest compared to reductions to pH 7.4 that have been observed in other woodrat species exposed to other PSMs (Dearing et al., 2000). Although urine pH was decreased as woodrats ingested greater amounts of resin (Figure 5), we reject the idea that they had to cease eating resin because they reached their capacity to buffer blood from organic acids or to excrete H^+ ions. Moreover, the small R^2 (0.22, Figure 5) suggests that some other factor might be involved in reducing of uirne pH, such as diuresis (García Matilla et al., 1999).

In conclusion, we studied the relationship between three detoxification mechanisms and urine pH and the tolerance of desert woodrats from two populations to a mixture of naturally occurring PSMs (mostly phenolics) in resin from creosote bush. The excretion rate of two phase II detoxification conjugates, glucuronides and sulfides, increased with increasing resin intake, while hippuric acid excretion was independent of resin intake. The molar proportion of glucuronides in urine was three times that of the other conjugates combined. Urine pH, a possible indicator of an animal's capacity to detoxify and eliminate metabolites, declined with increasing resin ingestion. Based on an evaluation of variation in the three detoxification pathways and urine pH in relation to resin intake, we rejected the hypotheses that tolerance to resin intake is related to woodrat capacity for amination, sulfation, or pH regulation. However, we could not reject the hypothesis that woodrats within and between populations that tolerate more resin do so because they have a greater capacity for glucuronide excretion. This hypothesis might be tested by feeding woodrats other compounds detoxified by this pathway, e.g., benzoic acid, and testing their ability to further increase glucuronide excretion rate. Another way to test whether glucuronidation can set the limits for tolerance to a PSM might be by using specific inhibitors of glucuronyl transferase.

Interactions between herbivores and the PSMs they ingest are complex. There may be other explanations for some of the patterns that we have observed. For example, we did not evaluate how differences in gut microbial community structure and function might influence detoxification, or whether different woodrats eating the same amount of resin excrete similar amounts of a conjugate (e.g., sulfates) even when they might actually differ in their metabolism of specific phenols in the resin. Other possible mechanisms that might explain differences in tolerance, such as differential absorption of PSMs, differences in other phase I and II detoxification pathways, differences in target site sensitivity, and differences in the capacity to habituate to aversive sensory properties of PSMs, remain to be studied.

Acknowledgments—We would like to thank two anonymous reviewers for the useful comments on this manuscript. We are grateful to Mark Larson, University of Wisconsin, Department of Ophthalmology, for letting us use the feed pelleting facilities and to Bruce Darken for his technical assistance during this study. Thanks to Perry Chang for introducing A.M.M to the HPLC world. Anna Weber assisted with care of the woodrats and the experiments. Funding was provided by a University of Wisconsin Hatch grant (142-3779) to WHK, and the Max McGraw Wildlife Fund.

REFERENCES

BELOVSKY, G. E. and SCHMITZ, O. J. 1991. Mammalian herbivore optimal foraging and the role of plant defenses, pp. 1–28, *in* R. T. Palo and C. T. Robbins (eds.). Plant Defenses Against Mammalian Herbivory. CRC Press, Boca Raton, Florida.

BLUMENKRANTZ, N. and ASBOE-HENSEN, G. 1973. New method for quantitaive determination of uronic acids. *Anal. Biochem.* 54:484–489.

BRATTSTEN, L. B. 1992. Metabolic defenses against allelochemicals, pp. 176–232 *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores. Their Interactions with Secondary Plant Metabolites. Vol. II Ecological and Evolutionary Processes, 2nd edn. Hercourt Brace Joanovich Publishers, Academic Press, San Diego, California.

CALDWELL, J. 1986. Conjugation mechanisms of xenobiotic metabolism: Mammalian aspects, pp. 3–28, *in* G. D. Paulson, J. Caldwell, D. H. Hutson, and J. J. Menn (eds.). Xenobiotic Conjugation Chemistry. American Chemical Society, Washington, D.C.

CHEN, X. B., PAGELLA, J. H., BAKKER, M. L., and PARRA, O. 1996. Determination of aromatic metabolites in ruminant urine by high-performance liquid chromatography. *J. Chromatogr. B: Biomed. Sci. Appl.* 682:210–208.

DEARING, D. M., MANGIONE, A. M., KARASOV, W. H., MORZUNOV, S., OTTESON, E., and ST. JEOR, S. 1998. Prevalence of hantavirus in four *Neotoma* species in the southwestern United States. *J. Mammal.* 79:1254–1259.

DEARING, D. M., MANGIONE, A. M., and KARASOV, W. H. 2000. Diet breadth of mammalian herbivores: Nutrient versus detoxification constraints. *Oecologia* 123:397–405.

DIETZ, A. B., HAGERMAN, A. E., and BARRETT, G. W. 1994. Role of condensed tannin on salivary tannin-binding proteins, bioenergetics, and nitrogen digestibility in *Microtus pensylvanicus*. *J. Mammal.* 75:880–889.

EPEL, D. 1998. Use of multidrug transporters as first lines of defense against toxins in aquatic organisms. *Comp. Biochem. Physiol.* 120:23–28.

FOLEY, W. J., MCLEAN, S., and CORK, S. J. 1995. Consequences of biotransformation of plant secondary metabolites on acid-base metabolism in mammals: The role of plant secondary compounds. *J. Chem. Ecol.* 21:721–743.

FREELAND, W. J. and JANZEN, D. H. 1974. Strategies in herbivory by mammals: The role of plant secondary compounds. *Am. Nat.* 108:269–289.

GARCÍA MATILLA, F., GARCIA ONTES, F., and RIBAS SERNA, J. 1999. Relationships between diuresis, urine pH and lithogenesis. *Actas Urol. Esp.* 23:202–213.

GOODMAN, T., GRICE, H. C., BECKING, G. C., and SALEM, F. A. 1970. A cystic nephropathy induced by nordihydroguaiaretic acid in the rat. *Lab. Invest.* 23:93–107.

GRICE, H. C., BECKING, G., and GOODMAN, T. 1968. Toxic properties of nordihydroguaiaretic acid. *Food Cosmet. Toxical.* 6:155–161.

GUGLIELMO, C. G., KARASOV, W. H., and JAKUBAS, W. J. 1996. Nutritional cost of a plant secondary metabolite explains selective foraging by ruffed grouse. *Ecology* 77:1103–1115.

HANSEN, L. G. and SHANE, B. S. 1994. Xenobiotic metabolism, pp. 49–105, *in* B. S. Shane and L. G. Cockerham (eds.). Basic Environmental Toxicology. CRC Press, Boca Raton, Florida.

HARBORNE, J. B. 1991. Flavonoid Pigments, pp. 389–426, *in* G. A. Rosenthal and M. R. Berenbaum (ed.). Herbivores. Their Interaction with Secondary Plant Metabolites. Academic Press, San Diego, California.

HARJU-ANU. 1996. Effect of birch (*Betula pendula*) bark and food protein level on root voles (*Microtus oeconomus*): Detoxification capacity. *J. Chem. Ecol.* 22:719–728.

HEWITT, D. G. and KIRKPATRICK, R. L. 1997. Ruffed grouse consumption and detoxification of evergreen leaves. *J. Wildl. Manage.* 61:129–139.

JAKUBAS, W. J., KARASOV, W. H., and GUGLIELMO, C. G. 1993. Ruffed grouse tolerance and biotransformation of the plant secondary metabolite coniferyl benzoate. *Condor* 96:625–640.

KONISHI, T., NISHIKAWA, H., KITAMURA, S., and TATSUMI, K. 1998. Stereoselective determination of *R,S*-2-[4-(3-methyl-2-thienyl) phenyl] propionic acid and its taurine conjugates in dog urine by high-performance liquid chromatography. *J. Chromatogr. B, Biomed. Sci. Appl.* 709:105–111.

LINDROTH, R. L. 1988. Adaptations of mammalian herbivores to plant chemical defenses, pp. 415–445, *in* K. C. Spencer (ed.). Chemical Mediation of Coevolution. Academic Press, San Diego, California.

LINDROTH, R. L. and BATZLI, G. O. 1983. Detoxification of some naturally occurring phenolics by prairie voles: A rapid assay of glucuronidation metabolism. *Biochem. Syst. Ecol.* 11:405–410.

LINDROTH, R. L., BATZLI, G. O., and AVILDSEN, S. L. 1984. Plant phenolics as chemical defenses: Effects of natural phenolics on survival and growth of prairie voles (*Microtus ochrogaster*). *J. Chem. Ecol.* 10:229–244.

LUNDQUIST, P., MARTENSSON, J., SORBO, B., and OHMAN, S. 1980. Turbidimetry of inorganic sulfate, ester sulfate and total sulfur in urine. *Clin. Chem.* 26:1178–1181.

MABRY, T. J., DIFEO, JR., D. R., SAKAKIBARA, M., BOHNSTEDT, JR., C. F., and SEIGLER, D. 1977. The natural products chemistry of *Larrea*, pp. 115–134, *in* T. J. Mabry, J. H. Hunziker, and D. R. DiFeo (eds.). Creosote Bush: Biology and Chemistry of *Larrea* in New World Deserts. Hutchinson and Ross, Stroudsburg, Pennsylvania.

MACCOUBREY, C., HAGERMAN, A. E., and KIRKPATRICK, R. L. 1997. Effects of tannins on digestion and detoxification activity in Gray Squirrels (*Sciurus carolinensis*). *Physiol. Zool.* 70:270–277.

MANGIONE, A. M., DEARING, M. D., and KARASOV, W. H. 2000. Interpopulation differences in tolerance to creosote bush resin in desert woodrats (*Neotoma lepida*). *Ecology* 81:2067–2076.

MCARTHUR, C. and SANSON, G. D. 1993. Nutrition effects and costs of a tanning in a grazing and a browsing macropodid marsupial herbivore. *Func. Ecol.* 7:690–696.

MCLEAN, S., FOLEY, W. J., DAVIES, N.W., BRANDON, S., DUO, L., and BLACKMAN, A. J. 1993. Metabolic fate of dietary terpenes from *Eucalyptus radiata* in common ringtail possum (*Pseudocheirus peregrinus*). *J. Chem. Ecol.* 19:1625–1643.

NANBO, T. 1993. Influence of dietary lipids on glucuronidation and sulfation of phenol and its para substituent in the rat. *Biol. Pharm. Bull.* 16:518–520.

PARKE, D. V. 1968. The Biochemistry of Foreign Compunds. Pergamon Press, New York.

PHIPPS, A. N., STEWART, J., WRIGHT, B., and WILSON, I. D. 1998. Effect of diet on the urinary excretion of hippuric acid and other dietary-derived aromatics in rat. A complex interaction between diet, gut microflora and substrate specificity. *Xenobiotica* 28:527–537.

PRICE, M. L., HAGERMAN, A. E., and BUTLER, L. C. 1980. Tannin in sorghum grain: Effect of cooking on chemical assays and on antinutritional properties in rats. *Nutr. Rep. Int.* 21:761–767.

PRICE, V. F. and JOLLOW, D. J. 1988. Mechanism of decreased acetaminophen glucuronidation in the fasted rat. *Biochem. Pharmacol.* 37:1067–1076.

PRICE, V. F. and JOLLOW, D. J. 1989. Effects of sulfur-amino acid deficient diets on acetaminophen metabolism and hepatotoxicity in rats. *Toxicol. Appl. Pharmacol.* 101:356–369.

RHOADES, D. F. and CATES, R. G. 1976. Towards a general theory of plant antiherbivore chemistry. *Recent. Adv. Phytochem.* 10:168–213.

ROBINSON, D., SMITH, J. N., and WILLIAMS, R. T. 1953. Studies in detoxication: 52. The apparent dissociation constants of some glucuronides, mercapturic acids and related compounds. *Biochem. J.* 55:151–155.

SIPES, I. G. and GANDOLFI, A. J. 1991. Biotransformation of toxicants, pp. 88–126, *in* M. O. Amdur, J. Doull, and C. D. Klaassen (eds.). Casarett and Doull's Toxicology. The Basic Science of Poisons. Pergamon Press, New York.

WILKINSON, L. 1992. SYSTAT for Windows: Statistics, Version 5.03. Edition Systat, Evanston, Illinois.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

PLANT PHENOLICS AS DIETARY ANTIOXIDANTS FOR HERBIVOROUS INSECTS: A TEST WITH GENETICALLY MODIFIED TOBACCO

KELLY S. JOHNSON[1,*] and GARY W. FELTON[2]

[1]*Department of Biological Sciences*
Ohio University
Athens, Ohio 45701

[2]*Department of Entomology*
Pennsylvania State University
501 ASI Building
University Park, Pennsylvania 16802

(Received March 26, 2001; accepted August 16, 2001)

Abstract—High foliar phenolics are generally assumed to increase resistance to insect herbivores, but recent studies show that tobacco lines modified to over– and underexpress phenolics do not exhibit higher constitutive resistance to caterpillars. This is contrary to the expectation that ingestion of tobacco phenolics, particularly chlorogenic acid, should cause oxidative stress in herbivores. We investigated free radical production and antioxidant capacity of fresh crushed leaves of tobacco lines exhibiting over a sixfold difference in chlorogenic acid content to test whether high phenolic concentrations are associated with increased production of reactive oxygen species (ROS). The effects of in planta phenolic levels on feeding behavior, growth, biochemical markers of oxidative stress, and the antioxidant capacity of midgut fluid and hemolymph were assessed in tobacco budworm, *Heliothis virescens*. The experiments showed that high phenolic foliage was more prooxidant than low phenolic foliage, but the net balance in crushed tissue was antioxidant in comparison to buffer and the commercial antioxidant standard, Trolox. In *H. virescens*, the antioxidant capacity of midgut fluid was also powerful, and caterpillars fed high phenolic foliage did not exhibit the expected markers of oxidative stress in midgut tissues (altered ascorbate ratios, disulfides, or total hydroperoxides). Instead, hemolymph of larvae fed high phenolic foliage exhibited improved total Trolox equivalent antioxidant capacity (TEAC). These results suggest that the elevated foliar phenolics in some plants may have beneficial antioxidant properties for herbivorous insects, much as dietary phenolics do in mammals.

*To whom correspondence should be addressed. E-mail: johnsok3@ohio.edu

Key Words—*Heliothis*, *Nicotiana*, phenolics, antioxidants, transgenic plants, insect resistance, midgut, genetically modified organism.

INTRODUCTION

Although plant phenolics are believed to play an important role in chemical defense against herbivores, their specific physiological effects on insects are variable and poorly understood (Harborne, 1985; Appel, 1993). They have been variously described as antifeedants (Bernays et al., 1989; Wrubel and Bernays, 1990), digestibility reducers (Feeny, 1976; but see Martin et al., 1987), and toxins (Bernays, 1981; Steinly and Berenbaum, 1985). One mechanism by which their toxicity is thought to arise is via their propensity to produce reactive by-products when oxidized (Felton et al., 1989; Ahmad, 1992; Appel and Schultz, 1992; Appel, 1993; Barbehenn and Martin, 1994). These products include semiquinone radicals and other reactive oxygen species (ROS) such as superoxide anion and hydroxyl radicals, hydrogen peroxide, and singlet oxygen (Hodnick et al., 1989; Ahmad and Pardini, 1990), that are generated during autooxidative processes and/or by the action of plant oxidases during maceration of leaf tissues.

Evidence for the prooxidant model of toxicity comes primarily from artificial diet experiments. When pure phenolics are presented in this manner, many reduce growth and survival of caterpillars (Reese and Beck, 1976; Elliger et al., 1981; Isman and Duffey, 1982a,b, 1983; Lindroth and Peterson, 1988; Felton et al., 1989, 1992; Stamp et al., 1994; Stamp and Yang, 1996; Yang et al., 1996; Ali et al., 1999). In the case of chlorogenic acid (CHA), a widespread *o*-dihydroxy phenolic, reduced insect performance is due at least in part to the antinutritive effects of oxidative by-products on dietary constituents. Aeration and addition of polyphenol oxidases to the diet enhances CHA oxidation and increases the binding of orthoquinones to nucleophilic groups of amino acids and proteins, which reduces their nutritional quality and/or bioavailability (Felton et al., 1989, 1992; Duffey and Felton, 1991; Bi et al., 1994).

It has generally been assumed that similar reactions occur in the insect digestive tract, particularly in caterpillar midguts where the alkaline pH and oxidizing redox potentials would promote the generation of harmful ROS. Such reactions would result in direct oxidative damage to midgut lipids and proteins (Appel and Martin, 1990; Summers and Felton, 1994; Appel, 1994). Summers and Felton (1994) found elevated levels of biochemical markers of oxidative stress (lipid peroxidation products, oxidized protein, and free iron) in midguts of caterpillars fed artificial diets containing chlorogenic and caffeic acid. Addition of the antioxidant ascorbic acid to the diet ameliorated some of these effects, suggesting that their toxicity was due to prooxidant behavior. Barbehenn and Martin (1994) showed that tannic acid is oxidized as it passes through the gut of *Malacosoma*

disstria, but not *Orygia leucostigma*, which is tolerant of high concentrations of this polyphenolic.

Several recent attempts to demonstrate prooxidant effects of phenolics in planta have produced results inconsistent with the prooxidant model and artificial diet experiments. In a series of experiments with transgenic tobacco (*Nicotiana tabacum*) lines modified to differentially express phenylpropanoid products, Bi et al. (1997a), Eichenseer et al. (1998), and Ali et al. (1999) found no negative effects of high foliar phenolics on growth and survival of *Manduca sexta* and *Heliothis zea*. These plants over- and underexpressed phenylalanine ammonia lyase (PAL) through sense suppression by an introduced bean PAL2 transgene (Elkind et al., 1990; Pallas et al., 1996; Howles et al., 1996) and exhibited a 6.8-fold difference in chlorogenic acid content and up to 84% difference in total flavonoid levels (Bi et al. 1997a). Since these modifications to constitutive phenolic levels have been shown to clearly influence resistance to fungal and viral pathogens (Maher et al., 1994; Pallas et al., 1996), the absence of an effect on chewing herbivores was unexpected.

The discrepancy between in planta experiments and artificial diet studies is probably due to the complex chemical interactions among phenolics, plant oxidases, and dietary nutrients and antioxidants that cooccur in fresh foliage (Bi et al., 1997b; Ali et al., 1999). High levels of phenolics in plant foliage may fail to induce prooxidant damage to herbivores if either oxidative reactions and ROS production in macerated plant tissue do not occur to the same extent as observed in artificial diets or if herbivores feeding on foliage are better able to withstand oxidative stress or damage than those on artificial diet. The purpose of the present study was to reassess the prooxidant nature of phenolics in the context of fresh foliage, rather than artificial diet. In order to appropriately interpret their effects on the herbivore, we simultaneously measured behavioral and physiological responses of *H. virescens*. We used transgenic tobacco lines modified to over- and underexpress foliar phenylpropanoids to manipulate in planta levels of phenolics. The effects of phenol content on radical production (hydroperoxides) and total antioxidant (quenching of the $ABTS^{\cdot+}$ radical) capacities of crushed foliage were measured and compared to the antioxidant capacities of *H. virescens* midgut fluid, hemolymph, and oxidative damage to the midgut. Feeding trials to measure effects of phenolics on preference, consumption, digestive efficiency and growth were conducted. Our goal was to assess the degree to which foliar phenolic levels correlate with free radical production and oxidative stress experienced by insect herbivores. A strong correlation would support the prooxidant model of phenolic toxicity and confirm the importance of herbivore traits such as gut physicochemistry and antioxidant defenses for circumventing plant chemical defenses. A weak correlation would suggest that oxidative activation of phenolics may be of less importance in antiherbivore defense.

METHODS AND MATERIALS

Insects and Plants

Heliothis virescens larvae were obtained from a colony maintained at the University of Arkansas and reared on a wheat germ-casein diet or on greenhouse grown transgenic tobacco (*Nicotiana tabacum*).

The generation of PAL modified tobacco lines and their phenotypic characteristics have been described in detail elsewhere (Elkind et al., 1990; Pallas et al., 1996; Howles et al., 1996). The 274-T4 line was developed from cv. Xanthi-nc and exhibits reduced accumulation of PAL transcripts through sense suppression (Elkind et al., 1990). The C-17 control lines have lost the bean PAL2 gene through segregation and are thus operationally wild type. In Ox lines, the gene silencing was lost following selfing, so the resulting offpring exhibit much higher PAL activity and elevated phenylpropanoid products compared to wild types (Howles et al., 1996). Two additional transgenic lines were included as part of a parallel study investigating the effects of salicylic acid (SA) on induced resistance to insects. The NahG-10 line contains the bacterial *Nah*G gene encoding salicylate hydroxylase (Gaffney et al., 1993; Delaney et al., 1994), which removes SA after it is synthesized. These plants have higher constitutive jasmonic acid levels and greater induced resistance to herbivores, apparently because the inhibitory effect of SA on JA has been removed (Felton et al., 1999). Crossing NahG-10 with Ox-434 plants produced the Ox-NahG line, which exhibits elevated phenolics and reduced SA.

Plants were propagated from stem cuttings and maintained in a greenhouse in Redi-Earth Peat-Lite soil mix (Scotts-Sierra Horticultural Products Co., Marysville, Ohio). They were watered daily and fertilized with Osmocote (N-P-K 14:14:20, Scotts-Sierra Horticultural Products Co.) each month. The greenhouse was lighted with high-pressure sodium lights (100 W) on 14 hr scotophase and a day–night temperature of $27{:}19 \pm 2^{\circ}$C. Plants were used before they reached the flowering stage.

Chemicals. Trolox (Hoffman-La Roche) (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], bovine methemoglobin and all other reagents were obtained from Sigma-Aldrich Chemical Co., St. Louis, Missouri.

Oxidizing Properties of Plant Foliage

Total Antioxidant Activity. The total antioxidant activity of aqueous leaf extract was measured relative to the antioxidant standard Trolox (TEAC assay) using a modified ferryl myoglobin/ABTS assay (Miller and Rice-Evans, 1994). This assay has been widely used as a general measure of antioxidant activity of food

extracts, pure compounds in solution, or body fluids, and it is appropriate for both aqueous and lipophilic systems (Rice-Evans et al., 1995, 1996). In brief, the ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation is generated by the activation of metmyoglobin with hydrogen peroxide. Suppression of the ABTS·+ blue-green chromophore by the sample of interest is monitored spectrophotometrically and compared to the scavenging capacity of Trolox, a standard antioxidant, under the same conditions. We used the assay as described by Miller and Rice-Evans (1994) but used bovine methemoglobin in place of metmyoglobin.

Total antioxidant activity of foliage of the six tobacco lines was assayed using the following procedure: Two leaf discs were collected from two leaves on each of 10 individual plants of each line. Discs (9 cm) were punched from the second and third fully expanded leaves below the apical terminal using a cork borer and placed on ice. After fresh weights of the discs were recorded, each was crushed in 1 ml of PBS buffer (0.005 M phosphate, 0.145 M NaCl, pH 7.4) using four strokes of the end of a heavy steel cylinder at room temperature. Then 20 μl aliquots of this aqueous extract were assayed immediately for antioxidant activity. The total time elapsed between the harvesting of the leaf discs and the assay was less than 45 min, and spectrophotometric measures were initiated within 10 min of the time plant tissue was crushed. Preliminary experiments showed that this method produced reproducible values among discs from a single leaf. The 20 μl of leaf extract, buffer, or Trolox standard was combined in a standard 96-well microplate with 110 μl of 0.02% methemoglobin solution (in PBS buffer at room temperature) and 110 μl of ABTS solution (0.8 mg/ml) in buffer. After the initial absorbance at 590 was recorded, the reaction was initiated by the addition of 40 μl of H_2O_2 in dH_2O (0.015%) and the increase in absorbance monitored for 20 min. All reactions were performed in duplicate and a standard curve of Trolox (0.3, 0.43, 0.6, and 3.0 mM) was run with each microplate.

Hydroperoxide Production in Macerated Leaves. To estimate free radical generation in high and low phenolic tobacco foliage, total hydroperoxide (ROOH) formation in macerated foliage of unwounded plants was measured using xylenol orange, which forms a complex with the $Fe_3{}^+$ produced by the hydroperoxide-based oxidation of $Fe_2{}^+$ that can be quantified spectrophotometrically (Jiang et al., 1992). Pairs of leaf discs were punched from fully expanded leaves and immediately placed in test tubes containing either 2 ml ice cold 0.1 M KPO_4 buffer (pH 7.4) or buffer plus 900 units of catalase. Because catalase removes H_2O_2, the difference between the values from the two tubes represents the portion of total hydroperoxides specifically attributable to H_2O_2.

One leaf from the upper portion of each plant was sampled, but samples were pooled such that each test tube contained three leaf discs from three plants and was paired with a second tube (+ catalase) that contained the three corresponding discs from the same plants. The tubes were left at room temperature for 1 hr before leaf

discs were homogenized for 7 sec with an electric tissue grinder. Samples were centrifuged at 10,000*g* for 4 min to settle the cellular debris.

Leaf sample supernatant (20 μl) was combined in a microplate well with 180 μl of a reagent solution containing 100 μM xylenol orange, 250 μM $Fe_2{}^+$, 25 mM H_2SO_4, and 4 mM BHT in 90% methanol (v/v). After 30 min at room temperature, the OD_{560} was recorded and compared to a standard curve prepared with *t*-butyl hydroperoxide. Thus, the measure is a combined measure of constitutive levels of hydroperoxides in leaf tissues as well as those produced by the leaf discs during the hour after damaging.

Data Analysis. A three-way analysis of variance was used to partition variation in antioxidant activity among leaves, individual plants, and the transgenic lines. Tukey's test was used to identify treatments that differed. Variation in hydroperoxides generated in the absence of catalase across tobacco lines was analyzed by one-way ANOVA followed by Tukey's test. Levels of hydroperoxides generated in the presence of catalase did not meet the assumptions for ANOVA so were analyzed separately using a Kruskal-Wallis analysis on ranks and a Student-Neuman Keuls test to separate means.

Insect Feeding Trials

Performance. Standard gravimetric nutritional budgets were calculated for fifth-instar *H. virescens* fed over- (Ox) and underexpressing (T4) tobacco. Larvae were reared on artificial diet until they reached the premolt stage (head capsule slipping and not feeding). They were then weighed, placed individually into plastic tubs containing a preweighed tobacco leaf, and maintained at 28°C on a 16L:8D cycle. Each tub was lined with moistened paper towel, and leaves were replaced every 48 hr or as needed. Only fully expanded leaves from the upper part of the plant were used. Uneaten leaf material and frass were dried and weighed to the nearest 0.1 mg. A wet/dry conversion factor was calculated from 10 randomly selected leaves from each tobacco line from which a 9-mm disc was punched from the tip of each leaf with a cork borer.

After 72 hr, larvae were harvested and hemolymph samples collected (see below), after which the larvae were killed by freezing. It is unlikely that hemolymph removal affected dry mass. Carcasses were oven-dried at 70°C and weighed when they reached constant weight. Growth, consumption, and approximate digestibility (AD = consumption/consumption-egested) were calculated on a dry weight basis.

Feeding Preference. A 12- to 36-hr feeding preference assay with paired choices of leaf discs was performed on the following: Ox vs. C17, Ox vs. T4, and C17 vs. T4 with 16–24 replicates per test. At least 10 plants per treatment were represented in each test. A pair of leaf discs was punched from a newly expanded leaf using a 9-mm-diam. cork borer and arranged alternately with another pair from a different treatment plant in a 10- × 1-cm plastic petri dish. Dishes were lined

with moistened filter paper, and discs were held in place by impaling them on the upright points of four thumbtacks inserted through the bottom of the filter paper. An individual fourth-instar larva was placed in the center of each arena and allowed to feed for 12–36 hr until it had eaten 30–80% of the available food. Uneaten leaf disc material was digitally scanned using a Hewlett Packard flatbed scanner and the area in pixels quantified with SigmaScan Image software (Jandel Corporation, San Rafael, California). Differences in the area of leaf material consumed were assessed using a *t* test.

Oxidative Status of Herbivore Tissues. Three biochemical indicators of oxidative stress were examined in midgut tissue: total and reduced ascorbic acid, hydroperoxide formation, and total disulfides. Larvae were reared on detached leaves until they reached the fourth and fifth instars; then they were transferred to potted plants in the greenhouse for an additional four days before midgut tissues were assayed. Five to 10 larvae were placed in net bags for a total of five to seven plants per treatment. After larvae were harvested, the midguts were dissected, separated from the lumen contents, rinsed with 0.01 M KPO_4 buffer, and pooled in groups of three midguts per replicate. The number of larval replicates per plant varied from 1 to 3. After tissue samples were weighed in pretared tubes, they were homogenized (3 midguts/ml 0.01 M KPO_4 buffer) and immediately assayed as described in Summers and Felton (1994).

For total and reduced ascorbic acid, 300 μl of midgut homogenate was added to a microcentrifuge tube containing 300 μl of 10% metaphosphoric acid [to stabilize the ascorbic acid (AA)]. For total AA, 100 μl of sample was then combined with 125 μl sodium phosphate buffer (0.05 M, pH 8.4, with 3 mM EDTA, and 25 μl of 10 mM dithiothreitol (DTT) to reduce dehydroascorbic acid (DHA) to AA. After 10 min, excess DTT was removed by adding 25 μl of 5.0 mM *N*-ethyl malemide (NAM), and 100 μl of 10% trichloroacetic acid, 100 μl of 44% *o*-phosphoric acid, 100 μl of 4% 2,2′-dipyridyl in 70% ethanol, and 50 μl of 3% $FeCl_3$ were added to the incubation mix. After 40 min at 40°C, tubes were centrifuged and the OD_{520} of the supernatant was measured against a standard curve of total ascorbic acid by using a Cambridge 7520 microplate reader. To determine reduced AA, a parallel incubation was performed, except that 175 μl of sodium phosphate buffer (without EDTA) was used and no DTT or NEM was added. DHA was calculated by subtracting reduced AA from total AA.

Total hydroperoxides (ROOH) were measured using methods modified from Jiang et al. (1992): 20 μl of midgut homogenate was combined in a microplate well with 180 μl of a reagent solution containing 100 μM xylenol orange, 250 μM $Fe_2{}^+$, 25 mM H_2SO_4, and 4 mM BHT in 90% methanol (v/v). After 30 min. at room temperature, the OD_{560} was recorded and compared to a standard curve prepared with *t*-butyl hydroperoxide.

Total disulfides were measured following the method of Anderson and Wetlaufer (1975) as modified in Summers and Felton (1994). In a microplate

well, 50 μl of homogenate was combined with 50 μl 6 N NaOH and incubated for 30 min at room temperature. Then 100 μl of 6 N H_3PO_4 was added, the plate was shaken thoroughly, and 20 μl of 2.5 mM DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid) in 0.05 M sodium acetate was added. After an additional 20 min, the OD_{420} was measured.

The antioxidant activity of midgut fluid was assayed using the ferryl myoglobin–ABTS assay. Intact midguts were rinsed with saline to remove any contaminating oxidases from hemolymph, then slit longitudinally and their contents (including peritrophic envelope) placed in a tared microcentrifuge tube. Preliminary experiments revealed that the midgut content preparations had very strong antioxidant activity, so after weighing to the nearest 0.1 mg, samples were diluted with 10 times their mass in volume (milliliters per milligram) with distilled water. After centrifugation for 2 min at 14,000*g*, 20 μl of the supernatant was used in the ABTS assay. Tubes were kept on ice until the time of the assay, which was performed at room temperature. The assay was replicated with nine larvae fed high phenolic (Ox) plants, nine led low phenolic (T4) plants, and six fed wild-type (C17) plants. Treatment effects on the quantity of ABTS radical formed after 10 min were tested using a one-way ANOVA.

Antioxidant activity of hemolymph from larvae fed high- (Ox) and low-phenolic (T4) plants for 72 hr (used in the nutritional performance experiment) was also measured by using the ABTS radical formation assay. Larvae were chilled and a drop of hemolymph collected from a proleg into a cold ceramic dish. Assays were initiated within 5–10 min because hemolymph tended to coagulate quickly. Undiluted hemolymph (20 μl) was used in the ABTS assay using the same methods described above for foliage and midgut fluid. Samples from nine larvae from the Ox treatment and 10 from the T4 treatment were assayed. A Mann-Whitney rank sum test was used to test for significant differences in the rate of radical formation after 20 min.

RESULTS

Antioxidant Properties of Plant Foliage. Aqueous leaf extracts from all the tobacco lines exhibited antioxidant activity that inhibited the rate of ABTS radical formation relative to the phosphate buffer control (Figure 1) over a 20-min incubation period. Three-way analysis of variance showed significant differences between tobacco lines ($P < 0.001$), individual plants ($P < 0.006$), and leaves ($P < 0.001$). An all pairwise multiple comparison (Tukey's test) showed that the low-phenolic line (T4) had the strongest antioxidant properties, particularly in comparison to the high phenolic lines (Ox and Ox-NahG) and the wild-type control (C17). Although the high phenolic line (Ox) was more proxidant than the wild-type, this difference was not statistically significant. The NahG plants exhibited an intermediate

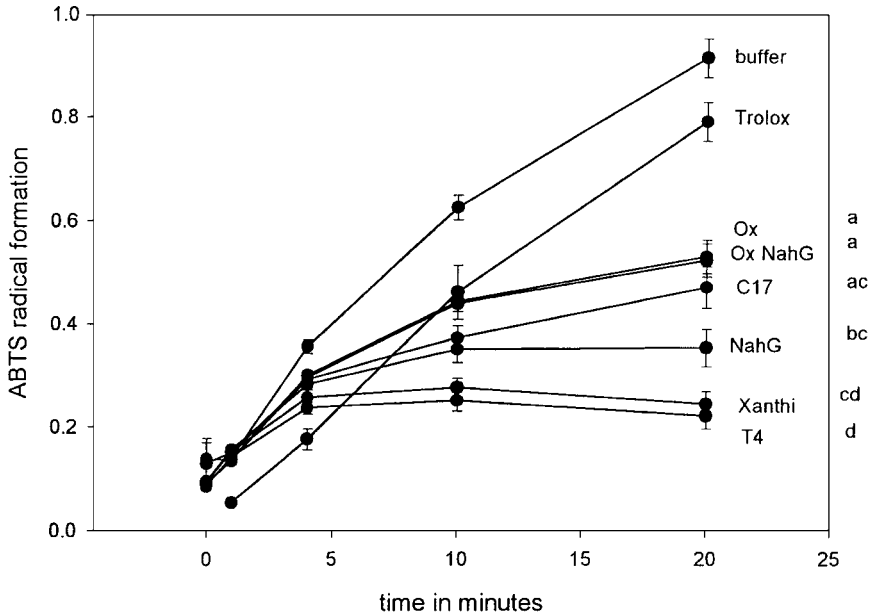


FIG. 1. Antioxidant capacity of macerated tobacco foliage as indicated by suppression of ABTS radical formation in vitro. The effects of saline buffer and 0.4 mM Trolox are included for comparison. The T4 tobacco line is lowest in phenolic content (PAL sense-suppressed), Xanthi, C17, and NahG are intermediate, and Ox and OxNahG are PAL overexpressing lines. Letters indicate significant differences at $P < 0.05$ (ANOVA followed by all pairwise multiple comparison Tukey's test).

antioxidant activity that was not significantly different from the wild-type control (Xanthi).

Total hydroperoxides were also lowest in the T4 underexpressing lines (Figure 2). The addition of catalase to the homogenization mixture did not uniformly decrease hydroperoxide levels in foliar samples, so no further attempt to estimate or analyze H_2O_2 production was made (values obtained from tubes containing catalase were analyzed separately from those without catalase and differed in significance levels, but otherwise produced comparable results). One-way analysis of variance of tubes without catalase (means separated by Tukey's test) showed the T4 underexpressing lines accumulated significantly fewer hydroperoxides than the Ox-NahG and NahG lines. Hydroperoxides appeared to be lower in the Ox and C17 lines but the differences were not significant at $P = 0.05$.

Caterpillar Feeding Trials. Larvae consumed more T4 leaf material than Ox during the trial (*t* test, $P < 0.001$), but did not differ in mass gained/day (*t* test,

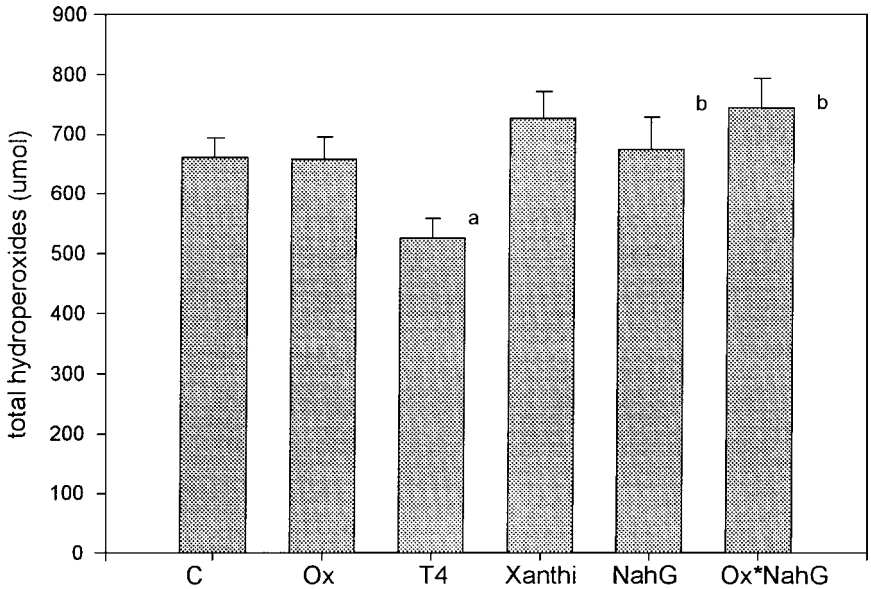


FIG. 2. Total hydroperoxides produced in macerated leaf tissues. The T4 tobacco line is lowest in phenolic content (PAL sense-suppressed), Xanthi, C17, and NahG are intermediate, and Ox and OxNahG are PAL overexpressing lines. Letters indicate significant differences at $P < 0.05$ (all pairwise multiple comparison (Tukey's test).

$P = 0.093$) and final mass of T4 larvae was only slightly higher (t test, $P = 0.064$) (Table 1). The underexpressing leaves were more digestible than overexpressing ($P = 0.003$). The dry masses of Ox and T4 leaf discs were not significantly different (3.59 ± 0.69 for Ox and 3.32 ± 0.89 for T4), suggesting that the ratio of water to lignin did not differ substantially.

In the choice trials, larvae also preferred T4 leaf discs over wild-type controls (C17) ($P < 0.001$, $N = 22$) and overexpressing (Ox) lines ($P < 0.00$, $N = 19$), indicating that the increased consumption of T4 foliage is due in part to

TABLE 1. FEEDING AND GROWTH PERFORMANCE OF FIFTH-INSTAR *H. virescens* FED PAL OVEREXPRESSING (OX) AND UNDEREXPRESSING (T4) TOBACCO FOR 72 HOURS

	Consumption (mg)	Final mass (mg)	Mass gained per day (mg)	Digestibility AD	*N*
Ox (overexpressed)	154.9 ± 20.0	47.5 ± 5.1	10.5 ± 1.7	0.49 ± 0.05	12
T4 (underexpressed	283.8 ± 22.9	58.6 ± 2.9	14.0 ± 1.0	0.68 ± 0.03	15

t test; $P < 0.001$; $P = 0.064$; $P = 0.093$; $P = 0.003$.

phagostimulatory effects. Interestingly, larvae did not discriminate between the wild-type control and overexpressed (Ox) lines ($P = 0.313$, $N = 23$).

Oxidative Status of Herbivore Tissues. Midguts of caterpillars fed different tobacco lines did not differ in total midgut ascorbic acid ($P = 0.235$), reduced ascorbic acid ($P = 0.104$), dehydroascorbic acid ($P = 0.578$), hydroperoxides ($P = 0.368$), or disulfides ($P = 0.491$) (Table 2). Undiluted midgut fluid exerted strong antioxidant properties, completely preventing formation of the ABTS radical (as strongly as Trolox) for over 40 min. There was no significant difference in antioxidant activity of diluted midgut fluid (1:10 dilution) of caterpillars fed wild-type, and over- and underexpressing tobacco (Figure 3). However, caterpillars fed high phenolic tobacco (Ox) for 72 hr had stronger hemolymph antioxidant activity than those fed the underexpressing plants (T4) ($P = 0.005$, Mann-Whitney Rank sum test, $N = 9$, Ox and $N = 10$, T4). (Figure 4).

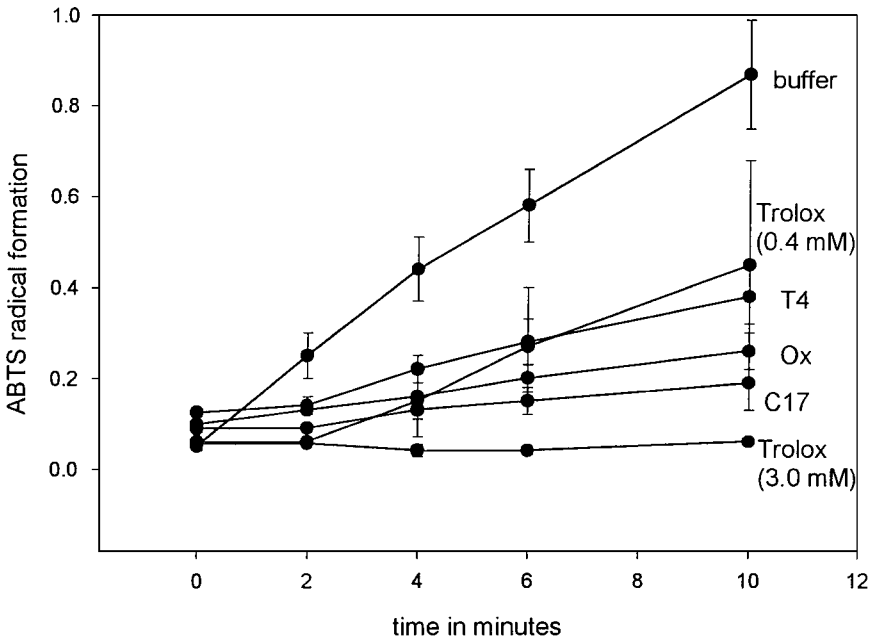


FIG. 3. Antioxidant capacity of *H. virescens* midgut fluid (diluted 1:10) as indicated by suppression of ABTS radical formation in vitro. The effects of buffer and two concentrations of Trolox (0.04 and 3.0 mM) are included for comparison. Larvae were fed detached leaves of T4 (low phenolic content, PAL sense-suppressed), C17 (intermediate), or Ox (high phenolic, PAL overexpressing) tobacco. There were no significant differences among caterpillars fed different tobacco lines.

TABLE 2. OXIDATIVE STATUS OF MIDGUT TISSUES OF INSECTS FED MODIFIED TOBACCO LINES

	T4	C14	Ox	Ox-NahG	NahG	Xanthi	*P*
Total ascorbic acid (μmol/mg protein)	0.926 ± 0.07	1.031 ± 0.11	0.904 ± 0.10	0.761 ± 0.05	0.738 ± 0.10	0.768 ± 0.14	0.235
Reduced (ASC) (μmol/mg protein)	0.743 ± 0.04	0.790 ± 0.08	0.698 ± 0.07	0.615 ± 0.06	0.584 ± 0.08	0.572 ± 0.06	0.104
Oxidized (DHA) (μmol/mg protein)	0.183 ± 0.04	0.241 ± 0.04	0.167 ± 0.06	0.159 ± 0.07	0.154 ± 0.03	0.196 ± 0.09	0.578
Hydroperoxides (μmol/mg protein)	11.83 ± 2.36	14.52 ± 1.71	12.28 ± 1.36	15.20 ± 1.45	19.78 ± 4.11	15.34 ± 2.00	0.368
Disulfides (ABS/mg protein)	5.40 ± 0.60	5.59 ± 0.54	4.99 ± 0.42	5.02 ± 0.45	8.17 ± 2.46	6.46 ± 0.88	0.491

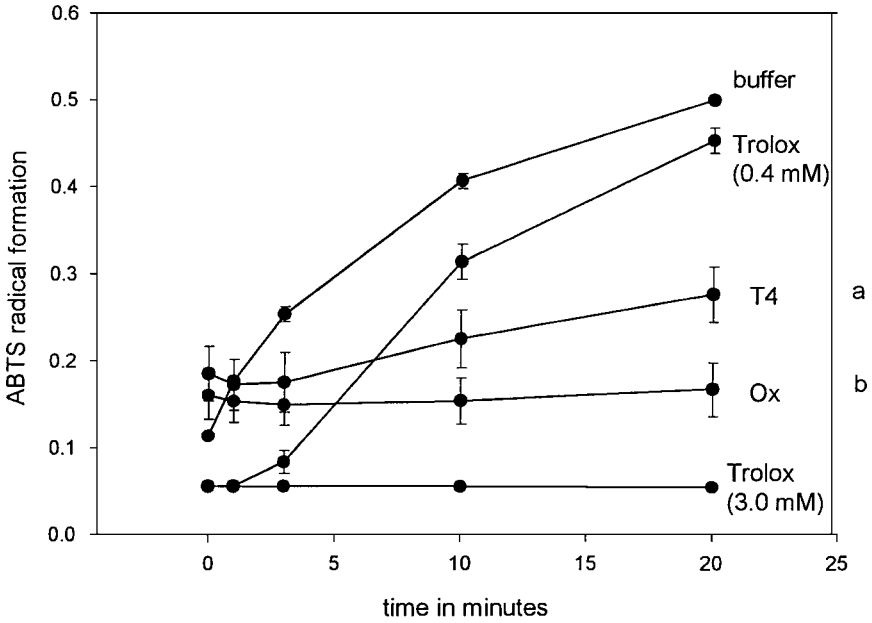


FIG. 4. Antioxidant capacity of *H. virescens* hemolymph as indicated by suppression of ABTS radical formation in vitro. The effects of buffer and two concentrations of Trolox (0.04 and 3.0 mM) are included for comparison. Larvae were fed detached leaves of T4 (low phenolic content, PAL sense-suppressed) or Ox (high phenolic, PAL overexpressing) tobacco for 72 hr prior to the assay. Letters indicate significant differences at $P < 0.05$ (*t* test).

DISCUSSION

Our experiments show that *H. virescens* larvae are relatively unaffected by the 8- to 10-fold range of variation in PAL (phenylpropanoid) expression in tobacco (Bi et al., 1997a; Ali et al., 1999). Although consumption and digestibility were lower on overexpressing plants, they resulted in very modest (not significant) reductions in growth with no indications of oxidative stress as a causal factor. These observations are in contrast to predictions from in vitro model systems and artificial diet experiments that chlorogenic acid, the predominant soluble phenolic in tobacco leaves should have strong negative, prooxidant effects on herbivores (Isman and Duffey, 1982a,b, 1983; Lindroth and Peterson, 1988; Felton et al., 1989, 1992; Stamp et al., 1994; Stamp and Yang, 1996; Yang et al., 1996). In fresh plant tissue, CHA's prooxidant effects appear to be ameliorated by the strong overall antioxidant nature of crushed foliage and the beneficial effects of dietary phenolics on antioxidant defenses of caterpillars. The demonstration that high

levels of oxidizable plant phenolics in foliage are not necessarily prooxidant when encountered by herbivores in their natural context has important implications for understanding the mode of action and biological activity of phenols that are often presumed to function in antiherbivore defense.

Our results indicate that high free radical activity in foliage does not necessarily translate into oxidative damage to herbivores. Although high phenolic lines (Ox, Ox-NahG) in our study exhibited measurably elevated hydroperoxide formation, all of the lines were strongly antioxidant compared to saline buffer, which is presumably neither pro- nor antioxidant. The overall antioxidant capacity of the chemical milieu of crushed foliage is clearly important for determining the biological impact of prooxidant phenols on herbivores. The net oxidative balance in crushed foliage depends on the predominant types of phenolics and phenolases present and can vary substantially among plant species. Hoover et al. (1998a,c) compared the effects of monophenols, catecholic phenols, tannins, peroxidase (POD), and polyphenoloxidase (PPO) activity on free radical generation in crushed cotton, tomato, and lettuce foliage. While some monophenols (ferulic and *p*-coumaric acids), the polyphenolic tannin quebracho, and mixtures of phenolics prone to redox cycling (CHA and rutin) enhanced free radical generation in the presence of POD, other combinations (e.g., monophenolics with catecholic phenolics) exhibited antioxidant properties. For example, chlorogenic acid, rutin, and catechin applied to foliage in mixtures had strong antioxidant properties and decreased free radical generation in crushed foliage by 30–60% (Hoover et al., 1998c). The tendency of this combination of phenolics to be antioxidant may explain why tomato fails to inhibit baculoviral disease, in spite of being rich in phenolics (Hoover et al., 1998b). Our results with tobacco differ somewhat in that we measured higher free radical production in high phenolic lines, but tobacco appears to be similar to tomato in that the overall nature of crushed foliage is strongly antioxidant. This may be a general pattern for solanaceous plants, which are rich in CHA and rutin, but also have smaller amounts of ferulic acid (Waiss et al., 1981; Isman and Duffey, 1982a,b; Bi et al., 1997a).

The powerful antioxidant capacity of *H. virescens* midgut fluid appears to represent an additional and significant barrier to ROS generation in the gut lumen, suppressing ABTS radical formation as strongly as the commercial antioxidant Trolox. Its antioxidant activity was much stronger than that of macerated leaf tissues, suggesting that caterpillars have biochemical mechanisms for enhancing the antioxidant properties of gut fluid. Possible mechanisms include the secretion of antioxidants such as glutathione, ascorbate, and uric acid (Summers and Felton, 1994; Mathews et al., 1997; Barbehenn et al., 2001) or enzymes such as catalase (Felton and Duffey, 1991), ascorbate peroxidase (Mathews et al., 1997) or glucose oxidase, which can deplete molecular O_2 from the gut lumen (Eichenseer et al., 1999; Johnson and Barbehenn, 2000).

One of the more surprising results was the positive effect of PAL overexpressing foliage on the total antioxidant capacity of caterpillar hemolymph. This is the first demonstration of a positive antioxidant benefit of dietary phenolics for herbivorous insects, although there is a huge body of literature demonstrating protective antioxidant effects of phenolics in green tea, red wine, and green vegetables in mammalian systems (reviewed in Kahl, 1991; Frei, 1994; Hertzog et al., 1997; Maxwell, 1997). Many phenols act as chain-breaking antioxidants (Takahama, 1985; Pincemail et al., 1986), prevent oxidation of lipids by chelating catalytic metal ions such as Fe and Cu (Afansas'e et al., 1989), and inhibit LOXs, POs, and other oxidases responsible for free radial production (Gryglewski et al., 1987; Moroney et al., 1988; Hoover et al., 1998a,b). They may also induce antioxidant defenses in caterpillars to levels that exceed the immediate need, resulting in higher overall antioxidant capacity of hemolymph and other tissues. In caterpillars, superoxide dismutase (SOD) is inducible and increases rapidly in response to dietary prooxidants, including the phenolic quercetin (Pritsos et al., 1988; Lee and Berenbaum, 1989; Ahmad and Pardini, 1990). Finally, it should be noted that the antioxidant benefit could be due to an unknown trait other than phenolic concentration that covaries with PAL expression. For example, high levels of the phenolic salicylic acid can suppress wound-induced responses by inhibiting the wound-signal cascade (Felton et al., 1999). This mechanism is not likely to have been important in our experiments because caterpillars were fed detached leaves or foliage from unwounded plants, but the possibility that PAL modification altered other biochemical pathways warrants further investigation.

Although our experiments do not support the hypothesis that high in planta concentrations of CHA have prooxidant effects on caterpillar tissues, PAL overexpression did alter feeding behavior and digestive responses of *H. virescens* in other ways. In the choice experiments, larvae showed a clear preference for low phenolic foliage. In this regard, *H. virescens* differs from *M. sexta*, which does not discriminate between PAL over- and underexpressing foliage (Eichenseer et al., 1998) and is not deterred by similar concentrations of CHA presented on artificial substrates (Boer and Hanson, 1987; Wrubel and Bernays, 1990). Early instar *H. virescens* larvae may respond differently; although late instar diet-reared larvae preferred and consumed more low phenolic foliage and grew at a slightly faster rate (this study), early instar growth and survival on underexpressing lines is poor (Ali et al., 1999). Possibly early instar larvae are more sensitive to host-specific phagostimulants than mature larvae.

Overexpression of PAL was also associated with reduced digestibility of leaves to caterpillars. Although reduced protein quality and inhibition of digestive enzymes are symptoms associated with ROS from phenolic oxidation, given our measures of the strong antioxidant capacity of crushed foliage, midgut fluid, and the positive oxidative balance of hemolymph and midgut epithelium, we believe the reduced digestibility is more likely related to changes in lignin content. Lignin

is formed by the polymerization of phenylpropanoids and is directly correlated with PAL expression (Elkind et al., 1990; Bate et al., 1994; Sewalt et al., 1997).

In summary, the results of this study show that the efficacy of prooxidant phenolics against herbivores is not easily predicted from foliar concentrations and artificial diet bioassays. Thus, from a plant's perspective, increased allocation to phenylpropanoid synthesis (through PAL) may not increase resistance to herbivores; in fact, it may have the opposite effect since some phenol mixtures can provide beneficial antioxidant properties. The demonstration of antioxidant, rather than prooxidant, effects on leaf-chewing caterpillars represents a previously unrecognized beneficial effect of phenolics on insects and emphasizes the need to examine their biological activity in the appropriate phytochemical context, rather than as single dietary additives, to fully understand their ecological role in plant herbivore interactions.

REFERENCES

AFANSAS'E, I. B., DOROZHKO, A. I., BRODSKII, V., KOSTYIK, A., and POTAPOVITCH, A. I. 1989. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol*. 38:1763–1769.

AHMAD, S. 1992. Biochemical defense of pro-oxidant plant allelochemicals by herbivorous insects. *Biochem. Syst. Ecol.* 20:269–296.

AHMAD, S. and PARDINI, R. S. 1990. Antioxidant defense of the cabbage looper, *Trichoplusia ni*: Enzymatic responses to the superoxide-generating flavonoid, quercetin, and photodynamic furanocoumarin, xanthotoxin. *Photochem. Photobiol.* 51:305–311.

ALI, M. I., BI, J. L., YOUNG, S. Y., and FELTON, G. W. 1999. Do foliar phenolics provide protection to *Heliothis virescens* from a baculovirus? *J. Chem. Ecol*. 25:2193–2204.

ANDERSON, W. L. and WETLAUFER, D. B. 1975. A new method for disulfide analysis of peptides. *Anal. Biochem*. 67:493–502.

APPEL, H. 1993. Phenolics in ecological interactions: The importance of oxidation. *J. Chem. Ecol.* 19:1521–1552.

APPEL, H. M. 1994. The chewing herbivore gut lumen: Physicochemical conditions and their impact on plant nutrients, allelochemicals, and insect pathogens, pp. 210–223, *in* E. A. Bernays (ed.). Insect-Plant Interactions, Vol V. CRC Press, Ann Arbor, Michigan.

APPEL, H. M. and MARTIN, M. M. 1990. Gut redox conditions in herbivorous lepidopteran larvae. *J. Chem. Ecol.* 16:3277–3290.

APPEL, H. M. and SCHULTZ, J. C. 1992. Activity of phenolics in insects: The role of oxidation, pp. 609–620, *in* R. W. Hemingway and P. E. Laks (eds.). Plant Polyphenols, Plenum Press, New York.

BARBEHENN, R. V. and MARTIN, M. M. 1994. Tannin sensitivity in larvae of *Malacosoma disstria* (Lepidoptera): Roles of the peritrophic envelope and midgut oxidation. *J. Chem. Ecol.* 20:1985–2001.

BARBEHENN, R. V., BUMGARNER, S. L., ROOSEN, E. F., and MARTIN, M. M. 2001. Antioxidant defenses in caterpillars: Role of the ascorbate-recycling system in the midgut lumen. *J. Insect Physiol.* 47:349–357.

BATE, N. J., ORR, J., NI, W., MEROMI, A., NADLER-HASSAR, T., DOERNER, P. W., DIXON, R. A., LAMB, C. J., and ELKIND, Y. 1994. Quantitative relationship between phenylalanine ammonia-lyase

levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. *Proc. Natl. Acad. Sci. USA* 91:7608–7612.

BERNAYS, E. A. 1981. Plant tannins and insect herbivores: An appraisal. *Ecol. Entomol.* 6:353–361.

BERNAYS, E. A., COOPER-DRIVER, G., and BILGENER, M. 1989. Herbivores and plant tannins. *Adva. Ecol. Res.* 19:263–275.

BI, J. L., FELTON, G. W., and MUELLER, A. J. 1994. Induced resistance in soybean to *Helicoverpa zea*: Role of plant protein quality. *J. Chem. Ecol.* 20:183–198.

BI, J. L., FELTON, G. W., MURPHY, J. B, HOWLES, P. A., DIXON, R. A., and LAMB, C. J. 1997a. Do plant phenolics confer resistance to specialist and generalist insect herbivores? *J. Agric. Food Chem.* 45:4500–4504.

BI, J. L., MURPHY, J. B., and FELTON, G. W. 1997b. Antinutritive and oxidative components as mechanisms of induced resistance in cotton to *Helicoverpa zea*. *J. Chem. Ecol.* 23:95–115.

BOER, G. DE and HANSON, F. E. 1987. Feeding responses to solanaceous allelochemicals by larvae of the tobacco hornworm, *Manduca sexta*. *Entomol. Exp. Appl.* 35:177–193.

DELANEY, T. P., UKNES, S., VERNOOIJ, B. FRIEDRICH, L., WEYMANN, K., NEGROTTO, D., GAFFNEY, T., GUT-RELLA, M., KESSMAN, H., WARD, and E., RYALS. 1994. A central role for salicylic acid in disease resistance. *Science* 266:1247–1250.

DUFFEY, S. S. and FELTON, G. W. 1991. Enzymatic antinutritive defenses of tomato plants against insects, pp. 167–197, *in* P. A. Hedin (ed.). Naturally Occurring Pest Bioregulators. American Chemical Society Symp. Ser. 449. Washington, D.C.

EICHENSEER, H., BI, J. L., and FELTON, G. W. 1998. Indiscrimination of *Manduca sexta* larvae to overexpressed and underexpressed levels of phenylalanine ammonia-lyase in tobacco leaves. *Entomol. Exp. Appl.* 87:73–78.

EICHENSEER, H., MATHEWS, M. C., BI, J. L., MURPHY, J. B., and FELTON, G. W. 1999. Salivary glucose oxidase: Multifunctional roles for *Helicoverpa zea*? *Arch. Insect Biochem. Physiol.* 42:99–109.

ELKIND, Y., EDWARDS, R., MAVANDAD, M., HEDRICK, S. A., RIBAK, O., DIXON, R. A., and LAMB, C. J. 1990. Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *Proc. Natl. Acad. Sci. USA* 87:9057–9061.

ELLIGER, C. A., WONG, Y., CHAN, B. G., and WAISS, A. C., JR. 1981. Growth inhibitor in tomato (*Lycopersicon*) to tomato fruitworm (*Heliothis zea*). *J. Chem. Ecol.* 5:753–758.

FEENY, P. P. 1976. Plant apparency and chemical defense, pp. 1–40, *in* J. Wallace and R. L. Mansell (eds.). Biochemical Interactions Between Plants and Insects. Recent Advances in Phytochemistry, Vol. 10, Plenum Press, New York.

FELTON, G.W. and DUFFEY, S. S. 1991. Protective action of midgut catalase in lepidopteran larvae against oxidative plant defenses. *J. Chem. Ecol.* 17:1715–1732.

FELTON, G. W., DONATO, K. K., DEL VECCHIO, R. J., and DUFFEY, S. S. 1989. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *J. Insect Physiol.* 38:277–285.

FELTON, G. W., DONATO, K. K., DEL VECCHIO, R. J., BROADWAY, R. M., and DUFFEY, S. S. 1992. Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore. *J. Insect Physiol.* 38:277–285.

FELTON, G. W., KORTH, K. L., BI, J. L., WESLEY, S. V., HUHMAN, D. V., MATHEWS, M. C., MURPHY, J. B., LAMB, C., and DIXON, R. A. 1999. Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Curr. Biol.* 9:317–320.

FREI, B. 1994. Natural Antioxidants in Human health and Disease. Academic Press, New York.

GAFFNEY, T., FRIEDRICH, L., VERNOOIJ, B., NEGROTTO, D., NYE, G., UKNES, S., WARD, E., KESSMAN, H., and RYALS, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261:754–756.

GRYGLEWSKI, R. J., KORBUT, R., ROBAK, J., and SWIES, J. 1987. On the mechanism of antithrombic action of flavonoids. *Biochem. Pharmacol.* 36:317–322.

HARBORNE, J. B. 1991. Flavonoid Pigments, pp 389–429, *in* G. A. Rosenthal and M. B. Berenbaum (eds.). Herbiveres: Their Interactions with Secondary Plant Metabolites, Vol I. Academic Press, New York.

HERTZOG, M. G. L., SWEETNAM, P. M., FEHILY, A. M., ELMWOOD, P. C., and KROMHOUT, D. 1997. Antioxidant flavonols and ischemic heart disease in a Welsh population of men: The Caerphilly study. *Am. J. Clin. Nutr.* 65:1489–1494.

HODNICK, W. F., KALYANARAMAN, B., PRITSOS, C. A., and PARDINI, R. S. 1989. The production of hydroxyl and semiquinone free radicals during the autooxidation of redox active flavonoids, pp. 149–152, *in* M. G. Simic, K. A. Taylor, J. F. Ward, and C. von Sonntag (eds.). Oxygen Radicals in Biology and Medicine. Plenum Press, New York.

HOOVER, K., KISHIDA, K. T., DIGIORGIO, L. A., WORKMAN, J., ALANIZ, S. A., HAMMOCK, B. D., and DUFFEY, S. S. 1998a. Inhibition of baculoviral disease by plant-mediated peroxidase activity and free radical generation. *J. Chem. Ecol.* 24:1949–2000.

HOOVER, K., STOUT, M., ALANIZ, S. A., HAMMOCK, B. D., and DUFFEY, S. S. 1998b. Influence of induced plant defenses in cotton and tomato on the efficacy of baculoviruses on noctuid larvae. *J. Chem. Ecol.* 24:253–271.

HOOVER, K., YEE, J. L., SCHULTZ, C. M., ROCKE, D. M., HAMMOCK, B. D., and DUFFEY, S. S. 1998c. Effects of plant identity and chemical constituents on the efficacy of a baculovirus against *Heliothis virescens*. *J. Chem. Ecol.* 24:221–252.

HOWLES, P. A., SEWALT, V. J. H., PAVIA, N. J., ELKIND, Y., BATE, N. J., LAMB, C., and DIXON, R. A. 1996. Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiol.* 112:1617–1624.

ISMAN, M. B. and DUFFEY, S. S. 1982a. Toxicity of tomato phenolic compounds to the fruitworm, *Heliothis zea*. *Entomol. Exp. Appl*. 31:370–376.

ISMAN, M. B. and DUFFEY, S. S. 1982b. Phenolic compounds in the foliage of commercial tomato cultivars as growth inhibitors to the fruitworm, *Heliothis zea*. *J. Am. Hortic. Sci.* 107:167–170.

ISMAN, M. B. and DUFFEY, S. S. 1983. Pharmacokinetics of chlorogenic acid and rutin in larvae of *Heliothis zea*. *J. Insect Physiol.* 29:295–300.

JIANG, Z., HUNT, J. V., and WOLFF, S. P. 1992. Ferrous iron oxidation in the presence of xylenol orange for detection of lipid hydroperoxides in low density lipoprotein. *Anal. Biochem.* 202:384–389.

JOHNSON, K. S. and BARBEHENN, R. V. 2000. Oxygen levels in the gut lumens of herbivorous insects. *J. Insect Physiol.* 46:897–903.

KAHL, R. 1991. Protective and adverse biological actions of phenolic antioxidants, pp. 245–273, *in* H. Seis (ed.). Oxidative Stress: Oxidants and Antioxidants. Academic Press, New York.

LEE, K. and BERENBAUM, M. R. 1989. Action of antioxidant enzymes and cytochrome P-450 monooxygenases in the cabbage looper in response to plant phototoxins. *Arch. Insect Biochem. Physiol.* 10:151–162.

LINDROTH, R. L. and PETERSON, S. S. 1988. Effects of plant phenols on performance of southern armyworm larvae. *Oecologia* 75:185–189.

MAHER, E. A., BATE, N. J., NI, W., ELKIND, Y., DIXON, R. A., and LAMB, C. J. 1994. Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. *Proc. Natl. Acad. Sci. USA* 91:7802–7806.

MARTIN, J. S., MARTIN, M. M., and BERNAYS, E. A. 1987. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores: Implications for theories of plant defense. *J. Chem. Ecol.* 13:605–621.

MATHEWS, M. C., SUMMERS, C. B., and Felton, G. W. 1997. Ascorbate peroxidase: A novel antioxidant enzyme in insects. *Arch. Insect Biochem. Physiol.* 34:57–68.

MAXWELL, S. R. J. 1997. Wine antioxidants and their impact on antioxidant activity in vivo, pp 150–165, *in* T. R. Watkins (ed.). Wine: Nutritional and Therapeutic Benefits. American Chemical Society, Washington, D.C.

MILLER, N. J. and RICE-EVANS, C. A. 1994. Total antioxidative status in plasma and body fluids. *Methods Enzymol.* 234:279–293.

MORONEY, M. A., ALCARAC, M. J., FORDER, R. A., CAREY, F., and HOULT, J. R. S. 1988. Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. *J. Pharm. Pharmacol.* 40:787–792.

PALLAS, J. A., PAIVA, N. L., LAMB, C., and DIXON, R. A. 1996. Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. *Plant J.* 10:281–293.

PINCEMAIL, J., DEBY, C., LION, Y., BRAQUET, P., HANS, P., DRIEU, K., and GOUTIER, R. 1986. Role of flavonoids in lipoperoxidation and radicalar reactions, pp 423–436, *in* L. Parkas, M. Gabor, and F. Kallay (eds.). Flavonoids and Bioflavonoids. Elsevier, Amsterdam, The Netherlands.

PRITSOS, C. A., AHMAD, S., BOWEN, S. M., ELLIOTT, A. J., BLOMQUIST, G. J., and PARDINI, R. S. 1988. Antioxidant enzymes of the black swallowtail butterfly, *Papilio polyxenes*, and their response to the prooxidant allelochemical, quercetin. *Arch. Insect Biochem. Physiol.* 8:101–112.

REESE, J. C., and BECK, S. D. 1976. Effects of allelochemicals on the black cutworm, *Agrotis ipsilon*; effects of catechol, L-DOPA, dopamine and chlorogenic acid on larval growth, development and utilization of food. *Ann. Entomol. Soc. Am.* 69:68–72.

RICE-EVANS, C. A., MILLER, N. J., BOLWELL, P. G., BRAMLEY, P. M., and PRIDHAM, J. B. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radic. Res.* 22:375–383.

RICE-EVANS, C. A., MILLER, N. J., and PAGANGA, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20:933–956.

SEWALT, V. J. H., NI, W., JUNG, H. G., and DIXON, R. A. 1997. Lignin impact on fiber degradation: Increased enzymatic digestibility of genetically engineered tobacco (*Nicotiana tabacum*) stems reduced in lignin content. *J. Agric. Food Chem.* 45:1977–1983.

STAMP, N. E. and YANG, Y. 1996. Response of insect herbivores to multiple allelochemicals under different thermal regimes. *Ecology* 77:1088–1102.

STAMP, N. E., TEMPLE, M., TRAUGOTT, M. S., and WILKENS, R. T. 1994. Temperature-allelochemical interactive effects on performance of *Manduca sexta* caterpillars. *Entomol. Exp. Appl.* 73:199–210.

STEINLY, B. A. and BERENBAUM, M. 1985. Histopathological effects of tannins on the midgut epithelium of *Papilio polyxenes* and *Papilio glaucus. Entomol. Exp. Appl.* 39:3–9.

SUMMERS, C. B. and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Mol. Biol.* 24:943–953.

TAKAHAMA, U. 1985. O_2^- dependent and independent photooxidation of quercetin in the presence and absence of riboflavin and effect of ascorbate on the photooxidation. *Photochem. Photobiol.* 42:89–91.

WAISS, A. C., CHAN, B. G., ELLIGER, C. A., DRYER, D. L., BINDER, R. G., and GULEDNER, R. C. 1981. Insect growth inhibitors in crop plants. *Entomol. Soc. Am. Bull.* 27:217–221.

WRUBEL, R. P. and BERNAYS, E. A. 1990. The relative insensitivity of *Manduca sexta* to non-host plant secondary compounds. *Entomol. Exp. Appl.* 54:117–124.

YANG, Y., STAMP, N. E., and OSIER, T. L. 1996. Effects of temperature, multiple allelochemicals and larval age on the performance of a specialist caterpillar. *Entomol. Exp. Appl.* 79:335–344.

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

CALL FOR PAPERS

SPECIAL ISSUE ON AQUATIC CHEMICAL ECOLOGY

There will be a special issue of the *Journal of Chemical Ecology* devoted entirely to papers dealing with "Aquatic Chemical Ecology." Publication is planned for late summer/early fall 2002. The issue will consist of invited mini-reviews and contributed papers. Those wishing to submit a paper for consideration for this special issue should do so by February 1, 2001.

Please send the original and two copies of the manuscript (including illustrations) and the names and addresses of three potential reviewers to:

John T. Romeo
Journal of Chemical Ecology
Department of Biology
University of South Florida
Tampa, Florida 33620-5200

0098-0331/01/1200-2599/0

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

AUTHOR INDEX TO VOLUME 27

0098-0331/01/1200-2601/0

Journal of Chemical Ecology, Vol. 27, No. 12, December 2001 (© 2001)

SUBJECT INDEX TO VOLUME 27

0098-0331/01/1200-2607/0